Alkylacylglycerolipid domain of GPI molecules of *Leishmania* is responsible for inhibition of PKC-mediated c-*fos* expression

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Abstract Glycosylphosphatidylinositols (GPIs) are the most abundant molecules present in the membranes of the parasitic protozoa *Leishmania* responsible for multiple forms of leishmaniasis. Among the prominent biological activities displayed by the major *Leishmania* GPIs [lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs)] is the inhibition of macrophage functions such as the protein kinase C (PKC)-dependent signaling pathway. The bioactivity of *Leishmania* GPIs is in contrast to *Trypanosoma brucei* and *Plasmodium falciparum* GPIs, which activate the macrophage functions. To address the question as to which structural domain of *Leishmania* GPIs is responsible for dramatic down-regulation of PKC-dependent transient c-*fos* expression, the chemically synthesized defined alkylacylglycerolipids domain of corresponding GPIs, and LPG and GIPLs isolated from *Leishmania donovani*, were evaluated for inhibition of PKC and c-*fos* expression in macrophages. The results presented here demonstrate that the unusual lipid domain of *Leishmania* GPIs is primarily responsible for inhibition of PKC-dependent transient c-*fos* expression.— Chawla, M. and R. A. Vishwakarma. **Alkylacylglycerolipid domain of GPI molecules of *Leishmania* is responsible for inhibition of PKC-mediated c-*fos* expression. *J. Lipid Res.* 2003.** 44: **594–600.**

Supplementary key words glycosylphosphatidylinositol • lipophosphoglycan • protein kinase C • c-*fos*

Leishmania is an intracellular parasite that colonizes the macrophage system of its vertebrate host, causing various types of leishmaniases in humans. Upon entry into the vertebrate host, the parasite has to encounter two normal host immune mechanisms, the destruction by macrophages, and lysis by complement system. *Leishmania* has evolved a unique approach to deal with both these innate immune mechanisms by learning not only to avoid early destruction but also to co-opt the opsonic properties of complement to enhance its interaction with macrophages (1). Once inside the macrophage, the parasite subverts and attenuates normal signaling of the cells to inhibit or reduce the impact of microbicidal mechanisms. For this purpose, the parasite has devised unique molecular strategies, and one such strategy involves the structurally distinct glycosyl phosphatidyl inositol (GPI) molecules, expressed abundantly on parasite cell surface.

The GPIs were first discovered as a novel mode of anchoring of a few specialized surface proteins, but subsequently a diverse family of "free" GPIs was isolated from protozoan *Leishmania*, *Trypanosoma*, and malaria parasites (2–5). Recent studies indicate that GPIs from the parasites play a significant role in manipulating normal macrophage functions, and may also be targets for early NK-T cell and humoral responses during acute infection (6). The emerging theme is that the GPIs are among the most important virulence and survival determinants for the protozoan parasites. There are mainly three types of GPIs that are expressed by *Leishmania*: GPI anchor for surface proteins, lipophosphoglycans (LPGs), and free GPIs called glycoinositol phospholipids (GIPLs). LPG and GIPLs are expressed in high copy numbers (more then 10^7/cell) by the parasite and are considered essential for parasite infectivity and survival (2–5). All GPIs contain a conserved structural motif, Manα1-4GlcNα1-6-PI, and differ from each other with respect to the composition of glycan head group and/or the lipid anchor (2). The lipid anchor of GIPLs is exclusively 1-*O*-alkyl-2-*O*-acyl-phosphatidylinositol (PI) or 1-*O*-alkyl-*lyso*-PI type, and alkyl chains of type I and hybrid GIPLs are $C_{18:0}$, while type II GIPLs have longer alkyl chains, $C_{24:0}$ and $C_{26:0}$. The lipid anchor of LPG is

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Abbreviations: GIPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; HI-FBS, heat-inactivated fetal bovine serum; LPG, lipophosphoglycan; PDBu, phorbol dibutyrate; PI, phosphatidylinositol; PKC, protein kinase C; PTK, protein tyrosine kinase; TCA, trichloroacetic acid.

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1-*O*-alkyl-2-*lyso*-PI with $C_{24/26}$ saturated unbranched aliphatic chains.

For macrophages to get activated for resistance and killing of the parasite, the cells must have the capability to respond to external signals by sending information from the surface to the nucleus. Obviously, the macrophage signaling pathways are appropriate targets for the intracellular parasites, as this strategy enables them to manipulate normal gene expression of the macrophages and the normal activity of the cell without even entering the nucleus. The c-*fos* is among the most studied key early competence genes that are expressed immediately after the macrophage activation by infection. The c-*fos* expression in macrophages is controlled by two mechanisms. Protein kinase C (PKC)-mediated c-*fos* expression leads to a transient and rapid increase in c-*fos* mRNA, peaking 30 min after stimulation, whereas protein kinase A-mediated expression leads to stable c-*fos* transcription lasting for several hours. For these reasons, c-*fos* has become a useful nuclear marker to study the role of parasitic GPIs in signaling in macrophages.

From various recent studies, it is abundantly clear that the nature of lipid domain of 'free' GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites. For example, in *Trypanosoma brucei*, the lipid structure of GPIs changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms (7), and unique parasite-specific myristic acid exchange occurs (8), which has also been exploited for drug design (9). More importantly, the differences in lipid structure of the GPIs of various parasites manifest in opposite biological activities. For example, GPIs of *T. brucei* and *Plasmodium falciparum* activate macrophage functions (10, 11) such as induction of proinflammatory cytokines and activation of PKC and protein tyrosine kinase (PTK) activity, whereas in contrast to above observations, the *Leishmania* GPIs (LPG and GIPLs) severely inhibit (12–16) macrophage functions (PKC and related downstream signal trasduction). At present there is no convincing explanation for these contradictory observations, however, the major structural differences among the parasite GPIs lie in their glycerolipid structure. Another factor, which complicates the issue further, is the high degree of microheterogeneity present in the lipid domains, and therefore most of the GPIs isolated from the parasites are heterogeneous and ill-defined. The heterogeneity lies in fatty acid chain lengths, alkyl versus acyl substitution mode, alkylacyl versus lysoalkyl, degree, and geometry of unsaturation in the fatty acids, and additional inositol lipidation, etc. Therefore, it is not really clear at present what really contributes to the opposite biological activities observed within same class of molecules. It is obvious that parasites have fine-tuned the GPI structure and function by sophisticated molecular mimicry. The only way these issues can be sorted out unambiguously is by using structurally defined GPIs and their domains obtained by chemical synthesis. In a program to address the above-mentioned issue, we have initiated efforts (17–22) toward chemical synthesis of structurally defined *Leishmania* GPIs (LPG and GIPLs) and their various structural domains-analogs to decipher the role of these GPIs in macrophage signaling and function using synthetic compounds. The results presented in this paper demonstrate that synthetic 1-*O*-alkyl$_{C18:0}$-2-*O*-acyl$_{C16:0}$-*sn*-glycerolipids [alkylacylglycerol (AAG) and alkyacylphosphatidic acid (AAP)] corresponding to the *Leishmania donovani* GPIs are responsible for inhibition of PKC-dependent transient c-*fos* gene expression in macrophages.

EXPERIMENTAL PROCEDURES

Materials

All materials were of analytical grade or higher. ATP, staurosporine, phorbol dibutyrate (PDBu), diacylglycerol, phosphatidylserine, phosphatidylcholine (from egg yolk), and chelex were obtained from Sigma. Medium 199, gentamicin sulfate, Hepes, fetal bovine serum, DMEM, random labeling kit, RNase-A, restriction enzymes (*Pvu*II, *Bgl*II, *Hind*III, *Eco*RI), and $\phi \times 174$ DNA marker (*Hae*III digest) were procured from Gibco-BRL. TRI-reagent-BD was from MRC. RNeasy RNA extraction kit was from Qiagen. Octyl-sepharose CL-4B, phenyl-sepharose CL-4B, MOPS, formamide, agarose, Hyperfilm-MR, and Hybond-N nylon membrane were procured from Amersham-Pharmacia. β-glycerophosphate was from Calbiochem. C-18 derivatized reverse phase silica beads were from Waters. P-81 phosphocellulose paper and DE-52 (DEAE-cellulose) were obtained from Whatman. Aluminium-backed HPTLC plates (Kieselgel 60 F254) were procured from Merck-BDH. X-ray film developer and fixer were from Kodak. Diethylpyrocarbonate was from Ameresco. The PKC-specific nonapeptide (VRKRTLRRL) was synthesized at the Protein Chemistry Lab. All the reagents used were of highest purity grade available. Molecular biology grade reagents were used for all procedures involving DNA and RNA. Cell culture grade reagents were used for all culture work.

Leishmania culture

A well-characterized pathogenic strain of *L. donovani* (DD8, WHO reference MHOM/IN/80/DD8) was provided by K-P. Chang (Chicago). The parasites were cultured in medium-199 (pH 7.2) containing 10% heat-inactivated fetal bovine serum (HI-FBS), 50 mg/l gentamicin sulfate, and 25 mM Hepes at 23°C in a cooling incubator. The parasites were harvested (20) in late log-phase for isolation of GIPLs and LPG.

Isolation and purification of GPIs from *L. donovani*

Parasites were harvested in late log phase, and PBS-washed pellet was extracted twice with chloroform-methanol-water (4:8:3, v/v/v). Pooled CMW extract was dried (nitrogen stream) and extracted into n-butanol-water (2:1, v/v), and individual GIPLs (iM2, iM3 and iM4) were purified by high-performance thin layer chromatography (HPTLC). The delipidated cell pellet was extracted twice with 9% n-butanol for extraction of LPG, which was further purified by octyl-sepharose column, passed through chelax resin, and characterized by ^{1}H- and ^{13}C-NMR experiments.

Chemical synthesis of alkylacylglycerolipids

Alkylacylglyceroipids 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycerol and 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycero-phosphate (alkylacyl phosphatidic acid) used in the present study were prepared by chemical synthesis as follows. The starting material isopropylidene-*sn*-glycerol was alkylated with 1-bromooctadecane using sodium

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hydride followed by deketalation with HCl to get 1-*O*-octadecyl-*sn*-glycerol. This was followed by selective protection of primary alcohol with t-butyldimethylsilyl group (TBDMSCl, imidazole), palmitoylation at C-2 position (palmitoyl chloride, pyridine), and TBDMS group removal (tetrabutylammonium fluoride) to get 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycerol. The phosphorylation of this lipid with diphenyl phosphorochloridate followed by catalytic hydrogenolysis (Adams catalyst) provided the desired 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycero-phosphate (alkylacylphosphatidic acid). The choice of alkyl and acyl chains was based on the structures of GPI molecules of *L. donovani*. The final lipid products, as well as all the intermediates, were characterized by NMR, IR, and mass spectrometry (17).

PKC assay in vitro

PKC was purified from rat brain tissue (male Wistar rats) using DE-52 and phenyl-sepharose chromatography (23), and assayed as described earlier (24) with minor modifications. The enzyme activity was measured as phosphorylation of a nonapeptide (VRKRTLRRL, 2.5 mg/ml) in a reaction mixture containing buffer (50 mM Tris-Cl, pH 7.5), 0.65 mM $CaCl_2$/0.5 mM EGTA, 10 mM $Mg(OAc)_2$, 10 mg/ml phosphatidylserine, 2 mg/ml diacylglycerol), and 100 μM [γ-^{32}P]ATP (10^3 cpm/pmol). The phospholipids dissolved in chloroform were dried under a stream of nitrogen and resuspended in buffer by sonication. Reaction was carried out at 30°C for 15 min before terminating by adding 25 μl of 20% trichloroacetic acid (TCA). A 25 μl aliquot was spotted onto a 2 × 2 cm P-81 strip, air dried, and washed with 75 mM phosphoric acid. Radioactivity incorporated in the peptide substrate was measured by scintillation counting. Nonspecific activity was measured in the absence of phospholipids and Ca^{2+}. Specific activity was the difference between total activity and nonspecific activity. Enzyme activity was calculated as pmol of phosphate transferred from ATP to substrate per min per milligram of protein.

Inhibition of PKC by synthetic alkyl-acyl-glycerolipids in vitro

Different concentrations of synthetic alkylacyl-*sn*-glycerol and alkylacylphosphatidic acid were prepared in chloroform, dried under nitrogen, and resuspended in buffer by sonication before use in in vitro PKC inhibition experiments as described above.

Culture of J774A.1 murine macrophage cell line

Murine macrophage cell line J774A.1 was cultured in DMEM (containing 2 mM glutamine, 50 mg/l gentamicin sulfate, 2 g/l sodium bicarbonate, and 15% HI-FBS; pH 7.2). Cells incubated at 37°C in 5% CO_2 atmosphere showed adherence within 2 h, and a confluent monolayer was obtained in 4 days.

Preparation of synthetic lipid-coated RP-C_{18} silica beads

Synthetic alkylacylglycerolipids were coated onto RP-C_{18}-derivatized silica beads according to the method first reported by Russell and Wright (25) and later applied by Descoteaux et al. (15) for presenting LPG-GIPLs to the macrophages. In a typical procedure, required aliquots of 2 mg/ml solution of synthetic alkylacylglycerolipid in $CHCl_3$ were mixed with 100 μl aliquots of 3 μm diameter beads (10 mg/ml) suspended in the same solvent (RP silica beads derivatized with C_{18} alkyl groups were from Waters). The mixture was dried under N_2 gas in an Eppendorf centrifuge tube and then under vacuum overnight. The beads were resuspended in 1 ml of PBS, sonicated for 2 min, washed twice with PBS, and resuspended in 2 ml of complete medium by sonication (3 cycles, 15 s each spaced by 10 s). The final concentration was made of 2 mg beads/ml medium. Efficiency of bead-coating and their uptake by macrophages was estimated using [^{14}C]palmitic acid as standard. For this, the 200 μl solution of labeled palmitic acid (2 mg/ml in $CHCl_3$, 100 μCi) was mixed with equal volume (200 μl) of beads (10 mg/ml) and suspended in $CHCl_3$. After mixing, the beads were dried, washed with PBS, resuspended in 2 ml of complete medium, and the labeled beads fed to confluent J774A.1 macrophages for 2 h. The monolayer was washed twice with the medium and washings saved. Cells were scraped in 5 ml of medium. Aliquot of [^{14}C]palmitic acid, coated beads, bead washings, monolayer washings, and treated macrophages were added to scintillation fluid, and radioactivity was measured. This analysis showed that efficiency of coating of the beads by [^{14}C]palmitic acid was 99%, and when these beads were fed to macrophages, virtually all of them were taken up. The reported concentrations of synthetic lipids are the concentrations of the lipid solutions used for coating beads. Controls were incubated with the same amount of uncoated beads, which were processed in the same way as experimental materials. The viability of macrophages after treatment with lipid-coated beads was routinely checked microscopically.

PKC assay in macrophages

Lipid-coated beads (uncoated/coated with alkylacylglycerolipids) suspended in the complete medium by sonication were added (200 μl per well) to macrophages (10^5 cells per well) in 96 well plates and incubated for 2 h at 37°C. The cells were washed twice with medium and incubated further in fresh complete medium for 3 h. These were then stimulated or not stimulated with 100 nM PDBu for 15 min before PKC activity was measured in digitonin-permeabilized cells as phosphorylation of peptide substrate (16). Medium was then replaced with 60 μl of assay buffer (20 mM Hepes, pH 7.2, 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1 mg/ml glucose), supplemented with 10 mM $MgCl_2$, 50 g/ml digitonin, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, 300 μM peptide substrate, and 100 μM [γ-^{32}P]ATP. The reaction was carried out at 30°C for 15 min, after which it was stopped by addition of 20 μl of 20% TCA. Aliquots (40 μl) were spotted onto 2 × 2 cm P-81 strips, air dried, and washed in 75 mM phosphoric acid (5 × 100 ml). Air-dried strips were placed in cocktail-W, and incorporated activity was measured by liquid scintillation counting. Specific activity was calculated as the difference between total activity (in the presence of peptide substrate) and nonspecific activity (in absence of peptide substrate). PKC activity was calculated as pmol of phosphate transferred from ATP to peptide substrate per min per 10^6 cells.

Analysis of c-*fos* expression in macrophages

Spent medium from confluent cultures was removed, and the monolayer was rinsed with 5 ml of medium. Then the synthetic lipids coated on beads and suspended in complete medium (2 ml per T-25 flask) were fed to macrophages for 2 h. Monolayers were then rinsed twice with medium and further incubated for 3 h in complete medium. These were either stimulated or not stimulated as per experimental requirement with 100 nM PDBu for 15 min. Cells were scraped, pelleted, and used for analysis of c-*fos* expression, as detailed below. Expression of β-actin was monitored as a housekeeping gene.

RNA extraction and Northern blot analysis

Total RNA was extracted from macrophages using Tri-reagent (MRC) or RNeasy kit (Qiagen). Samples were denatured in formamide at 65°C for 10 min, electrophoresed in 1% agarose gels (containing formaldehyde), and transferred to Hybond-N nylon membranes using 20×-SSC (175.3 g NaCl, 88.2 g sodium citrate, pH 7.0, volume made to 1 liter) as the transfer buffer. Prehybridization and hybridization were carried out at 42°C in 50% formamide with the probes previously random-labeled with [γ-^{32}P] dCTP to 1 × 10^8 cpm/μg DNA. Membranes were then washed once in 2×-SSPE (20×-SSPE = 175.3 g NaCl, 27.6 g NaH_2PO_4,

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7.4 g EDTA, pH 7.4, volume made to 1 liter), 0.1% SDS at 42°C for 10 min, twice in 2×-SSPE, 0.1% SDS at room temperature for 15 min, twice in 2×-SSPE, 0.1% SDS at 37°C for 10 min, and finally in 0.1×-SSPE, 0.1% SDS at 42°C. While still damp, membranes were autoradiographed at −70°C with an intensifying screen using Hyperfilm-MR. The relative abundance of the transcripts in Northern blots was quantitated using an optically enhanced densitometer (420 oe) with Diversity One software, version 1.6 (PDI Inc). The c-*fos* DNA probe containing a 1.3 kb *Pvu*II-*Bgl*II fragment from *pFBH*-1 was from Albert Descoteaux (Insitut Aramand Frappier, Canada), and the β-actin probe (0.6 kb *Eco*RI fragment from pAS-6) was provided by Anu Bashamboo of the Institute.

RESULTS AND DISCUSSION

Leishmania GPIs (LPG and GIPLs) have been implicated in a number of functions, including the parasite attachment and entry into macrophages, and its intracellular survival (3, 6). The most important target for *Leishmania* survival strategy is severe inhibition (nM range) of PKC, the enzyme responsible for initiation of normal oxidative mechanisms of the macrophages. In macrophages, these activation-associated events are under the control of PKC, an important enzyme and a key molecule in many signal transduction pathways. PKC comprises a family of at least 12 serine-threonine kinases, which are activated by turnover of membrane phospholipids (26). Since PKCs have a key role in the development of a protective host immune response by activation of various effector cells, it is not surprising that intracellular pathogens target this family of enzymes. This appears to be the case with *Leishmania*, which inhibits PKC directly as well as indirectly (via inhibition of PKC-mediated functions). The major cell surface molecules of *Leishmania* (LPG and GIPLs) have been shown to inhibit PKC and the downstream expression of related genes (12–16). While some studies have proposed that the majority of inhibition of LPG lied in its lipid anchors, other suggested that it was not required at all (14). The contribution of lipid anchors of GIPLs is not known. To ascertain this, one would require chemically defined molecules free from any contaminating domains. We used for the present study synthetic alkylacylglycerolipids (alkylacylglycerol and alkylacylphosphatidic acid) corresponding to Leishmania GPIs (17), and studied their effect on the PKC activity and the expression of key early competence gene, c-*fos*, in macrophages.

PKC inhibition in vitro by Leishmania GPIs and synthetic alkylacylglycerolipids

In order to assess the contribution of lipid domains of GPIs (LPG and GIPLs), synthetic and structurally defined alkylacylglycerolipids, 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycerol and 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycero-phosphate (alkylacylphosphatidic acid), were prepared by chemical synthesis by the following steps. First, the isopropylidene-*sn*-glycerol was alkylated with 1-bromooctadecane (NaH, DMF) followed by deketalation (HCl) to get 1-*O*-octadecyl-*sn*-glycerol. Selective protection of primary alcohol with t-butyldimethylsilyl group (TBDMSCl, imidazole), palmitoylation at C-2 position (palmitoyl chloride, pyridine), and TBDMS group removal (tetrabutylammonium fluoride) to get 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycerol. Phosphorylation of this lipid with $(PhO)_2POCl$ followed by catalytic hydrogenolysis provided desired 1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0)}$-*sn*-glycero-phosphate (alkylacylphosphatidic acid).

For measurement of PKC activity in vitro, a highly specific arginine-rich peptide substrate (27) was used. The synthetic lipids were suspended in chloroform, dried under nitrogen, and resuspended in buffer by sonication. PKC assay was done in the presence as well as absence of diacyl-*sn*-glycerol, the natural physiologic activator of PKC, at various concentrations (10 to 400 μM). Interestingly, the synthetic alkylacyl-*sn*-glycerol showed significant PKC inhibition (**Fig. 1**), and more then 50% inhibition was observed at the 50 μM to 100 μM range. Increasing the concentrations further did not lead to further increase in inhibition and reached a plateau. Similarly, synthetic alkylacylphosphatidic acid showed a 50% inhibition at 50 μM. These results, in comparison with the reported PKC inhibition activity of GIPLs isolated from *Leishmania* species (10, 13) and our results on GIPLs from *L. donovani*, demonstrate that the synthetic alkylacylglycerolipids without sugar residues of intact GPIs showed almost similar inhibition (μM range). This clearly indicates that the alkylacylglycerolipid domain of the *Leishmania* GPIs contributes primarily to PKC inhibitory activity. For comparisons to PKC inhibitory activity of synthetic lipids with intact LPG, we isolated LPG from *L. donovani* promastigotes (with average 10 phosphoglycan repeats) and evaluated it for PKC assay, which showed 80% inhibition at 100 μM (data not shown).

PKC assay in macrophages

To assess the role of lipids or lipid-containing molecules on enzyme activity in an aqueous system, it is necessary that the molecules are available to the cells by uptake. For

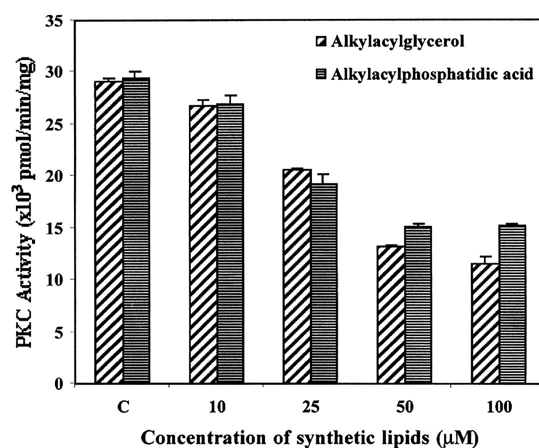

Fig. 1. Effect of varying concentrations of synthetic alkylacylglycerolipids on protein kinase C (PKC) activity in vitro. Different concentrations of alkylacylglycerol (AAG) or alkylacylphosphatidic acid (AAP) dried under nitrogen and resuspended in buffer by sonication were added to the assay mixture. PKC activity was then measured as detailed in Experimental Procedures.

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this, the synthetic lipid domains were coated onto RP-18 beads by the published method (15, 25) and then fed to macrophages. The efficiency of the coating procedure was assessed by using ^{14}C-labeled palmitic acid as control, which showed that almost 99% of the material was loaded onto the beads. The labeled beads, when fed to macrophages, were efficiently taken up. In each set of experiments, basal as well as agonist-induced (stimulation with 100 nM PDBu) PKC activity was measured. The controls were devoid of the peptide substrate for measurement of nonspecific activity, which was subtracted from total activity observed in the presence of peptide substrate, the specific activity expressed in pmol of PI transferred from [γ-^{32}P]ATP to substrate per min per 10^6 cells.

Evaluation of PKC activity and c-*fos* expression in macrophages

For measurement of both the PKC activity as well as the expression of c-*fos* in macrophages, synthetic lipids were coated onto RP-18 beads and fed to macrophage (J774A.1) cells. Efficiency of coating of beads and their uptake by macrophages was determined using [^{14}C]palmitic acid as a standard that showed that coating of the beads was 99% and lipid-coated beads were efficiently taken up by macrophages. The reported concentrations of synthetic lipids are the concentrations of the lipid solutions used for coating beads. The controls were incubated with the same amount of uncoated beads that were processed the same way as the experimental materials. The viability of macrophages after treatment with lipid-coated beads was routinely checked microscopically. After incubation for 2 h, the lipid-coated beads were removed and macrophage monolayers washed and further incubated in complete medium for 3 h. Macrophages were then either stimulated with 100 nM PDBu, or not stimulated as for the control, before replacing the medium with assay buffer for PKC assay or cells were harvested for RNA extraction. The synthetic lipids showed a similar pattern of PKC inhibition in macrophages, as shown in the in vitro experiments (data not shown), and an increase in inhibition as the concentration was increased up to 100 μM, beyond which a plateau was observed. Northern blot and densitometric analysis showed that the synthetic lipid 1-*O*-alkyl-2-*O*-acyl-*sn*-glycerol dramatically inhibited c-*fos* gene expression by 60% at 100 μM, which remained static at higher concentrations (**Fig. 2**). A similar inhibition level was observed with alkylacylphosphatidic acid (data not shown). Significantly, the intact LPG, isolated from *L. donovani,* showed significant inhibition (90%) of PKC activity in macrophages at 100 μM, and almost complete inhibition of c-*fos* expression at the same concentration. On comparison with intact LPG and GIPLs of *L. donovani*, the synthetic alkylacylglycerolipid domains showed significant PKC inhibition (both in vitro as well as in macrophages) and transient c-*fos* expression.

The question as to how parasitic cell-surface GPIs (LPG and GIPLs) bring about the extremely high levels of inhibition of PKC-dependent c-*fos* gene expression inside macrophages without even entering the cell, and which structural domain is responsible for this dramatic inhibition, has been a subject of speculation and debate (3, 6). A recent biophysical study (28) proposed that LPG brings about conformational changes in the lipid bilayer of macrophage plasma membrane, and these conformational changes directly or indirectly affect the binding of PKC to the membrane and/or prevent it from folding correctly in an active conforma-

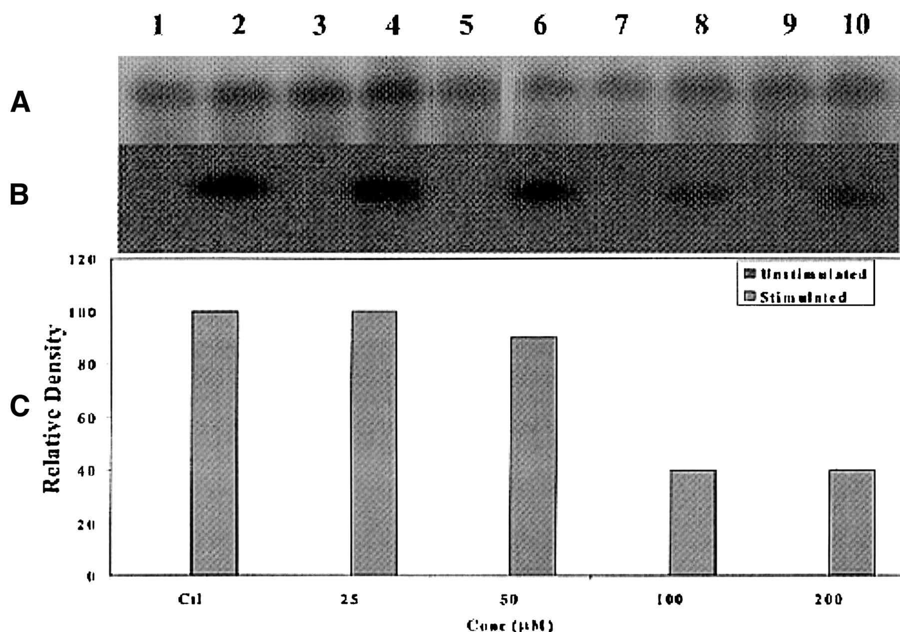

Fig. 2. Effect of varying concentrations of alkylacylglycerol on c-*fos* expression in macrophages: total RNA extracted from macrophages was subjected to c-*fos* analysis as detailed in Experimental Procedures. Samples in even-numbered lanes are from cells stimulated with 100 nM phorbol dibutyrate. A: β-actin m-RNA, (B) c-*fos* m-RNA, and (C) densitometric analysis of c-*fos* signals.

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tion. In either case, the outcome would be inhibition of PKC-dependent downstream signaling cascade. Since it is not known as yet whether LPG-GIPLs can flip across the lipid bilayer, another possible mode of action could be through their association with GPI-sphingolipid-cholesterol-rich lipid "rafts" and inhibition of normal signal transduction. An alternative hypothesis is that the GPIs are first hydrolyzed by secretory GPI-specific phospholipase (PI-PLC or PI-PLD types) generating alkylacylglycero lipids, which then can easily flip across the plasma membrane. Due to their structural similarity with diacyl-*sn*-glycerol (natural physiologic activator), the released alkylacyl glycerolipids can compete for the binding pocket of PKC and act as competitive DAG analog inhibitors. The first pointer toward such a possibility is a recent study (29) showing that several proteases of *Leishmania* play an important role in the secretion of gp63, a major GPI-anchored surface metalloprotease believed to promote parasite attachment and entry into host macrophages, and proteolytic cleavage of gp63 by membrane GPI-PLC contributes to disease pathogenesis. It is likely that these GPI-PLC enzymes also promote the release of lipid anchors of LPG-GIPLs, and such mechanisms would promote a parasite's entry into and survival thereupon in macrophages. Our results presented in this paper show that alkylacylglycerolipid domain alone is sufficient for observed inhibition of PKC-dependent c-*fos* gene transcription by *Leishmania* GPIs.

It is also intriguing that minor differences in lipid structure of the GPIs of various parasites manifest in opposite biological activities. For example, GPIs of *T. brucei* and *P. falciparum* containing diacyl-glycerolipid structural domain activate (10, 11, 30) macrophage functions (induction of proinflammatory cytokines, and activation of PKC and PTK), whereas *Leishmania* GPIs containing alkylacyl- or alkyllysoglycerolpid domain severely inhibit (12–16) macrophage functions (PKC and downstream signaling). Another factor complicating the issue further is the microheterogeneity present in the lipid domains, and most of the GPIs isolated from the parasites are heterogeneous, and it is not clear as to what really contributes to the opposite biological activities observed within the same class of molecules from different parasites. We have addressed this by using synthetic and chemically defined glycerolipids corresponding to *Leishmania* GPIs, which unambiguously demonstrate that signaling inhibitory activities of GPIs reside in their unusual lipid domains. Our ongoing efforts focus on the chemical synthesis of full-length alkylacyl- and diacyl-GPIs of *Leishmania* and *P. falciparum*, respectively, to address the issue of the opposite biological activities.

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