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Simplified Analogs of Bryostatin with Anticancer Activity Display Greater Potency for Translocation of PKCδ-GFP

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Summary

Structurally simplified analogs of bryostatin 1, a marine natural product in clinical trials for the treatment of cancer, have been shown to be up to 50 times more potent than bryostatin 1 at inducing the translocation of PKCδ-GFP from the cytosol of rat basophilic leukemia (RBL) cells. The end distribution of the protein is similar for all three compounds, despite a significant difference in translocation kinetics. The potency of the compounds for inducing the translocation response appears to be only qualitatively related to their binding affinity for PKC, highlighting the importance of using binding affinity in conjunction with real-time measurements of protein localization for the pharmacological profiling of biologically active agents.

Introduction

The bryostatins are a structurally novel family of marine natural products that exhibit a unique and potent combination of biological activities [1]. In addition to its extraordinary potency against murine P388 leukemia, the lead member of this family, bryostatin 1, has been found to stimulate immune system responses [2], regulate apoptotic function [3], reverse multidrug resistance [4, 5], and act synergistically with other oncolytic agents [6]. While the mode of action of bryostatin 1 is not established, it binds to protein kinase C (PKC) with high affinity and modulates its function [1, 7]. Other proteins containing C1 domains, such as RasGRP, Unc/Munc, and the chimaerins, have also been implicated as possible receptor targets for bryostatin [8, 9]. Despite its impressive biological profile, the advancement of bryostatin 1 as a therapeutic agent has been limited by its low natural abundance and chemical complexity, which render it and its derivatives chemically inaccessible.

Bryostatin, along with the phorbol esters and the endogenous ligand diacyl glycerol (DAG), modulate PKC activity by binding to the C1 domains of the protein and inducing translocation from the cytosol to cellular membranes. While PKC can be activated without translocation, under most conditions translocation is an integral part of the activation process and can be used as a surrogate measure for activation [10]. Translocation of PKC isozymes to different subcellular locations is believed to be at least partly responsible for controlling the substrate selectivity of PKC by localizing the enzyme near its substrates but away from other proteins [11].

In an effort to create compounds with bryostatin-like activity that are readily accessible through chemical synthesis, the Wender group has designed and developed simplified analogs of bryostatin (Figure 1) [12–14]. When tested for binding to PKC, these analogs displayed equal or greater potency than bryostatin 1. Further testing against several human cancer cell lines demonstrated that the analogs are up to two to three orders of magnitude more potent than the natural product against several cancer types [15]. Taken together, these data suggest that the biological activity of these compounds in complex systems might not be directly related to their binding affinity to PKC mixtures. A lack of correlation between binding affinity and biological activity is well precedented in the diverging effects of the phorbol esters and bryostatin. Both are potent activators of PKC, but bryostatin is an antagonist for the tumor promoting effects of the phorbol esters [16]. A possible explanation for this divergence is that the activation of PKC by bryostatin might result in differences in PKC localization or isozyme specificity from that of the phorbol esters.

Due to the coupling of PKC translocation to its activation, PKC-green fluorescent protein (GFP) fusion proteins provide an excellent tool for studying the behavior of PKC isozymes in response to various stimuli [12, 17–19]. We chose this system to further study the functional and kinetic effects of our analogs on PKC activation and translocation in a cellular system. The PKC-GFP translocation assay allows the direct observation of the protein response in living cells, quantification of the kinetics of translocation, and determination of the cellular destination of translocation. From these data, a measurement of the relative functional performance of these agents in a cellular environment can be determined. This type of measurement is likely to be more relevant to physiological effects than a measure of binding affinity since it measures directly the *action* of the ligand on its target in vitro as a function of time rather than a simple noncellular affinity as is the case for binding measurements. In addition to providing a quantifiable output, this system allows the localization of the protein to be observed. If localization is important for activity and specificity of the enzyme, as is believed for PKC, this could be a significant factor in the response.

Results

The abilities of analogs **1** and **2** and bryostatin 1 to induce translocation of PKCδ-GFP in RBL cells were compared at various concentrations. When expressed in this cell line, PKCδ-GFP is present throughout the cytosol but not in the nucleus prior to treatment (Figure 2).

Exposure of RBL cells overexpressing PKCδ-GFP to bryostatin 1 or the analogs resulted in a rapid transloca-

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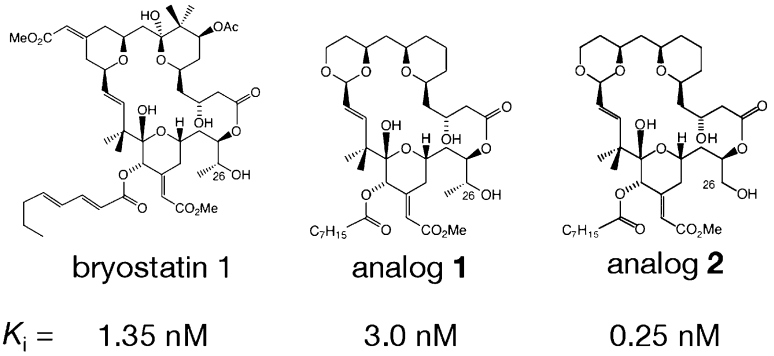

Figure 1. Bryostatin 1 and the Structures of Analogs 1 and 2

Shown are the binding affinities to a PKC mixture isolated from rat brain.

tion of fluorescence from the cytosol to the plasma membrane and the perinuclear region. Shown in Figures 2 and 3 is the response of the cells to each of the three agents at a concentration of 200 nM. For all three compounds at 200 nM, the ratio of the average fluorescence intensity measured at the plasma membrane to that at the nuclear membrane once translocation was complete was approximately 1:1. All three ratios were identical within error.

In order to ensure that the observed nuclear membrane localization was not a result of translocation of the kinase domain-GFP fragment after proteolysis, RBL cells were transfected with PKCδ-GFP and exposed to bryostatin 1 (200 nM) or analog **2** (200 nM) for 30 min. The lysates of these cells were examined by Western blot with both a monoclonal anti-GFP antibody and a polyclonal antibody to PKCδ. There was no increase in the amount of cleavage product in either the cells exposed to bryostatin 1 or analog **2** when compared to cells that had not been exposed to any agent (data not shown). This result is consistent with that obtained by Blumberg and coworkers in PKCδ-GFP-overexpressing CHO cells and indicates that the observed localization to the nuclear membrane is induced by the test molecules [18, 19].

At a concentration of 200 nM, translocation to the plasma and nuclear membranes was complete in less than 5 min for all three agents, as can be seen in Figure 2. Despite their difference in binding potency, analog **1** and **2** displayed essentially identical rates and degrees of translocation at this concentration. An additional set of experiments was performed at 37°C to determine the extent to which temperature influenced the relative rates. The results at 37°C were indistinguishable from those at room temperature (data not shown). Figure 4 shows the decrease in cytosolic fluorescence after the addition of bryostatin 1, analog **1**, or analog **2** (200 nM). The data was measured as the decrease in cytosolic fluorescence to avoid artifacts and difficulties related to ruffling and movement of the membrane, which was observed in several of the experiments.

For all three compounds, both the rate of translocation and the overall degree of translocation was dose dependent, with lower doses resulting in slower and less complete translocation (Figures 5 and 6). At a given concentration, analog **1** and **2** induced much faster translocation than bryostatin 1. Shown in Figure 6 are plots of the measured half-lives for the translocation of PKCδ-GFP in response to the analogs and bryostatin 1 as well as the degree of translocation in response to each agent. The measured half-life was calculated as the amount of time for the translocation to proceed halfway to its observed end point. The degree of translocation is the ratio of the amount of fluorescence in the

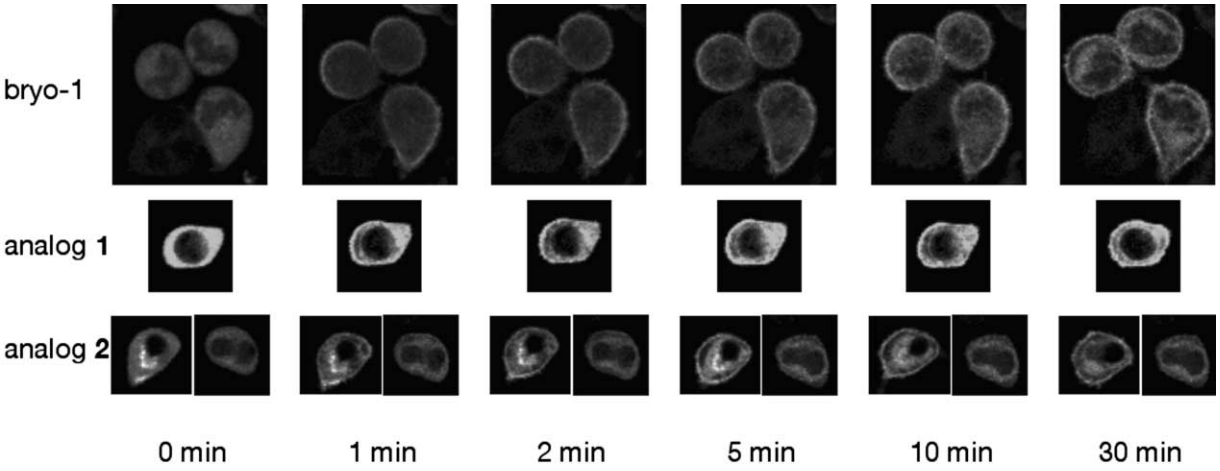

Figure 2. Confocal Images Showing the Response of PKCδ-GFP in Transfected RBL Cells to Treatment with 200 nM Bryostatin 1, Analog **1**, or Analog **2** during a 30 min Time Course

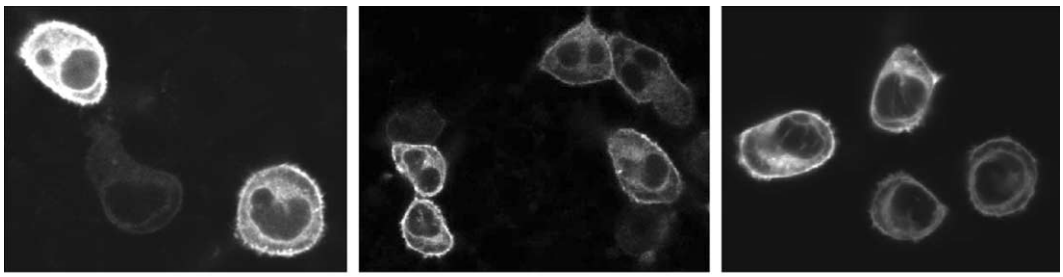

Figure 3. Confocal Images Displaying the Localization of PKCδ-GFP at Both the Plasma Membrane and the Nuclear Membrane of RBL Cells after Treatment with 200 nM Bryostatin 1, Analog **1**, or Analog **2** for 30 min

cytosol at the end point of the experiment to the amount present before the addition of any agent. At concentrations of 5 nM or greater, both analogs were found to have the same rate of translocation. Both analogs induced a translocation response that was approximately twice as fast as that induced by bryostatin 1. At 1 nM, however, the rates diverged and analog **2** had a substantially faster rate of translocation compared to both analog **1** and bryostatin 1.

A similar effect was seen when the degree of translocation was compared. At concentrations of 50 and 200 nM, all three agents induced a similar degree of translocation. At concentrations of 5 nM, the degree of translocation induced by analog **1** and analog **2** remained similar, but was significantly greater than that induced by bryostatin 1. When dosed at 1 nM, the response of PKCδ-GFP to analog **1** and analog **2** differed, with analog **2** resulting in greater translocation than analog **1** or bryostatin 1.

In addition to the observed differences in kinetics, the analogs also retained the ability to induce translocation of PKCδ-GFP at lower doses than bryostatin 1. Figure 7 shows images of the cells after exposure to the agents. Bryostatin 1 induced very little translocation at 5 nM, while both analogs **1** and **2** were still effective at inducing translocation of PKC to the plasma membrane. While treatment with analog **1** resulted in marginal translocation at 1 nM, analog **2** induced translocation even at a concentration of 0.5 nM and was active, albeit marginally, even at a concentration of 0.1 nM. As can be seen in Figure 5, a 100 pM concentration of analog **2** was as effective at inducing translocation of PKCδ-GFP as 1 nM of analog **1**, which was as effective as 5 nM of bryostatin 1.

Discussion

An understanding of why equipotent activators of PKC, such as bryostatin and the phorbol esters, differ in their pharmacological responses is an important focus of PKC research. While binding affinities to PKC can serve as indicators of potential interactions in biological systems, they often do not correlate with specific biological function. This is not surprising, as binding, a thermodynamic parameter, is not always connected to function, a kinetic parameter. For example, bryostatin and analog **1** have comparable binding affinities to PKC, whereas both analogs **1** and **2** are orders of magnitude more potent at inhibiting the growth of many human cancer cell lines [15]. In order to elucidate the cascade of events that culminate in a cellular response to the phorbol esters, bryostatin, and analogs of bryostatin, a more comprehensive understanding of the response of PKC and other proteins that contain C1 binding domains is re-

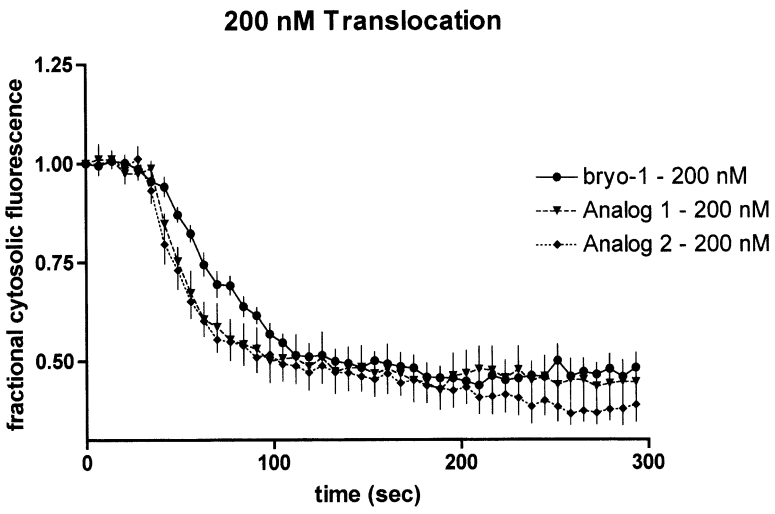

Figure 4. Decrease in Cytosolic Fluorescence of RBL Cells Transfected with PKCδ-GFP and Treated with 200 nM Bryostatin 1, Analog **1**, or Analog **2**

The degree of fluorescence was normalized to that present at the beginning of the experiment, and the agents were added after the fifth measurement at time = 35 s.

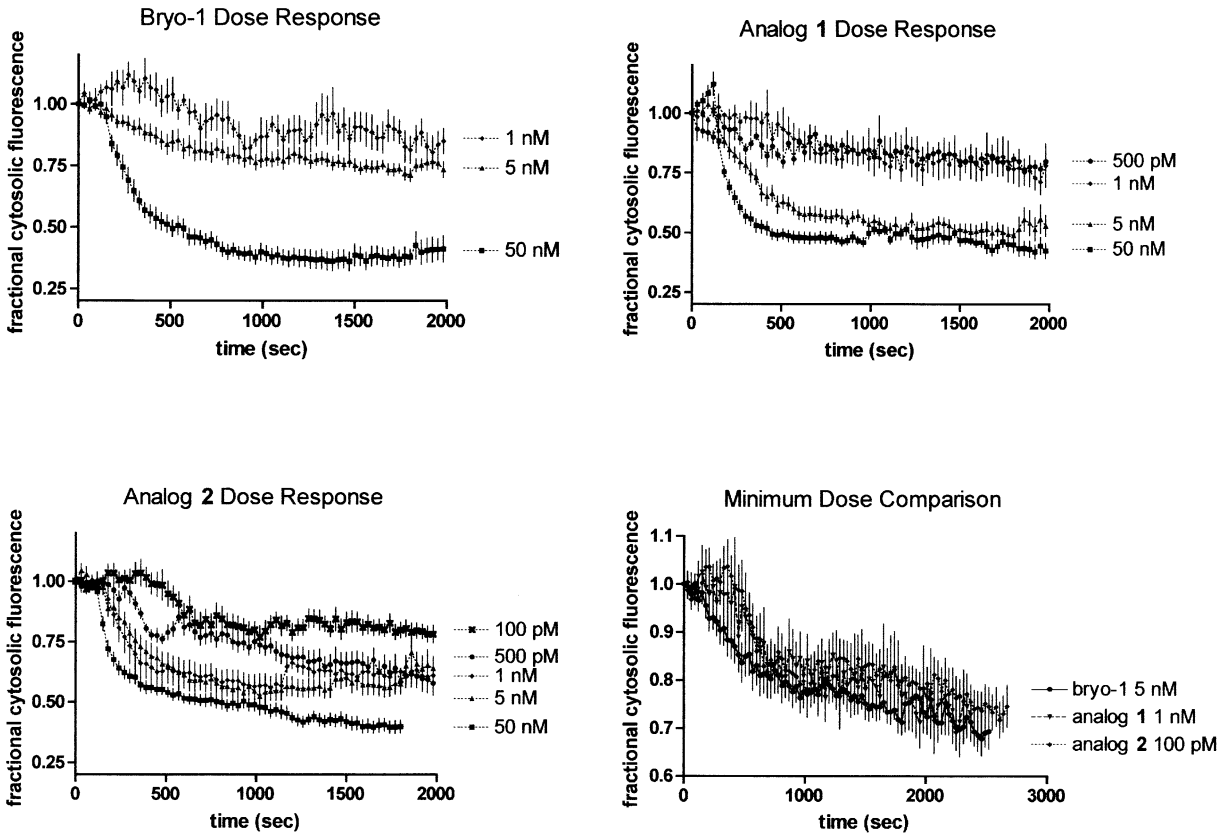

Figure 5. The Decrease in Cytosolic Fluorescence of PKCδ-GFP-Transfected RBL Cells after Treatment with Either Bryostatin 1, Analog 1, or Analog 2 at Varying Concentrations

In all experiments, the agent was added after the 5th image at time = 150 s. The degree of fluorescence is expressed relative to that present at the start of the experiment.

quired. The use of fluorescently tagged proteins and confocal microscopy enable one to observe the earliest stages of such a functional response in real time.

PKCδ is implicated as an important isozyme of PKC for determining the response of living systems to tumorogenesis induced by the phorbol esters [20]. PKCδ is integral to certain apoptosis pathways and might also play a role in the metastatic potential of cancer cells [21]. Blumberg and coworkers have also shown that the localization of PKCδ-GFP constructs in response to the tumor promoting phorbol esters differs from that seen in response to bryostatin 1 or nontumor promoting analogs of PMA [19]. Current understanding of PKC activity attributes the substrate specificity of PKC at least in part to its subcellular localization upon activation [22]. Therefore, different patterns of localization of PKC isozymes might account for the differing cellular responses to PKC activation. For these reasons, PKCδ-GFP fusion

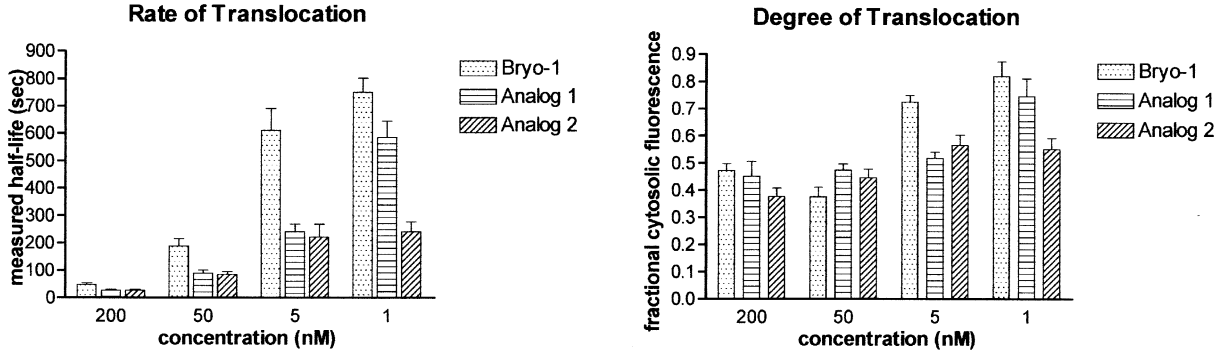

Figure 6. Comparison of the Measured Half-Life and Degree of Translocation of PKCδ-GFP in Response to Varying Doses of Bryostatin 1, Analog 1, or Analog 2 in RBL Cells

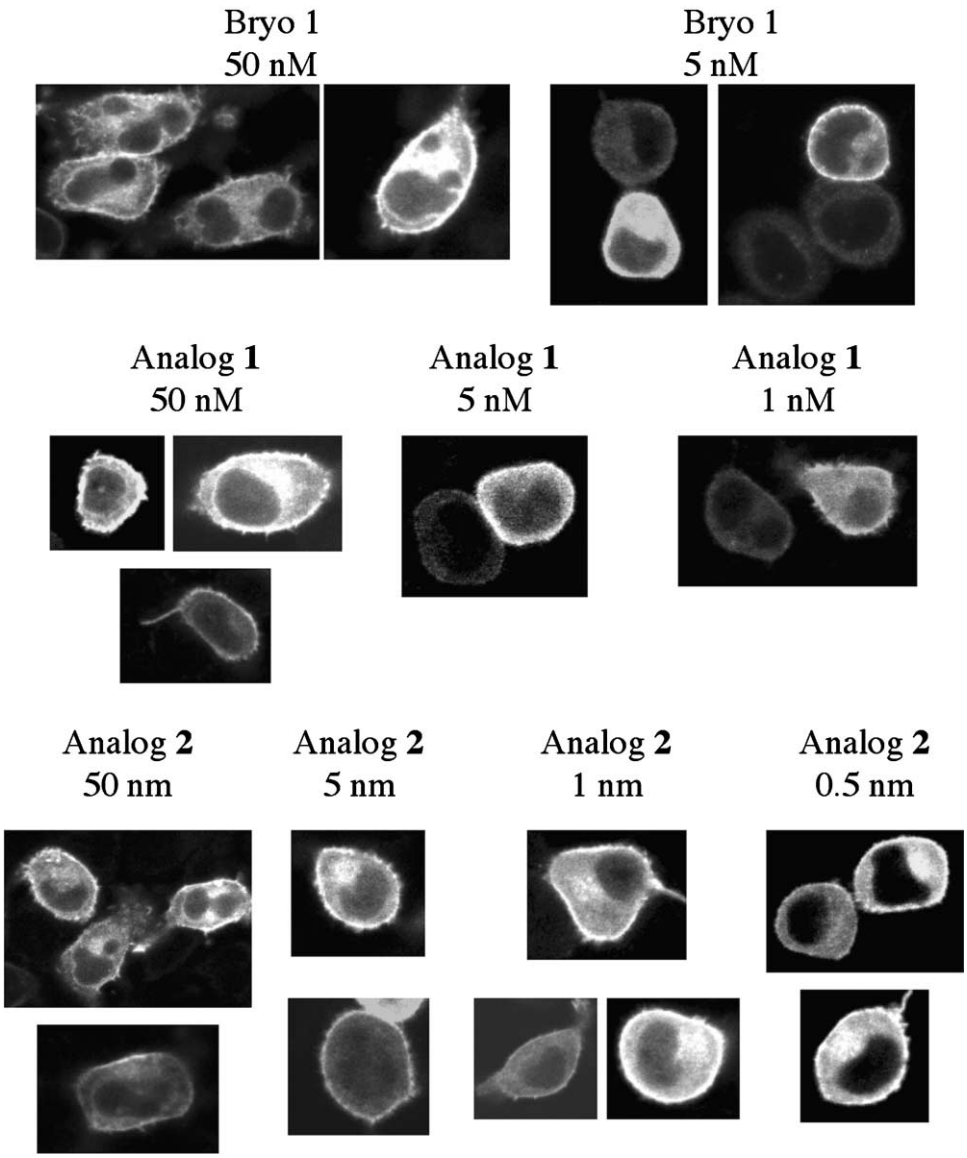

Figure 7. Images of PKCδ-GFP-Transfected RBL Cells Treated with Varying Concentrations of Bryostatin 1, Analog 1, or Analog 2 Acquired at the End of the Experiment, t = 30–50 min

proteins were chosen for characterizing the relative function of bryostatin and its analogs.

The results of these experiments show that analogs **1** and **2** are significantly more potent than bryostatin at inducing translocation of PKCδ-GFP but induce a similar pattern of translocation. At concentrations of 200 and 50 nM, bryostatin 1 induces an initial translocation of PKCδ-GFP to the plasma membrane. Translocation of PKCδ-GFP to the nuclear membrane in response to bryostatin 1 is slower and is not observed until approximately 5–10 min after administration (Figure 2). In response to 200 or 50 nM concentrations of analogs **1** and **2**, however, there is a rapid localization of PKCδ-GFP to the plasma and nuclear membranes simultaneously.

Another interesting result is that the rate of PKCδ-GFP translocation at higher concentrations (≥5 nM) is the same for analog **1** and **2**, but that these analogs are both faster than bryostatin 1. However, between the ranges of 5 nM and 1 nM, the rate at which analogs **1** and **2** induce translocation PKCδ-GFP becomes different.

A possible explanation for the difference in translocation potency between bryostatin 1 and the analogs is that the lipophilicity of these molecules might play a role in determining the potency and rate of the cellular response. The modified spacer domains present in both **1** and **2** might alter their lipophilicity, which in turn could affect their diffusion through cells and partitioning among various cellular compartments. As the binding event with PKC and bryostatin 1 or the analogs involves a tertiary complex of membrane, ligand, and protein, changing the interaction of the ligand with the membrane could significantly alter the kinetics and thermodynamics of the interaction.

Previous work in our group has suggested that the structure of bryostatin 1 could be thought of in terms of a recognition domain and a spacer domain [12–14]. The recognition domain, so-called because it contains all of the requisite functionality for recognition by, and binding to, PKC, consists of the C-ring moiety along with the macrolactone carbonyl. It was thought that the spacer domain, which comprises the A and B rings of the molecule, served to reduce the conformational freedom of the recognition domain and keep the recognition elements in the proper orientation for binding to PKC. It appears from the data presented here that changes to this region of the molecule also influence the translocation response of PKC.

The translocation of PKC isoforms is only the initial response of these proteins to activators, and measuring the translocation rate in response to different agents provides information on only the initial stage of their function. In many cases, treatment with PKC activators is followed by protein degradation, and the depletion of the kinase from the cells might also account for some of the observed action of these molecules. There is also growing evidence that for activators of PKC that bind to the C1 domains of the protein, including bryostatin and the phorbol esters, other proteins containing homologous C1 domains, such as the chimaerins, RasGRP, and Munc, might play a role in regulating the cellular response. However, these studies help to underscore the large disconnect between potency in simple binding models and activity in more complex cellular systems. Such methods as used here might help in the more detailed understanding of structure and function which can then be used in the design of better, more specific, and more potent agents with desired therapeutic functions.

We have shown that, in contrast to their binding affinities, synthetic analogs of bryostatin are more potent at inducing translocation of PKCδ-GFP in RBL cells. Analog **1**, which is comparable to bryostatin 1 in affinity for PKC, is effectively 5-fold more potent and analog **2** is effectively 50 times more potent than bryostatin 1 at inducing translocation of PKCδ-GFP.

Significance

Analogs of the marine natural product bryostatin are shown to be potent inducers of translocation of PKCδ-GFP. In both cases, the analogs were more potent than the natural product. Analog 1, which binds PKC comparably to bryostatin 1 (K_i = 3.0 nM for analog 1, K_i = 1.3 nM for bryostatin 1) was approximately 5-fold more potent than bryostatin 1 at inducing translocation, being as effective at 1 nM as bryostatin 1 at 5 nM. In the case of analog 2 (K_i = 0.25 nM), translocation of PKCδ-GFP was observed even at a concentration of 100 pM, making analog 2 approximately 50 times more potent than bryostatin 1. The final pattern of protein distribution was the same for both analogs as was observed for bryostatin 1.

Experimental Procedures

Cell Culture and Transfection

Rat basophilic leukemia 2H3 (RBL) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, GIBCO) containing 20% fetal calf serum with 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine (GIBCO). Cells were maintained at 37°C in an atmosphere of 10% CO_2. Two hours prior to transfection, cells were plated onto sterile glass coverslips. The mRNA encoding GFP-tagged full-length PKCδ was electroporated into the cells 12 hr before experiments according to the procedure described previously [23].

Fluorescence Microscopy

Fluorescence images were obtained using the 488 nm excitation line of a laser scanning confocal microscope (Pascal, Zeiss), and emission was collected through a 505–550 nm band-pass filter. Cells were imaged on the stage of an inverted microscope (Axiovert 100M) using a 40× 1.2 NA Zeiss Plan-apo oil immersion objective. For each experiment, a coverslip to which the cells adhered was used to form the base of a metal cell chamber (Molecular Probes). Cells were washed and maintained in Dulbecco's phosphate buffered saline (GIBCO) supplemented with 10 mM glucose. Bryostatin, analog **1**, and analog **2** were dissolved in DMSO and then diluted to the desired concentration in the extracellular buffer shortly before being added to the cells. The final concentration of DMSO to which the cells were exposed did not exceed 0.1%. Unless otherwise stated, each time series lasted 30 min, and images were acquired every 30 s. Reagents were added to the cell chamber after the fifth image in each time series. For experiments performed at 37°C, an air stream incubator (ASI400, Nevtek) was used to heat the stage and microscope objectives, and the extracellular buffer was warmed to 37°C before use.

Analysis

Images were exported as 12 bit files and analyzed using Metamorph data analysis software (Universal Imaging). To monitor the translocation of PKC-delta, a small region of interest was selected in the cytosol of each cell, and fluorescence intensity values were graphed against time following background subtraction and normalization.

Bryostatin and Bryostatin Analogs

The bryostatin analogs used have been reported previously. The C26 methyl analog [12–14] reported in 1998 is referred to as analog **1**, and the C26 des-methyl analog [15] reported in 2002 is referred to as analog **2**.

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