Synthetic Bryostatin Analogues Activate the RasGRP1 Signaling Pathway

James C. Stone,*,† Stacey L. Stang,† Yong Zheng,† Nancy A. Dower,‡ Stacey E. Brenner,§ Jeremy L. Baryza,§ and Paul A. Wender§

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, Department of Pediatrics, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, and Department of Chemistry, Stanford University, Stanford, California 94305

Received June 22, 2004

The functional properties of four diacylglycerol (DAG) analogues were compared using cell-signaling assays based on the protein RasGRP1, a DAG-regulated Ras activator. Compounds **1** and **2**, synthetic analogues of bryostatin 1, were compared to authentic bryostatin 1 and phorbol 12-myristate-13-acetate (PMA). The two "bryologues" were able to activate RasGRP1 signaling rapidly in cultured cells and isolated mouse thymocytes. They elicited expression of the T cell activation marker CD69 in human T cells. DAG analogues promptly recruited RasGRP1 to cell membranes, but they did not induce RasGRP1 proteolysis. Bryostatin 1 and compounds **1** and **2** appeared to be less potent than PMA at inducing aggregation of mouse thymocytes, a PKC-dependent, RasGRP1-independent response. In addition to sharing potential anticancer properties with bryostatin 1, compounds **1** and **2** might be clinically useful as modulators of the immune system.

The bryostatins are promising anticancer compounds isolated from the bryozoan *Bugula neritina*.[1] They were isolated on the basis of their potent growth inhibitory properties when tested in cultured cancer cells and were subsequently shown to bind and activate protein kinase C (PKC).[2] Although the bryostatins and the tumor promoting phorbol esters bind competitively to the diacylglycerol-binding sites within the C1 domain of PKC, the bryostatins lack the tumor-promoting properties of the phorbol esters.[3] The bryostatins are structurally complex macrocyclic lactones, and their availability both from natural sources and from chemical synthesis is severely limited. Recently, we reported the synthesis of two bryostatin analogues, designated as compounds **1** and **2**, that demonstrated comparable or improved in vitro binding to PKC and cell growth inhibitory properties relative to bryostatin 1[4,5] (Figure 1A).

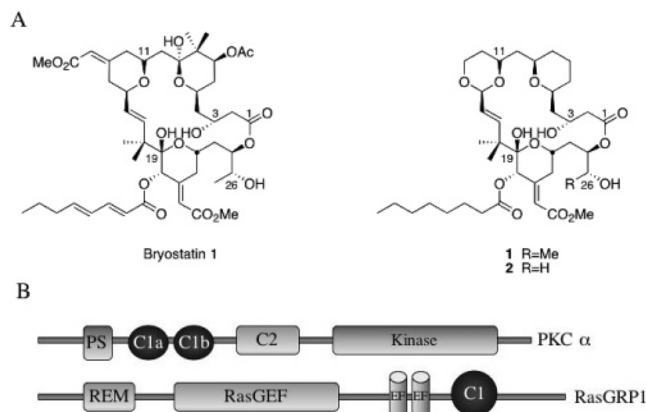

Figure 1. Schematic structures of DAG analogues and representative C1 domain proteins. (A) bryostatin 1, **1**, and **2**. (B) PKC α domains include: PS (pseudosubstrate), C1a and C1b (DAG binding), C2 (phospholipid and calcium binding), and kinase (catalytic). RasGRP1 domains include: REM (Ras-exchange motif), RasGEF (Ras–guanyl-exchange factor), EF (calcium-binding EF hands), and C1 (DAG binding).

In normal cells, receptor-mediated hydrolysis of phosphatidyl inositol bis-phosphate generates diacylglycerol (DAG) and calcium second messengers. Binding of DAG to the C1 domains of conventional (PKC α, β, or γ) or novel (PKC δ or θ) isoforms of PKC is thought to activate these enzymes. Only the former class is sensitive to calcium activation. DAG activation of PKC involves recruitment to cellular membranes as well as allosteric mechanisms.[6] Phosphorylation of diverse downstream targets by PKC and attendant changes in cellular properties then ensues. The process can be attenuated by the activity of DAG-metabolizing enzymes and protein phosphatases. Additionally, after long-term stimulation of cells by DAG analogues, PKC undergoes proteolytic degradation by an ubiquitin-dependent mechanism. It is not clear whether the persistent activation or the subsequent downregulation of PKC isoforms is responsible for the PKC-dependent effects of DAG analogues, and both effects might be required for some of the observed activities.

Over the past few years, several non-PKC classes of proteins that contain DAG-binding C1 domains have been discovered. The properties of these proteins have necessitated a revision of our view of both the normal DAG signaling and the actions of DAG analogues. One such protein, RasGRP1 (Figure 1B), possesses a DAG-binding C1 domain in addition to a catalytic domain that activates Ras at the membrane.[7] Unlike the ubiquitously expressed SOS proteins, RasGRP proteins exhibit very limited tissue expression. RasGRP1 is expressed in the T cell lineage, as well as some brain neurons, and select cells in the kidney and skin.[7–14] Our analysis of RasGRP1 function in the cultured Jurkat T cell line and

* Author to whom correspondence should be addressed. Phone: 1-780-492-7976. Fax: 1-780-492-9556. E-mail: jim.stone@ualberta.ca.
† Department of Biochemistry, University of Alberta.
‡ Department of Pediatrics, University of Alberta.
§ Department of Chemistry, Stanford University.

10.1021/jm0495069 CCC: $27.50 © 2004 American Chemical Society
Published on Web 11/19/2004

in knockout mutant mice revealed that this protein plays a critical role in transducing T cell receptor (TCR) signals.[15,8] Importantly, the C1 domain of RasGRP binds DAG analogues such as phorbol 12,13-dibutyrate and bryostatin 1 in vitro.[16] In the cell, the interaction of the RasGRP1 C1 domain with DAG and DAG analogues recruits this nucleotide-exchange factor to cellular membranes, where it can activate Ras by facilitating conversion of Ras−GDP to Ras−GTP.[7,17,8]

Because RasGRP1 is a likely target for bryostatin 1 in a clinical situation and the bryologues are likely candidates to replace natural bryostatins, we examined the potency of analogues **1** and **2** in RasGRP1 signaling assays.

Materials and Methods

Chemicals. Bryostatin 1 was a gift from Peter Blumberg, National Institutes of Health. Phorbol 12-myristate-13-acetate (PMA) was purchased from Sigma Aldrich (St. Louis, MO). Analogues **1** and **2** were synthesized as described.[4,5] All four DAG analogues were dissolved in dimethyl sulfoxide (DMSO) at 10 μM, and this solvent alone was used as a negative control treatment. The Mek inhibitor U0126 was purchased from Cell Signaling (Beverly, MA) and was used at a final concentration of 10 μM. Bisindolylmaleimide I was purchased from Calbiochem (San Diego, CA) and was used at a concentration of 4.5 μM.

Cells and Gene Transfer. Rat2 fibroblasts and HEK-293 cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum. Rat2 cells were engineered to express the retrovirus vector pBabePuro with or without the human RasGRP1 cDNA, as previously described.[7] The Jurkat T cell line and the mouse T cell hybridoma A1.1 were maintained in Roswell Park Memorial Institute (RPMI) cell culture media containing 10% fetal bovine serum, as previously described.[8] Mouse thymocytes were isolated in RPMI 1640 containing 10% fetal bovine serum, as previously described.[15] Peripheral blood lymphocytes were isolated from the blood of a healthy volunteer using Ficoll−hypaque gradient centrifugation (171440-02 from Amersham Biosciences Inc., Baie d'Urfe, Quebec).

Signaling and Protein Expression Assays. Ras−GTP assays were performed using the Ras binding domain (RBD) pulldown assay, as we have described.[8] Immunoblotting was performed for Ras with the pan-Ras clone 10 antibody from Upstate Biotechnology (Lake Placid, NY), phospho-Erk p44/p42 (9101 from Cell Signaling, Beverly, MA), and RasGRP1 m199 (sc-8430 from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as we have described.[15,18]

Fluorescence-Activated Cell Sorting (FACS) Analysis of Lymphocyte Surface Marker Expression. Jurkat T cells or freshly isolated peripheral blood lymphocytes were incubated overnight in RPMI containing DMSO or 100 nM DAG analogue. After fixation in 2% paraformaldehyde, cells were stained with phycoerythrin (PE)-labeled anti-CD69 (555531), FITC-labeled anti-CD4 (555346), and PE-labeled anti-CD8 (555367), all from BD Biosciences (Mississauga, ON). Fluorescence was detected using Cellquest software, as we have described.[15]

Intracellular Imaging of RasGRP1. HEK-293 cells were plated overnight on glass cover slips and then transfected with 5 μg of pEF-GFP or pEF-GFP-RasGRP1. After 48 h of incubation, cells were treated with DMSO or 100 nM DAG analogue followed by incubation at 37 °C for 10 min. After fixation in 4% paraformaldehyde, cells were mounted in Cytoseal-60 from Richard-Allan Scientific (Kalamazoo, MI). Fluorescent images were taken with a Zeiss 510 inverted laser scanning confocal microscope (LSM; Carl Zeiss, Oberkochen, Germany). A minimum of five 0.45 μm slices were acquired for each cell. Tiff images were processed with Adobe Photoshop 7.0.

Thymocyte Aggregation Assay. Mouse thymocytes at a concentration of 3.5×10^6 cells per mL were incubated at 37 °C in RPMI containing 10% fetal bovine serum and various concentrations of DAG analogue with or without inhibitors. Aggregates were photographed after 8 h of incubation.

Results

Bryologues Activate Ras−Erk Signaling in Mammalian Cells but Do Not Promote RasGRP1 Breakdown. To determine whether bryologues can activate RasGRP1, we studied rat2 cells that were engineered to express the human RasGRP1 cDNA. As controls, we studied cells that expressed the empty vector. Rat2 cells do not exhibit Ras activation in response to DAG analogue treatment, although they do show some activation of Erk. This latter process may reflect a PKC-dependent activation of Raf that depends on low basal Ras−GTP levels.[19] As expected, treatment of empty vector cells resulted in no accumulation of Ras−GTP after treatment with solvent, PMA, bryostatin 1, analogue **1**, or analogue **2**. In contrast, all four DAG analogues induced a rapid increase in Ras−GTP levels and a more robust phosphorylation of Erk in Rat2 cells expressing RasGRP1 (Figure 2A).

To examine the action of DAG analogues in cells that normally express RasGRP1, we studied Jurkat T cells and mouse thymocytes. We previously demonstrated that overexpression of RasGRP1 in Jurkat T cells resulted in enhanced TCR- and PMA-induced Ras−Erk signaling. Such signaling was associated with translocation of RasGRP1 to the membrane fraction.[8] The present studies show that Jurkat T cells respond similarly to each of the DAG analogues at the level of Ras−GTP accumulation and Erk phosphorylation (Figure 2B). A lower level of Ras activation was observed after treatment with OKT3. This antibody recognizes a component of the T cell receptor complex leading to activation of phospholipase C and subsequent DAG production in the plasma membrane.

We previously showed that wild-type mouse thymocytes express RasGRP1 and that PMA and bryostatin 1 induced activation of Ras and Erk. These responses were absent in thymocytes from RasGRP1 null mutant mice.[15] As expected, all four analogues activated Erk in wild-type thymocytes but not in mutant thymocytes (Figure 2C). Each compound was active in wildtype cells at 100 nM, but activity at 10 nM and 1 nM was negligible (Figure 2D). Collectively these studies show that, in addition to PMA and bryostatin 1, the compounds **1** and **2** can activate RasGRP1.

Prolonged treatment of cells with PMA has been reported to result in downregulation of conventional PKC isoforms by proteolysis. In contrast, RasGRP1 was

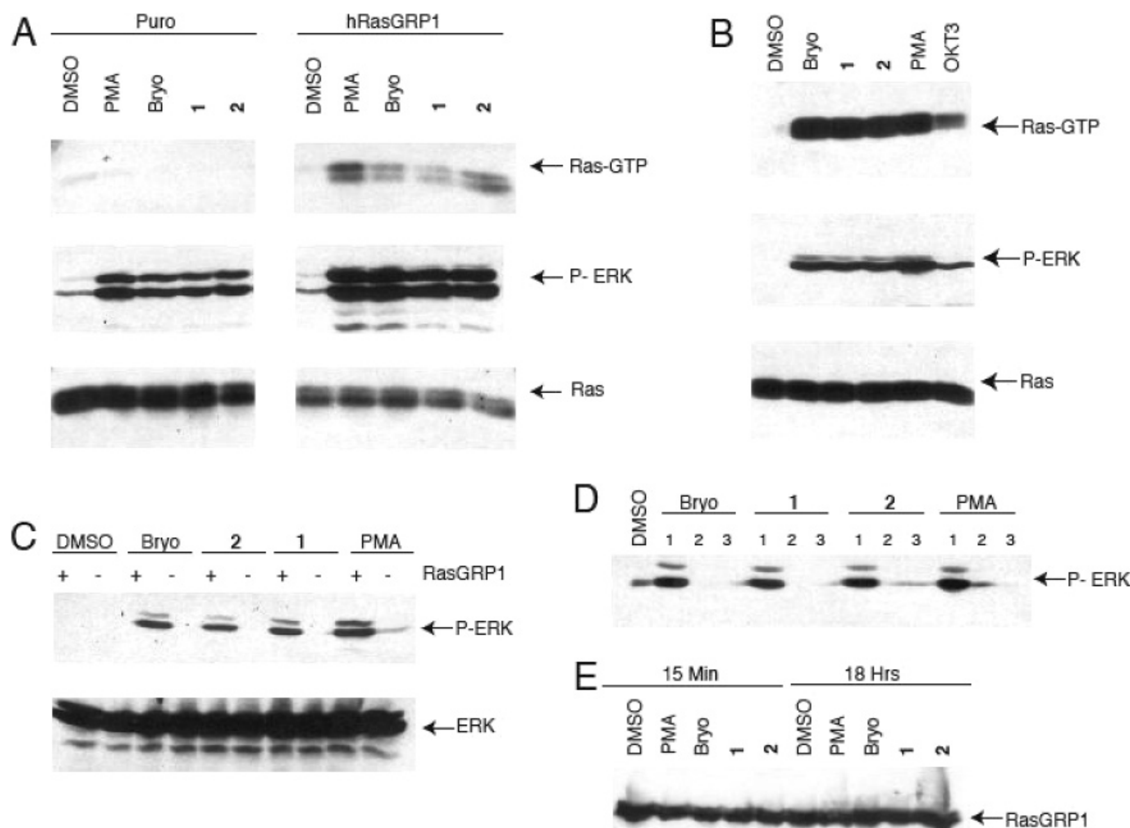

Figure 2. DAG analogues activate RasGRP1 in cultured cells and mouse thymocytes, but do not induce protein downregulation. (A) Rat2 cells expressing the empty pBabePuro vector or human RasGRP1 were treated for 10 min with DMSO or 100 nM DAG analogue and then assayed for Ras-GTP, phospho-Erk, or total Ras. The two forms of phospho-Erk represent the activated versions of the p42 and p44 Erk species. (B) Jurkat T cells were treated and assayed as above. Additionally, one cell sample was treated with the anti-TCR (CD3) antibody OKT3 (10 μg/mL). (C) Mouse thymocytes were isolated from young wild-type (+) or RasGRP1 knockout mutant (−) mice. After treatment with DMSO or DAG analogues for 10 min, cells were lysed and assayed for phospho-Erk. (D) Wild-type mouse thymocytes were treated at various concentrations of DAG analogue for 10 min and then lysed and assayed for phospho-Erk (lanes 1, 2, and 3 represent 100, 10, and 1 nM DAG analogue treatment, respectively). (E) Mouse T cell hybridoma A1.1 was treated with DAG analogues (100 nM) for 15 min or 18 h and then assayed for RasGRP1 expression using the m199 antibody.

shown to be stable under these conditions.[18] Like other T cell lines, the A1.1 T cell hybridoma constitutively expresses RasGRP1. We treated A1.1 cells with DAG analogues for 15 min and for 18 h, followed by examination of RasGRP1 expression levels by immunoblotting. As controls, we treated cells with solvent. RasGRP1 levels were unaffected by prolonged DAG analogue treatment (Figure 2E). These conditions do not appear to induce proteolysis of RasGRP1.

Bryologues Induce Translocation of RasGRP1 to the Plasma Membrane. RasGRP1 is recruited to cell membranes both by DAG analogue treatment and by normal stimulatory mechanisms that generate DAG.[7,17,8,20] To examine the ability of the bryologues to induce translocation of RasGRP1, we expressed a GFP−RasGRP1 construct in HEK-293 cells and observed its localization in response to treatment with DAG analogues. In unstimulated cells, the recombinant protein was distributed throughout the cytoplasm, with no particular concentration on membranous structures. After brief treatment with each of the DAG analogues, fluorescence signal was concentrated at the cell periphery as well as in the perinuclear region (Figure 3). The free GFP protein was distributed throughout the cell, and this distribution was unaffected by DAG treatment (data not shown). Thus, like PMA and authentic bryostatin 1, the bryologues **1** and **2** appear to bind RasGRP1 and facilitate its recruitment to cell membranes.

Bryologues Induce Lymphocytes To Express the Activation Marker CD69. To complement the short-term signaling studies, we examined the effects of DAG analogue treatment on expression of the T cell surface antigen CD69. CD69 is a T cell activation marker that is induced by TCR engagement as well as by treatment of T cells by PMA. This response can be mimicked by introduction of activated forms of either Ras or Raf-1.[21,22] Thus, CD69 induction is a manifestation of prolonged signaling through the Ras−Raf−Mek−Erk kinase cascade. As shown in Figure 4A, overnight treatment of Jurkat T cells with each of the DAG analogues separately induced strong surface expression of CD69.

To confirm this result in more normal cells, peripheral blood lymphocytes (PBL) were prepared and incubated overnight in the presence of each DAG analogue. These PBL (Figure 4B) exhibited normal ratios of $CD4^+$ (T helper lymphocytes), $CD8^+$ (cytotoxic T lymphocytes), and $CD4^-CD8^-$ (non-T lymphocytes). Exposure overnight to each DAG analogue resulted in overt expression

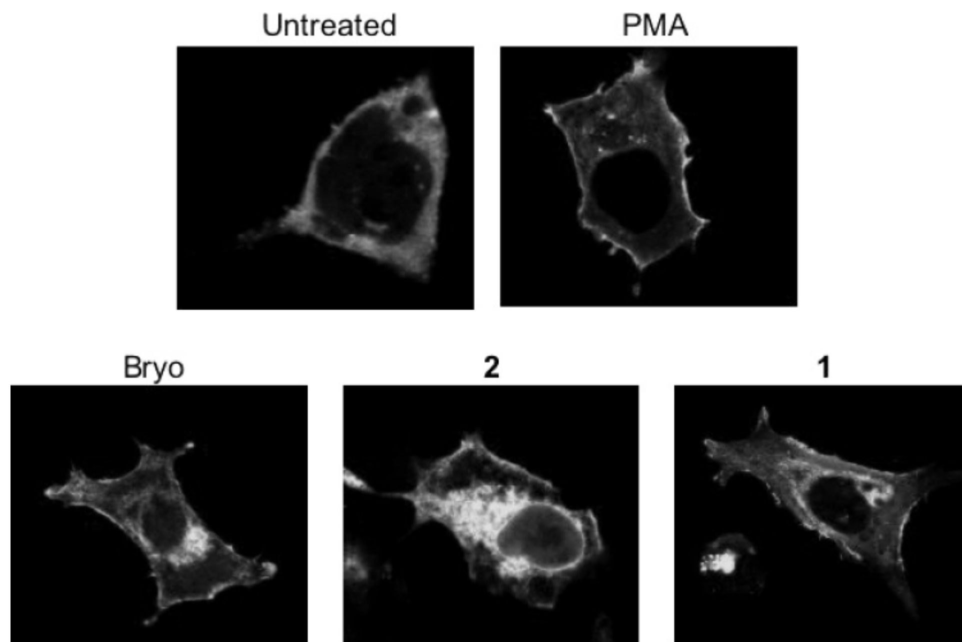

Figure 3. DAG analogues induce similar patterns of RasGRP1 membrane recruitment. HEK-293 cells were transfected with a GFP−RasGRP1 plasmid or a GFP plasmid (not shown). After 48 h, cells were treated with DMSO or DAG analogues at 100 nM for 10 min and then fixed with paraformaldehyde before analysis by confocal microscopy.

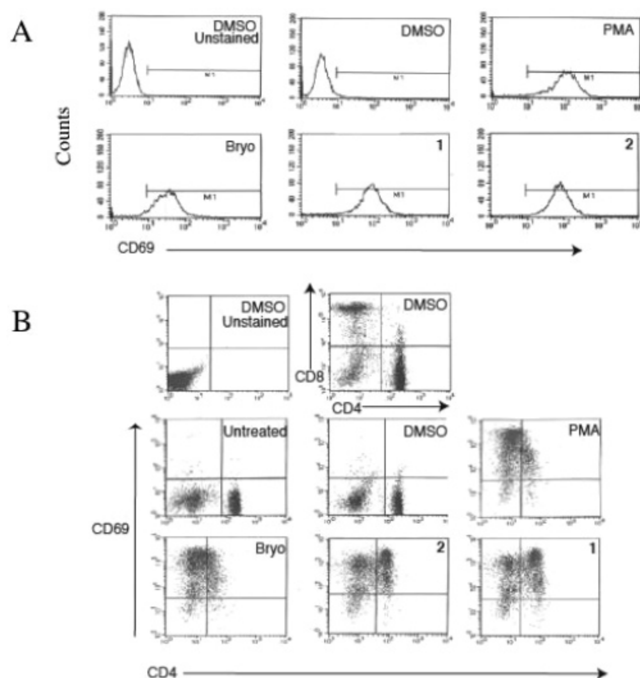

Figure 4. DAG analogue treatment induces expression of the T cell activation marker CD69. (A) Jurkat T cells were treated with DMSO or 100 nM DAG analogue overnight and then fixed in 2% paraformaldehyde and stained with PE-labeled CD69 followed by FACS analysis. (B) Freshly isolated peripheral blood lymphocytes were incubated overnight with various treatments and then stained with CD4 and CD69 (bottom six panels). In the top two panels, unstimulated cells were analyzed either without staining (left) or stained with anti-CD4 and anti-CD8 (right) to demonstrate specificity of our FACS signal and normal PBL profile, respectively.

of surface CD69. This expression involved both the $CD4^+$ population and the $CD4^-$ population. Independent experiments with concanavalin-A activated mouse splenocytes confirmed the induction of CD69 surface expression on both $CD4^+$ and $CD8^+$ lymphocytes by all four DAG analogues (data not shown).

Bryologues Are Less Effective Than PMA at Inducing a PKC-Dependent Pathway That Leads to Thymocyte Aggregation. Thymocytes and lymphocytes form multicellular collections or clumps when they are treated with PMA, and this response is also often seen with more physiological stimuli. Some leukemia cell lines also form aggregates after DAG analogue treatment. The basis for this homotypic aggregation appears to be activation of integrin receptors such as LFA-1 by PKC-induced clustering on the plasma membrane and binding of LFA-1 to intercelluar adhesion molecule (ICAM) proteins on the surface of other cells.[23]

We studied mouse thymocytes to compare the bryologues in an aggregation assay. Because various links between Ras signaling and cell adhesion receptors have been posited, we expected that thymocytes from RasGRP1 null mice would be defective at PMA-induced aggregation. Surprisingly, we found that RasGRP1 deficient thymocytes showed normal PMA-induced aggregation, indicating that a non-RasGRP1 target was involved (data not shown).

When we examined wild-type thymocytes in detail, we found that PMA, bryostatin 1, and the two bryologues all induced thymocyte aggregation at 100 nM, although PMA appeared to be more potent, as judged by the relative size of the aggregates. The PMA response was blocked by Bisindolylmaleimide I, Ro 31-8220, and Gö 6976, three PKC inhibitors but not by U0126, a specific inhibitor of Mek.[24] These results show that thymocyte aggregation is a PKC-mediated response but is independent of RasGRP1 and Mek. Interestingly, when we used lower doses of DAG analogue (25 nM), we were able to distinguish between PMA, which still elicited substantial aggregation, and the other DAG analogues (Figure 5).

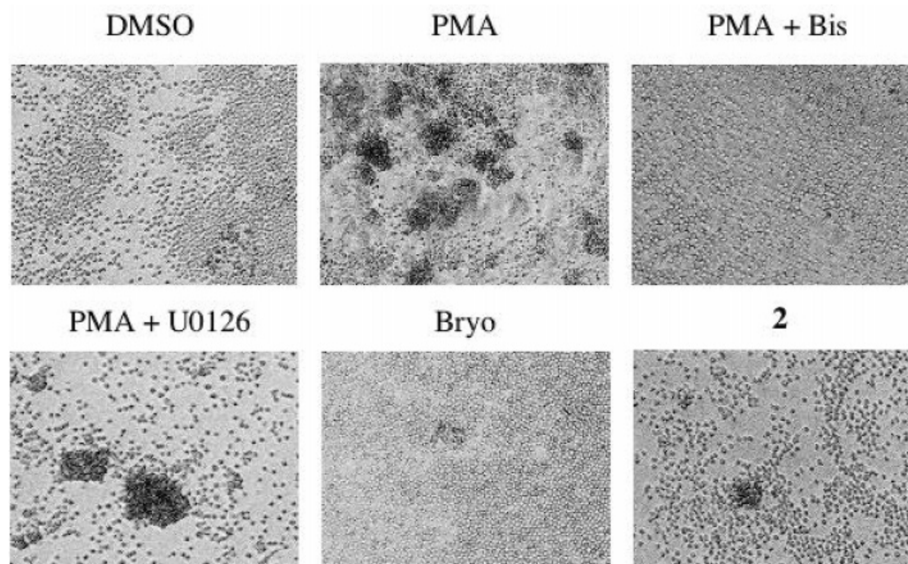

Figure 5. Homotypic aggregation of mouse thymocytes is preferentially induced by PMA and is a PKC-dependent, Ras−Erk-independent process. Wild-type mouse thymocytes were incubated for 8 h in the presence of DMSO, 25 nM DAG analogue, and inhibitors of PKC (Bisindolylmaleimide I) and Mek (U0126), as indicated. Typical PMA-induced aggregates are shown whereas the largest bryostatin 1- and **2**-induced aggregates were selected. Compound **1** behaved like bryostatin 1 and **2** (not shown).

Discussion

Conventionally, PKC has been assumed to be the major physiological target of both DAG and DAG analogues. However, work in recent years on non-PKC proteins that contain DAG-binding C1 domains has changed our view of the normal functions of DAG signaling and the effects of DAG analogue treatment in experimental systems.[25−28] DAG-binding C1 domains have been discovered in chimaerins, which regulate the small GTPase rac and in unc-13 homologues, which function to regulate vesicle exocytosis. DAG kinase γ,[29] an enzyme involved in attenuation of DAG signaling and protein kinase D, also possesses DAG-binding C1 domains. Thus, DAG responses could involve regulation of multiple independent signaling events. However, we also provided evidence that DAG-activated PKC can phosphorylate RasGRP3, which functions in B cells downstream of the B cell receptor[30,31] Thus, in addition to distinct DAG regulated pathways, cross talk between DAG pathways may be used in some cells.

The use of bryostatin 1 as an antitumor agent in clinical trials emphasizes the need for a more thorough understanding of DAG signaling systems. In addition to arresting the growth of certain tumor derived cell lines, bryostatin has a number of cellular effects that may be relevant to its potential as an antineoplastic agent (reviewed in ref 4). These include the ability to enhance differentiation and apoptosis as well as influence sensitivity to conventional chemotherapeutic agents and ionizing radiation. In addition to these cancer cell intrinsic effects, bryostatin has the ability to activate tumor-specific lymphocytes.[32−35] The clinically observed dose-limiting toxicity of bryostatin 1 is myalgia, which may be a reflection of immune stimulation and cytokine release. At least in T cells, many of the effects of bryostatin 1 might be better explained in terms of RasGRP1-dependent, rather than exclusively PKC-dependent, mechanisms.

The limited availability and cost of natural bryostatins, as well as their structural complexity, which precludes their ready access via chemical synthesis, motivated our studies aimed at synthesizing simplified analogues. The bryologues maintain the pharmacophore associated with C1 binding while incorporating only a modified spacer domain.[4] It was anticipated that the bryologues would maintain the same functional properties as bryostatin 1. In the present study, we compared two promising bryologues to bryostatin 1 in terms of their action in RasGRP1 signaling assays.

Our Ras and Erk activation assays demonstrate that both **1** and **2** can activate RasGRP1 in cultured cells. Ectopic expression of the RasGRP1 cDNA in rat2 fibroblasts is sufficient to endow these cells with the ability to respond separately to all four DAG analogues. Furthermore, all four DAG analogues can activate Ras−ERK signaling in Jurkat T cells, in which we have extensively characterized an endogenous RasGRP1 signaling function downstream of the TCR. Finally, wild-type but not RasGRP1 mutant thymocytes activate Erk in response to DAG analogue treatment. Our long-term stimulation studies failed to induce RasGRP1 breakdown, similar to previous studies with PMA treated T cells.[18] This may reflect a fundamental difference between RasGRP1 and PKC family members. However, PMA-dependent RasGRP1 downregulation in mouse skin cells has been reported.[14]

On the basis of our microscopic observations, all four DAG analogues function, at least in part, by recruiting RasGRP1 to membranes. The simplest explanation for our results is that the pharmacophore of each compound binds directly to the RasGRP1 C1 domain, while the hydrophobic spacer region partitions into various lipid bilayers within the cell. We have not ruled out the possibility that RasGRP1 undergoes allosteric regulation upon DAG analogue binding.

The prevalent expression of RasGRP1 in the T cell lineage and the abundant genetic evidence that RasGRP1 functions to amplify TCR signaling[8,15,36] has encouraged us to propose that DAG analogues might find uses in the clinical manipulation of the immune

system. To this end, we examined whether overnight treatment with the bryologues can elicit the surface expression of CD69, a marker of T cell activation that is regulated by Ras−Erk signaling. This issue was explored with the Jurkat T cell line, with normal human PBL treated immediately after isolation, and with mouse splenocytes that had been stimulated to undergo proliferation for a few days in culture. The four DAG analogues behaved similarly in all three of these assay systems.

DAG analogues also activate non-RasGRP1 signaling systems. Our analysis of homotypic aggregation in mouse thymocytes showed that this response is insensitive to RasGRP1 genotype and to a Mek inhibitor but sensitive to three different PKC inhibitors. In this assay, PMA was clearly more potent that bryostatin and the bryologues.

In summary, we have shown that two bryologues behave like bryostatin 1 in a variety of RasGRP1-based assays. Compounds **1** and **2** may prove useful for inhibiting tumor cell growth by interacting with various C1 domain proteins. However, a thorough understanding of DAG analogue action requires a more complete appreciation of the expression and functional properties of various C1 domain proteins in different target cells. The bryologues might also be used to modulate the immune system by activating RasGRP1 in T cells. The availability of potent synthetic bryologues provides the starting point for the development of additional compounds that affect toxicity, bioavailability, or C1 domain selectivity.

Acknowledgment. This work was supported by grants from The Alberta Cancer Board and The Alberta Heritage Foundation for Medical Research (J.C.S.) and The National Institutes of Health (CA31845) (P.W.).

References

(1) Pettit, G. R.; Herald, C. L.; Doubek, D. L.; Herald, D. L.; Arnold, E.; Clardy, J. Isolation and Stucture of Bryostatin 1. *J. Am. Chem. Soc.* **1982**, *104*, 6848−6848.

(2) Smith, J. B.; Smith, L.; Pettit, G. R. Bryostatins: Potent, New Mitogens That Mimic Phorbol Ester Tumor Promoters. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 939−45.

(3) Hennings, H.; Blumberg, P. M.; Pettit, G. R.; Herald, C. L.; Shores, R.; Yuspa, S. H. Bryostatin 1, An Activator of Protein Kinase C, Inhibits Tumor Promotion by Phorbol Esters in SENCAR Mouse Skin. *Carcinogenesis* **1987**, *8*, 1343−6.

(4) Wender, P. A.; Hinkle, K. W.; Koehler, M. T. F.; Lippa, B. The Rational Design of Potential Chemotherapeutic Agents: Synthesis of Bryostatin Analogues. *Med. Res. Rev.* **1999**, *19*, 388−407.

(5) Wender, P. A.; Baryza, J. L.; Bennett, C. E.; Bi, C. F.; Brenner, S. E.; Clarke, M. O.; Horan, J. C.; Kan, C.; Lacôte, E.; Lippa, B.; Nell, P. G.; Turner, T. M. The Practical Synthesis of a Novel and Highly Potent Analogue of Bryostatin. *J. Am. Chem. Soc.* **2002**, *124*, 13648−9.

(6) Newton, A. C. Protein Kinase C: Structural and Spatial Regulation by Phosphorylation, Cofactors, and Macromolecular Interactions. *Chem. Rev.* **2001**, *101*, 2353−64.

(7) Ebinu, J. O.; Bottorff, D. A.; Chan, E. Y. W.; Stang, S. L.; Dunn, R. J.; Stone, J. C. RasGRP, A Ras Guanyl Nucleotide-Releasing Protein with Calcium- and Diacylglycerol-Binding Motifs. *Science* **1998**, *280*, 1082−6.

(8) Ebinu, J. O.; Stang, S. L.; Teixeira, C.; Bottorff, D. A.; Hooton, J.; Blumberg, P. M.; Barry, M.; Bleakley, R. C.; Ostergaard, H. L.; Stone, J. C. RasGRP Links T-cell Receptor Signaling to Ras. *Blood* **2000**, *95*, 3199−203.

(9) Pierret, P.; Dunn, R. J.; Djordjevic, B.; Stone, J. C.; Richardson, P. M. Distribution of Ras Guanyl Releasing Protein (RasGRP) mRNA in the Adult Rat Central Nervous System. *J. Neurocytol.* **2000**, *29*, 485−97.

(10) Yamashita, S.; Mochizuki, N.; Ohba, Y.; Tobiume, M.; Okada, Y.; Sawa, H.; Nagashima, K.; Matsuda, M. CalDAG-GEFIII Activation of Ras, R-Ras, and Rap1. *J. Biol. Chem.* **2000**, *275*, 25488−93.

(11) Pierret, P.; Vallee, A.; Mechawar, N.; Dower, N. A.; Stone, J. C.; Richardson, P. M.; Dunn, R. J. Cellular and Subcellular Localization of Ras Guanyl Nucleotide-Releasing Protein in the Rat Hippocampus. *Neuroscience* **2001**, *108*, 381−90.

(12) Toki, S.; Kawasaki, H.; Tashiro, N.; Housman, D. E.; Graybiel, A. M. Guanine Nucleotide Exchange Factors CalDAG-GEFI and CalDAG-GEFII Are Colocalized in Striatal Projection Neurons. *J. Comp. Neurol.* **2001**, *437*, 398−407.

(13) Pierret, P.; Mechawar, N.; Vallee, A.; Patel, J.; Priestley, J. V.; Dunn, R. J.; Dower, N. A.; Stone, J. C.; Richardson, P. M. Presence of Ras Guanyl Nucleotide-Releasing Protein in Striosomes of the Mature and Developing Rat. *Neuroscience* **2002**, *111*, 83−94.

(14) Rambaratsingh, R. A.; Stone, J. C.; Blumberg, P. M.; Lorenzo, P. S. RasGRP1 Represents a Novel Nonprotein Kinase C Phorbol Ester Signaling Pathway in Mouse Epidermal Keratinocytes. *J. Biol. Chem.* **2003**, *278*, 52792−801.

(15) Dower, N. A.; Stang, S. L.; Bottorff, D. A.; Ebinu, J. O.; Dickie, P.; Ostergaard, H. L.; Stone, J. C. RasGRP Is Essential for Mouse Thymocyte Differentiation and TCR Signaling. *Nat. Immunol.* **2000**, *1*, 317−21.

(16) Lorenzo, P. S.; Beheshti, M.; Pettit, G. R.; Stone, J. C.; Blumberg, P. M. The Guanine Nucleotide Exchange Factor RasGRP Is a High-Affinity Target for Diacylglycerol and Phorbol Esters. *Mol. Pharmacol.* **2000**, *57*, 840−6.

(17) Tognon, C. E.; Kirk, H. E.; Passmore, L. A.; Whitehead, I. P.; Der, C. J.; Kay, R. J. Regulation of RasGRP Via a Phorbol Ester-Responsive C1 Domain. *Mol. Cell. Biol.* **1998**, *18*, 6995−7008.

(18) Puente, L. G.; Stone, J. C.; Ostergaard, H. L. Evidence for Protein Kinase C-Dependent and -Independent Activation of Mitogen-Activated Protein Kinase in T cells: Potential Role of Additional Diacylglycerol Binding Proteins. *J. Immunol.* **2000**, *165*, 6865−71.

(19) Marais, R.; Light, Y.; Mason, C.; Paterson, H.; Olson, M. F.; Marshall, C. J. Requirement of Ras−GTP−Raf Complexes for Activation of Raf-1 by Protein Kinase C. *Science* **1998**, *280*, 109−12.

(20) Jones, D. R.; Sanjuan, M. A.; Stone, J. C.; Merida, I. Expression of a Catalytically Inactive Form of Diacylglycerol Kinase α Induces Sustained Signaling through RasGRP. *FASEB J.* **2002**, *16*, 595−7.

(21) D'Ambrosio, D.; Cantrell, D. A.; Frati, L.; Santoni, A.; Testi, R. Involvement of p21ras Activation in T cell CD69 Expression. *Eur. J. Immunol.* **1994**, *24*, 616−20.

(22) Taylor-Fishwick, D. A.; Siegel, J. N. Raf-1 Provides a Dominant But Not Exclusive Signal for the Induction of CD69 Expression on T cells. *Eur. J. Immunol.* **1995**, *25*, 3215−21.

(23) McDowall, A.; Leitinger, B.; Stanley, P.; Bates, P. A.; Randi, A. M.; Hogg, N. The I Domain of Integrin Leukocyte Function-Associated Antigen-1 Is Involved in a Conformational Change Leading to High Affinity Binding to Ligand Intercellular Adhesion Molecule 1 (ICAM-1). *J. Biol. Chem.* **1998**, *273*, 27396−403.

(24) Favata, M. F.; Horiuchi, K. Y.; Manos, E. J.; Daulerio, A. J.; Stradley, D. A.; Feeser, W. S.; Van Dyk, D. E.; Pitts, W. J.; Earl, R. A.; Hobbs, F.; Copeland, R. A.; Magolda, R. L.; Scherle, P. A.; Trzaskos, J. M. Identification of a Novel Inhibitor of Mitogen-Activated Protein Kinase Kinase. *J. Biol. Chem.* **1998**, *273*, 18623−32.

(25) Kazanietz, M. G. Eyes Wide Shut: Protein Kinase C Isozymes Are Not the Only Receptors for the Phorbol Ester Tumor Promoters. *Mol. Carcinog.* **2000**, *28*, 5−11.

(26) Kazanietz, M. G.; Caloca, M. J.; Eroles, P.; Fujii, T.; Garcia-Bermejo, M. L.; Reilly, M.; Wang, H. Pharmacology of the Receptors for the Phorbol Ester Tumor Promoters: Multiple Receptors with Different Biochemical Properties. *Biochem. Pharmacol.* **2000**, *60*, 1417−24.

(27) Brose, N.; Rosenmund, C., Move Over Protein Kinase C, You've Got Company: Alternative Cellular Effectors of Diacylglycerol and Phorbol Esters. *J. Cell. Sci.* **2002**, *115*, 4399−411.

(28) Kazanietz, M. G. Novel "Nonkinase" Phorbol Ester Receptors: The C1 Domain Connection. *Mol. Pharmacol.* **2002**, *61*, 759−67.

(29) Shindo, M.; Irie, K.; Ohigashi, H.; Saito, N. Diacylglycerol Kinase γ Is One of the Specific Receptors of Tumor-Promoting Phorbol Esters. *Biochem. Biophys. Res. Commun.* **2001**, *289*, 451−6.

(30) Teixeira, C.; Stang, S. L.; Zheng, Y.; Beswick, N. S.; Stone, J. C., Integration of DAG Signaling Systems Mediated by PKC-Dependent Phosphorylation of RasGRP3. *Blood* **2003**, *102*, 1414−20.

(31) Oh-hora, M.; Johmura, S.; Hashimoto, A.; Hikida, M.; Kurosaki, T., Requirement for Ras Guanine Nucleotide Releasing Protein 3 in Coupling Phospholipase C-$\gamma 2$ to Ras in B Cell Receptor Signaling. *J. Exp. Med.* **2003**, *198*, 1841−51.

(32) Tuttle, T. M.; Inge, T. H.; Wirt, C. P.; Frank, J. L.; McCrady, C. M.; Bear, H. D., Bryostatin 1 Activates T Cells That Have Antitumor Activity. *J. Immunother.* **1992**, *12*, 75–81
(33) Fleming, M. D.; Bear, H. D.; Lipshy, K.; Kostuchenko, P. J.; Portocarero, D.; McFadden, A. W. J.; Barrett, S. K. Adoptive Transfer of Bryostatin-Activated Tumor-Sensitized Lymphocytes Prevents or Destroys Tumor Metastases without Expansion In Vitro. *J. Immunother. Emphasis Tumor Immunol.* **1995**, *18*, 147–55.
(34) Baldwin, N. G.; Rice, C. D.; Tuttle, T. M.; Bear, H. D.; Hirsch, J. I.; Merchant, R. E. Ex Vivo Expansion of Tumor-Draining Lymph Node Cells Using Compounds Which Activate Intracellular Signal Transduction. 1. Characterization and In Vivo Antitumor Activity of Glioma-Sensitized Lymphocytes. *J. Neurooncol.* **1997**, *32*, 19–28.
(35) Chin, C. S.; Graham, L. J.; Hamad, G. G.; George, K. R.; Bear, H. D. Bryostatin/Ionomycin-Activated T Cells Mediate Regression of Established Tumors. *J. Surg. Res.* **2001**, *98*, 108–15.
(36) Priatel, J. J.; Teh, S.-J.; Dower, N. A.; Stone, J. C.; Teh, H.-S. RasGRP1 Transduces Low-Grade TCR Signals Which Are Critical for T Cell Development, Homeostasis, and Differentiation. *Immunity* **2002**, *17*, 617–27.

JM0495069