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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 125I-labeled RANTES indicated the presence of a receptor for RANTES on these cells, which has a KD 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 125I-labeled RANTES was displaced by macrophage-inflammatory protein-1β (but not macrophage-inflammatory protein-1α), monocyte-chemotactic protein-1 (MCP-1), MCP-3, MCP-4, and eotaxin. RANTES-induced PLD activation was augmented by GTPγS, but not GDPβS, and inhibited by the protein kinase C inhibitor bisindoylmaleimide, 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.


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2 Abbreviations used in this paper: MIP, macrophage-inflammatory protein; ARF, adenosine diphosphate-ribosylation factor; BFA, Brefeldin A; 125I-RANTES, 125I-labeled RANTES, MCP, monocyte-chemotactic protein, PA, phosphatidic acid, PEI, polyethylenimine; PKC, protein kinase C; PLA, phospholipase A; PLD, phospholipase D; PEI, phosphatidylethanol.

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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), DMEM, dialyzed FCS, and BFA (Pencillium brefeldiun) were obtained from Life Technologies (Grand Island, NY), and gentamicin, GTP, and GDP were purchased from Sigma Chemical Co. (St. Louis, MO). GTP·S and GTP·BS were from Boehringer Mannheim Corp. (Indianapolis, IN); bisindolylmaleimide (GF109203X), Staphylococcus aureus a-toxin, and C3 exoenzyme (C. botulinum) were from Calbiochem (San Diego, CA). [3H]oleic acid and [32P]orthophosphate (10 mCi/ml, 370 MBq/ml) was from Amersham (Arlington Heights, IL); and PEI cellulose plates were from Selecto Scientific (Norcross, GA). RhoA mAb (IgG1) was from Santa Cruz Biotechnology (Santa Cruz, CA), and [3H]oleic acid and [32P]RANTES were from DuPont NEN (Boston, MA); and [3 H]oleic acid and 125 I-galactose-3-phosphoethanol as a tracer was followed by centrifugation to enable lipid-phase extraction. The dried phospholipids were resuspended in 20 μl of phosphate-buffered saline (PBS), and nucleotides were added to permeabilized cells at the same time as RANTES. In contrast, permeabilized cells were preincubated for 20 min with ARF peptide, C3 exoenzyme, or PLD inhibitors before RANTES stimulation. Again, cell viability, as assessed using trypan blue exclusion, was consistently greater than 98%.

Chemokine-binding assay

Equilibrium binding of 125I-RANTES using a filtration protocol was employed. Briefly, 1 × 10^5 cells were incubated with 0.1 nM 125I-RANTES in the presence of various unlabelled chemokines in the following buffer for 3 h at 22°C: 25 mM HEPES, 80 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, and 0.5% BSA, adjusted to pH 7.4. The reactions were then aspirated onto PEI-pretreated GF/C filters (Packard, Meriden, CT) using a 96-well cell harvester (Packard). The reactions were washed twice with the following buffer: 25 mM HEPES, 500 mM NaCl, 1 mM CaCl2, and 5 mM MgCl2, adjusted to pH 7.4. After the filters dried, 30 μl of scintillant (Scintiscan (ICN Pharmaceuticals, Costa Mesa, CA). In experiments using BFA or PKC inhibitor (GF109203X), cells were preincubated with increasing concentrations of inhibitor for 20 min before assay. Cell viability, as assessed using trypan blue exclusion, was always greater than 98%.

Permeabilization of cells was conducted in glutamate buffer (120 mM potassium glutamate, 20 mM potassium acetate, 3 mM magnesium chloride, 20 mM sodium HEPES, pH 7.4, 1 mM EGTA, and 1 mg/ml BSA) using a-toxin (34, 35). Cells were pelleted and resuspended in 20 ml glutamate buffer containing 250 U/ml a-toxin and incubated (37°C) for 20 min with periodic shaking. Permeabilization was assessed using trypan blue. Pelleted cells were then resuspended in glutamate buffer containing 0.1 μM free calcium and 130 mM ethanol and added to siliconized glass tubes for assay. In assays incorporating GDP·S or GTP·BS, nucleotides were added to permeabilized cells at the same time as RANTES. In contrast, permeabilized cells were preincubated for 20 min with ARF peptide, C3 exoenzyme, or PLD inhibitors before RANTES stimulation. Again, cell viability, as assessed using trypan blue exclusion, was consistently greater than 98%.

Assays of RhoA GDP/GTP exchange

To assess the activity of the small GTP-binding protein RhoA in the RANTES-induced PLD activation, assays of GDP/GTP exchange were performed (36). Jurkat T cells were resuspended at 5 × 10^5/ml in phosphate-free RPMI 1640 (10% dialyzed FCS and incubated overnight with 0.5 mM of [32P]hydrophosphatide. Cells were pelleted and resuspended in 2 × 10^5/ml in assay buffer (PBS, 1 mM CaCl2, 1 mM MgCl2, and 1 mg/ml BSA). Samples (0.5-ml) were stimulated with RANTES for the indicated times, and cell supernatants were rapidly centrifuged at 4°C. Cells were lysed (50 mM HEPES buffer, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl2, 1 mg/ml BSA, containing protease and phosphatase inhibitors; 1 mM PMSF, 10 μg/ml aprotinin and leupeptin, 1 mM orthovanadate, 1 mM EGTA, 100 μM β-glycerophosphate, 10 mM sodium fluoride, 1 mM tetradsodium pyrophosphate). Lysates containing 100 μg of protein (as assessed using the bichinchoninic acid reagent (Pierce, Rockford, IL) were incubated with 2 μg of anti-RhoA mAb (4°C, 1 h). Immune complexed Rho was captured using goat anti-mouse IgG-agarose (Sigma Assay). In assays incorporating GDP·S or GTP·BS, nucleotides were added to the following buffer (10 min): 25 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, and 0.05% SDS, and nucleotides were eluted with 5 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, and 0.5 mM GTP for 20 min at 68°C. Eluted nucleotides were separated on PEI cellulose plates run in 1.2 M ammonium formate/0.8 M HCl and visualized by autoradiography. Following removal of eluate, Laemmli sample buffer was added to the immunoprecipitates. After boiling for 5 min and electrophoresis on 16% Tris-glycine gels (NOVEX, San Diego, CA), the equivalence of immunoprecipitates was assured using trypan blue exclusion.

Results

RANTES stimulates PLD activation in Jurkat cells

RANTES stimulation of Jurkat cells fails to induce a rapid initial transient of Ca^{2+} (not shown), unlike classical chemokine receptor-mediated signaling described for RANTES in other T cell systems (15, 37). RANTES stimulation of Jurkat cells did, however, result in a dose-dependent increase in [3H]Pi (Fig. 1A), an indicator of PLD activation (38). Initial experiments to determine the time course of activation of PLD indicated that maximal cumulative titration of tritiated product (PiE) occurred after 40 min (Ref. 18 and not shown); thus, all additional experiments were performed for
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

PKC inhibitor, with tion) were reduced to basal levels in a dose-dependent manner by [3H]PTE induced by 1 nM RANTES (maximal agonist concentration, was no significant difference between the analogues. These compounds were analyzed over the concentration range (0.1 nM-3 M) was used in subsequent assays (43). Nonmyristylated ARF N-terminal peptide, but not the same sequence 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, MIP-1α, MCP-1, MCP-3, lymphotactin) that induces as robust a response as seen 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, MIP-1α, MIP-1β, MCP-1, MCP-3, lymphotactin) that induces as robust a response as seen in this study, although the others listed were capable of low levels of activity (not shown).

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, MIP-1α, MIP-1β, MCP-1, MCP-3, lymphotactin) 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 > MIP-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 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 10−6 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.

SCH42909 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 SCH42909 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.

![Graph](http://www.jimmunol.org/)
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]PJE 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.
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.

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References
RANTES-MEDIATED PLD ACTIVATION IN JURKAT T CELLS


