This information is current as of April 14, 2017.

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J Immunol 1998; 160:1894-1900; ;
http://www.jimmunol.org/content/160/4/1894

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RANTES Activation of Phospholipase D in Jurkat T Cells: Requirement of GTP-Binding Proteins ARF and RhoA

Kevin B. Bacon, Thomas J. Schall, and Daniel J. Dairaghi

The chemokine RANTES is a potent agonist of T cell activation. In an investigation of signal-transduction events activated by this chemokine, we have shown that RANTES stimulates dose-dependent phospholipase D (PLD) activity in Jurkat cells. Equilibrium-binding analyses using \(^{125}\)I-labeled RANTES indicated the presence of a receptor for RANTES on these cells, which has a \(K_d\) of 0.1 nM, is expressed at approximately 600 sites per cell, and a binding specificity that was not comparable with that of any of the known chemokine receptors, since \(^{125}\)I-labeled RANTES was displaced by macrophage-inflammatory protein-1\(\beta\) (but not macrophage-inflammatory protein-1\(\alpha\)), monocyte-chemotactic protein-1 (MCP-1), MCP-3, MCP-4, and eotaxin. RANTES-induced PLD activation was augmented by GTP\(\gamma\)S, but not GDP\(\beta\)S, and inhibited by the protein kinase C inhibitor bisindolylmaleimide, as well as the fungal metabolite brefeldin A, and C3 exoenzyme (Clostridium botulinum), implicating the activation of RhoA. RANTES also induced GTP-GDP exchange of immunoprecipitated RhoA. RANTES-stimulated PLD activity was dependent on an ADP-ribosylation factor(s), as assessed by inhibition studies using a synthetic inhibitory peptide of the N-terminal 16 amino acids of ADP-ribosylation factor 1. These studies indicate the potential existence of a novel receptor-mediated mechanism for activation of T cells by the chemokine RANTES. The Journal of Immunology, 1998, 160: 1894–1900.

Chemokines are a superfAMILY of small molecular mass proteins, related in primary structure by the conservation of a 4-cysteine motif (1–3). The superfamily is subdivided into four subfamilies, based on whether the second and fourth cysteines are missing (C family), or whether the first and second cysteines are separated by one or more intervening amino acids (C-C, C-X-C, and C-X\(\alpha\)-C-X\(\alpha\)) (1–4). Biologically, the chemokines are potent activators of leukocyte migration and activation. Lymphotactin, the prototype of the C class, is primarily an activator of T and NK cell migration, as well as having some anti-tumor activity (5–7). Members of the C-C class display overlapping, yet distinct activities on leukocytes other than neutrophils, while C-X-C family members primarily activate neutrophils and T lymphocytes. Despite the wealth of knowledge concerning neutrophil activation by C-X-C members such as IL-8, there has been little advance in the elucidation of signal-transduction pathways stimulated by the C and C-C family members. To date, four receptors have been cloned that bind the C-X-C family members, CXC-1, binding IL-8 with high affinity; CXC-2, binding IP-10 and monokine induced by IFN-\(\gamma\); and CXC-4/fusin/LESTR, binding SDF-1\(\alpha\) (2, 8–10). Receptors binding members of the C-C family include CCR-1 (affinity hierarchy: MIP-1\(\alpha\), RANTES, CCR-2 (MCP-1 through 5), CCR-3 (eotaxin, RANTES, MCP-2, 3, 4), CCR-4 (TARC) and CCR-5 (MIP-1\(\beta\), RANTES, MIP-1\(\alpha\)) (11–14). While certain virally encoded receptors may also bind a number of these chemokines (1–3, 11), a receptor for lymphotactin remains to be identified.

We have demonstrated recently that the chemokine RANTES is capable of stimulating T lymphocyte activation via direct receptor-mediated activation of dual signal-transduction pathways involving pertussis toxin-sensitive heterotrimeric G proteins and tyrosine kinase enzymes (15). Initially, RANTES stimulates an immediate transient of calcium mobilization, followed by a more sustained flux through specific membrane channels. Concurrently, there is rapid tyrosine phosphorylation of intracellular proteins, including pp125\(^{FAK}\), paxillin, and ZAP-70, but not TCR-\(\zeta\) (16). One manifestation of these signals is the secretion of IL-2 and the up-regulation of numerous cell surface adhesion receptors (15, 17). The Jurkat T cell line, however, does not respond to RANTES activation with a rapid \(Ca^{2+}\) transient, nor does it migrate in response to this chemokine (unpublished), making it a model system to use in investigations of other RANTES-mediated signal-transduction events.

In our prior investigations of chemokine-induced signal transduction, we had demonstrated the potent induction of phospholipase D (PLD) activity by the C-X-C chemokine IL-8 in human T lymphocytes and T cell clones (18). PLD activation is now recognized as an important component of numerous cellular functions, including vesicular trafficking and cell transformation (19–21). While the existence of multiple isoforms of PLD has been postulated, the identification and cloning of the PLD enzyme in mammalian cells have only recently been described (22, 23). Membrane-associated PLD catalyzes the hydrolysis of phosphatidylcholine and is dependent on calcium, phosphoinositol bisphosphate, and the GTP-binding proteins ARF (ADP-ribosylation factors) and RhoA (21, 24, 25). Other nonmammalian isoforms of PLD have also been shown to preferentially hydrolyze phosphatidylcholine and phosphatidylethanolamine (19–21, 26). Most recently, Mayr et al. have demonstrated a novel phosphatidylethanolamine and phosphatidylserine-specific PLD in yeast that exhibits absolute dependence on calcium (27). While hydrolyzing phosphatidylcholine to phosphatic acid (PA) and choline is known to...
be a direct consequence of numerous ligand-receptor interactions, the exact role of PA is as yet unclear. Of major importance is the finding that PLD is a vital component of the Golgi membrane and absolutely required in vesicular transport. Indeed the major function of the family of small GTP-binding proteins, the ARFs, is the activation of PLD (28–30). It is suggested that the generation of negatively charged lipid metabolites, including PA, is necessary for the docking of coatamer proteins to the lipid membrane in vesicle formation (31). These findings suggest a vital role for PLD activation in intracellular protein transport and vesicle formation.

In this study, we report on the activation of PLD in Jurkat cells by the C-C chemokine RANTES. We show that RANTES stimulates a potent and specific PLD activation that is dependent on prior activation of PKC. Additionally, the requirement for GTP, the reduction in PLD activation by the fungal metabolite Brefeldin A (BFA), and an inhibitory N-terminal peptide of mammalian ARF-1 indicate that the chemokine-induced activation of PLD in Jurkat is dependent on one or more ARFs. Evidence inferring a role for the activation of the monomeric GTP-binding protein RhoA is detailed from experiments using the C3 exoenzyme (Clostridium botulinum) as well as GDP/GTP exchange assays with immunoprecipitated RhoA. The activation of ARF- and Rho-regulated PLD suggests a potentially important role for RANTES not only as a proinflammatory stimulus, but also in the general physiology of T lymphocytes.

Materials and Methods

Materials

RPMI 1640, phosphate-free RPMI (11877-032), DEMEM, dialyzed FCS, and BFA (Pencellium brefeldiannum) were obtained from Life Technologies (Grand Island, NY), and gentamicin, GTP, and GDP were purchased from Sigma Chemical Co. (St. Louis, MO). GTP-γS and GTPβS were from Boehringer Mannheim Corp. (Indianapolis, IN); bisindolylmaleimide (GF109203X), Staphylococcus aureus α-toxin, and C3 exoenzyme (C. botulinum) were from Calbiochem (San Diego, CA). [3H]oleic acid and [32P]orthophosphate (10 mCi/ml, 37 MBq/ml) was from Amersham (Arlington Heights, IL), and PEI cellulture plates were from Selecto Scientific (Norscor, GA). RhoA mAb (IgGl) was from Santa Cruz Biotechnology (Santa Cruz, CA), and the PLD inhibitors SCH49209 and SCH50672 (32) were kindly provided by Drs. R. Bryant and K. Fu (Scherer Plough Research Institute, Ken- linworth, NJ). Inhibitory ARF peptide (2–17 ARF1p; GNIFANLFKGLF GKKE) (33), a similar peptide, but with scrambled peptide sequence (FLKEKGKFALFGNKGN), and an N-terminal peptide of Gso (GCLGNK TEDQRNNEK) were synthesized by Research Genetics (Huntsville, AL). The chemokines RANTES, MIP-1β, MIP-1α, MCP-1, and MCP-3 were purchased from PeproTech (Rocky Hill, NJ). All other reagents were obtained through standard suppliers.

PLD activity

The cell line Jurkat was maintained in RPMI 1640 containing 10% heat-inactivated FCS and gentamicin (50 μg/ml). Activation and analysis of PLD were performed as previously described (18). In brief, cells in DMEM containing dialyzed FCS were incubated overnight (37°C) with 50 nCi/ml of [3H]oleic acid. Labeled cells were harvested, washed twice, and resuspended in DMEM containing 130 mM ethanol and left to equilibrate for 15 min. Following equilibration, cells (5 × 10⁷) were aliquoted into siliconized glass tubes containing agonists and/or antagonist and incubated for 40 min at 37°C with periodic shaking. After incubation, cells were rapidly pelleted and the medium was removed. Pellets were lysed with methanol: HCl (100:1, v/v); chloroform:0.25 M HCl (1:1.1, v/v) and vortexed for 10 to 15 s to disrupt the cell pellet. Addition of 100 μl 1, 2-dioleoyl-sn-glycero-3-phosphoethanol as a tracer was followed by centrifugation to enable lipid-phase extraction. The dried phospholipids were resuspended in 20 μl of chloroform, and the reaction products were separated by TLC on silica gel 60 plates (Merck, Rahway, NJ) using a mobile phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (12:2.3:3:10, v/v). The radiolabeled phosphatidylethanol was located by staining the plate with iodine vapour (using tracer). The product was scraped from the plate, and radioactivity was assessed by liquid scintillation counting using Cytoscin (ICN Pharm.
this length of time. RANTES-induced PLD activity was observed following stimulation over the concentration range of 0.03 to 3 nM RANTES, maximal activation being between two- and fourfold above resting (unstimulated) controls (Fig. 1, A and B). Concentrations greater than 1 nM RANTES failed to give consistently higher values of [3H]PtE. PMA, as expected, induced a large increase in [3H]PtE (14,017 ± 2,317 cpm in unpermeabilized cells; 12,592 ± 2,006 cpm increase in permeabilized cells); however, there was little, if any, difference between cells from the two experimental conditions.

GTPγS augments RANTES-induced PLD activation

There was little, if any, difference between nonpermeabilized and permeabilized cells (Fig. 1, A and B), which was to be expected due to the mild nature of the permeabilization protocol and the rapid reversal of permeabilization in the presence of free Ca2+. PLD has been shown to be dependent on the activation of cofactors, including PIP2, ARFs, and the GTP-binding protein Rho. In the presence of increasing concentrations of the nonhydrolyzable analogue of GTP, GTPγS, there was a concomitant increase in the [3H]PtE product formed following RANTES stimulation (Fig. 1C). Specificity of this response was determined by the lack of increase in [3H]PtE when cells were incubated in the presence of GDPβS. Interestingly, the PLD activity was decreased in the presence of this analogue.

RANTES dissociable binding to Jurkat cells

We used equilibrium binding to assess the presence and affinity of a putative chemokine receptor for RANTES on these cells. Preliminary experiments indicated that we could see dissociable RANTES binding using 125I-RANTES and unlabeled chemokine competitors (A, RANTES, MIP-1α, and MIP-1β; B, MCP-1, -3, -4, and eotaxin). The displacement profile and Scatchard plots are shown. These plots are representative of three independent experiments.

**FIGURE 1.** RANTES-induced PLD activation in Jurkat cells. A, RANTES was tested over a concentration range of 0.03 to 3 nM on unpermeabilized cells for 40 min. PMA was used as a positive control. Results represent mean ± SEM cpm of [3H]PtE from n = 6 experiments performed in duplicate. B, RANTES induced increase in [3H]PtE in permeabilized cells over the same concentration range. Cells were permeabilized in calcium-free glutamate buffer using S. aureus α-toxin for 20 min, as outlined in Materials and Methods, washed, then resuspended in buffer containing 0.1 μM free calcium for assay. Results represent mean ± SEM increase in [3H]PtE (cpm) over background from n = 6 experiments performed in duplicate. C, Effect of guanine nucleotides on RANTES-induced PLD activity. GTPγS or GDPβS, from 0.1 to 10 μM, were added simultaneously with 1 nM RANTES to permeabilized cells. Results represent mean ± SEM cpm for [3H]PtE from n = 3 experiments performed in duplicate.

**FIGURE 2.** Expression of RANTES-binding receptors on Jurkat cells. Equilibrium-binding analyses using 125I-RANTES and unlabeled chemokine competitors (A, RANTES, MIP-1α, and MIP-1β; B, MCP-1, -3, -4, and eotaxin). The displacement profile and Scatchard plots are shown. These plots are representative of three independent experiments.
increased the maximal [3H]PtE recovered in the presence of each concentration (Fig. 3). Although addition of 10 nM 50% inhibition occurring with 1 nM RANTES increased the maximal [3H]PtE recovered in the presence of each concentration of GF109203X, the maximal PLD activation was never greater than 75% of control, and there was still almost complete inhibition in the presence of 100 nM inhibitor (not shown).

RANTES-induced PLD activation is PKC dependent

The activation of PLD by the chemokine IL-8 was shown to be PKC dependent (18). To assess whether RANTES (a C-C chemokine) stimulates PLD through a common mechanism, we analyzed the PKC dependence using the specific, cell-permeant PKC inhibitor bisindolylmaleimide (GF109203X; 50% inhibition occurring with 1 nM RANTES, IC50 = 10 nM). Increases in [3H]PtE induced by 1 nM RANTES (maximal agonist concentration) were reduced to basal levels in a dose-dependent manner by the PKC inhibitor, with >50% inhibition occurring with 1 nM concentration (Fig. 3). Although addition of 10−8 M RANTES increased the maximal [3H]PtE recovered in the presence of each concentration of GF109203X, the maximal PLD activation was never greater than 75% of control, and there was still almost complete inhibition in the presence of 100 nM inhibitor (not shown).

BFA inhibits RANTES-induced PLD activation

Using 1 μg/ml of the inhibitor BFA as an initial indication for the role of small GTP-binding proteins in the activation of RANTES-induced PLD activity resulted in complete inhibition of the [3H]PtE levels (Fig. 4A). There was a slight increase in the basal levels of PLD activity in control (unstimulated) samples in the presence of 1 μM BFA, possibly as a result of the structural disintegration of the Golgi apparatus and other organelles in the cytoplasm by BFA (39, 40). Inhibition of [3H]PtE accumulation occurred with concentrations of BFA as low as 30 nM, with maximal inhibition occurring in the presence of 1 μM BFA.

N-terminal ARF peptide inhibits RANTES-induced PLD activity

The activity of BFA and GTPγS, in the inhibition and activation of PLD, respectively, was consistent with a role for the ARF family of small GTP-binding proteins (28–30). To assess the contribution of ARFs to the PLD activation stimulated by RANTES, an inhibitory peptide of human ARF1 (IC50 = 75 μM in ARF-dependent assay of ADP ribosylation of Gsα) was used in subsequent assays (33). Nonmyristylated ARF N-terminal peptide, but not the same peptide with scrambled sequence, was able to inhibit the RANTES-induced PLD activity dose dependently at concentrations from 1 to 100 μM (Fig. 4B). In contrast, a control peptide corresponding to the N terminus of Gsα was ineffective in inhibiting RANTES-induced PLD (not shown).

RhoA activation mediates the RANTES-induced PLD response

Since Rho has been shown to be a necessary cofactor for PLD activity, we analyzed the effect of C3 exoenzyme (C. botulinum), a specific inhibitor of Rho (41, 42), on PLD activation induced by 1 nM RANTES. Figure 4C demonstrates that preincubation of cells with C3 exoenzyme dose dependently inhibited the RANTES-induced PLD activation. To further characterize the role of Rho, GDP/GTP exchange on immunoprecipitated Rho was investigated. Figure 4D demonstrates that there is a basal level of [32P]GTP present in the immunoprecipitates from unstimulated cells. This is most likely a reflection of the intrinsic activation of this immortalized cell line. RANTES stimulates rapid exchange of GDP for GTP on RhoA, with significant elevation in [32P]GTP as early as 30 s. The increase in GTP continued to increase up to a maximum at 5 min in this assay system. In addition, this increase was seen with concentrations of 10−8 M, the concentration most effective in stimulating PLD activation. The loading of equal amounts of RhoA in the samples was indicated by Western blot analysis of eluted immune complexed Rho from the samples, as described in Materials and Methods.

SCH49209 and SCH56072 inhibit PLD activity in Jurkat

In a final analysis, the specificity of the PLD activity was determined by the use of PLD inhibitors SCH49209 and SCH56072. Reported as inhibitors of receptor-stimulated PLD and PLA2 (IC50 = 2 μM for FMLP-stimulated PLD activity in HL-60 cells) (32), these compounds were analyzed over the concentration range (0.1 nM–3 μM). Figure 5 shows that there was a potent dose-dependent inhibition of PLD activity stimulated by 1 nM RANTES. Half-maximal inhibition occurred using 1 μM of compound, and there was no significant difference between the analogues.

Discussion

We have demonstrated previously the potent activation of PLD by the chemokine IL-8 in T lymphocytes (18). We have now extended our findings to include the C-C chemokine RANTES in demonstrating that in the Jurkat T cell line, the activation of this enzyme occurs at subnanomolar concentrations and is dependent on the activation of small GTP-binding protein cofactors. RANTES-induced PLD activation is consistently maximal at 1 nM, a concentration corresponding to the optimal chemotaxis-inducing dose in normal T lymphocytes. Interestingly, PLD activation in T lymphocytes and Jurkat T cells appears to be an important biologic consequence of chemokine action and more readily measurable (at nanomolar concentrations) than readouts of receptor activation such as calcium flux. It was also apparent that RANTES is the only chemokine tested to date (RANTES, MCP-1, MCP-1, MCP-3, lymphotacticin) that induces as robust a response as seen in this study, although the others listed were capable of low levels of activity (not shown).

Binding analyses have revealed a receptor for RANTES, expressed at a low level on these Jurkat cells. Heterologous competition analyses demonstrated inhibitory activity in the order of potency of RANTES > MCP-1β = MCP-3 > MCP-4 > eotaxin > MCP-1, indicating a unique specificity in ligand binding. Additionally, the buffer conditions utilized in these experiments are inconsistent with binding to known receptors. In addition, only CCR-4 has been shown to be present on Jurkat, while CCR-1, 2, 3, and 5 have not been detected (43). It is feasible that there is
more than one receptor, although the complete displacement obtained with each ligand in isolation points away from this hypothesis. Alternatively, Jurkat cells may express cell surface moieties that modify the binding characteristics (hence, the experimental procedures required to observe them) of the known receptors. Finally, it is not inconceivable that there is a novel, previously unrecognized CCR, which may explain this unique binding pattern and the relative specificity of RANTES over other chemokines in eliciting this signal-transduction pathway in Jurkat cells.

We have demonstrated that activation of PLD in Jurkat conforms to the standard requirements for the activity of the cofactors ARF and RhoA. Use of the fungal metabolite BFA as an inhibitor clearly indicated a role for intact Golgi and ARF proteins. The mode of activation of BFA is thought to be through the destruction of vesicular and Golgi membranes (39, 40), in addition to inhibition of ARF-guanine nucleotide exchange factor activity on ARFs (44). Our studies have demonstrated that BFA can completely inhibit the RANTES-induced PLD activation. Exactly which ARF family members are activated and/or the most important cofactors awaits further characterization in this system. Use of the N-terminal peptide of ARF1 does, however, implicate this family member in the activation of Jurkat PLD. In addition, the potency of the ARF peptide in these experiments is similar to that reported (33), indicating the utility of this inhibitory peptide in determining ARF dependency. Further information concerning the identity of ARF family members in Jurkat may help to determine the specificity of the mechanisms involved in RANTES-mediated PLD activation.

RhoA has been shown to be necessary for PLD activation; however, there have been reports that Rac family members may also be important as cofactors (23, 45). In our investigations of RANTES-induced activation of cytoskeletal components (19), we consistently immunoprecipitated RhoA and Rac with activated pp125FAK, suggesting activation of these small GTP-binding proteins. Our preliminary evidence from the assays reported in this study suggests that Rac is not activated (in terms of GDP/GTP exchange; not shown). In addition, Rac is a poor substrate for C3

![FIGURE 4. Inhibition of RANTES-induced PLD activity by A, BFA; B, N-terminal ARF1 peptide and scrambled control; and C, C3 exoenzyme. BFA was added to unpermeabilized cells for 20 min, then washed from the cells before addition of RANTES (1 nM). ARF1 peptide and C3 exoenzyme were added to permeabilized cells for 20 min, then washed from the cells before addition of calcium-containing buffer (to reseal cells) and RANTES (1 nM). Each histogram represents mean ± SEM cpm for [3H]PE from n = 4 (BFA), n = 6 (peptides), and n = 3 (C3 exoenzyme). D, Activation of RhoA GDP/GTP exchange by RANTES. Cells were loaded overnight with [32P]orthophosphate according to Materials and Methods. Following stimulation for the indicated times, RhoA was immunoprecipitated with specific mAb and nucleotides resolved on PEI cellulose TLC plates. The positions of GDP and GTP were visualized under UV light and are indicated. The equal loading of Rho onto the gels is indicated by Western blot analysis using the same immunoprecipitating Ab. These blots are representative of n = 4 individual experiments.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/Downloadedfrom)
Since PLA2 has been shown to stimulate Rho activation in bradykinin-stimulated cell lines (53), but not PLD activity due to tyrosine kinase activation (32), it is interesting to speculate that these inhibitors are functioning upstream of the phospholipases themselves, possibly at the GTP-binding proteins or their guanine nucleotide exchange factors.

Perhaps of greater relevance, when one considers the activation of ARFs and Rho, is the control of vesicular trafficking. Little is known to date of the receptor fate following agonist (chemokine) ligation. It is not improbable that ligand-mediated receptor endocytosis of chemokine receptors is linked somehow to the efficient assembly of coatamer proteins. Efficient coatamer binding to membranes has been linked to the presence of high concentrations of negatively charged lipids in the membrane bilayer. The highly negatively charged PA has been shown to provide such an environment (31). Indeed, constitutive PLD activity in certain membrane preparations has been shown to be sufficient to allow vesicle formation in the absence of ARF activation (54). Furthermore, the ARF family of proteins has been implicated in the retrograde transport of proteins between Golgi and endoplasmic reticulum (44), making this RANTES-induced activation of PLD a likely candidate mechanism for receptor down-regulation. Interestingly, numerous chemokines of the C-C family (MIP-1α, MIP-1β, MCP-1, MCP-3) and lymphotactin of the C family are capable of inducing PLD activation in normal peripheral blood T and B lymphocytes (K.B.B., unpublished). It is not unlikely, therefore, that chemokine-induced PLD activation may be responsible for receptor down-regulation as a common mechanism.

In conclusion, we have demonstrated the potent and specific activation of PLD by a C-C chemokine, RANTES, in a model T cell system, the Jurkat T cell line. While the receptor(s) mediating this effect awaits characterization, it is clear that activation of this signal-transduction pathway, either in physiologic or inflammatory contexts, by RANTES, will have profound effects on the regulation of cell activity. Additionally, activation of this pathway in vivo may have important implications in chronic inflammation.

Acknowledgments

I am extremely grateful for advice from Drs. Richard Kahn (National Institutes of Health), K. M. Coggeshall (Ohio State University), and R. Bryant and J. K. Pai (Schering-Plough Research Institute). DNAX Research Institute is funded by Schering-Plough Corporation.

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