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BINDING OF [^{3}H]BRYOSTATIN 4 TO PROTEIN KINASE C

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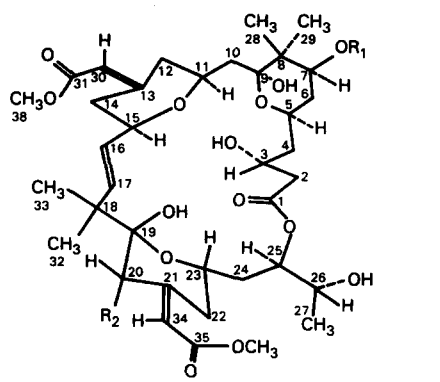
Abstract—The bryostatins represent a unique class of activators of protein kinase C (PKC) which induce only a subset of the responses typical of the phorbol esters and block those responses to the phorbol esters which they themselves do not induce. To better understand the interaction of the bryostatins with PKC, we have synthesized [26-^{3}H]bryostatin 4 and characterized its binding to PKC. [^{3}H]Bryostatin 4 and [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) differed markedly in their binding to PKC reconstituted with phosphatidylserine (PS). The binding affinity of [^{3}H]bryostatin 4 under these conditions was too high to measure and the rate of release of bound bryostatin was much slower than that of the phorbol esters, with a half-time of several hours. These properties caused bryostatin 1 to appear to inhibit [^{3}H]PDBu binding under these conditions in a non-competitive fashion. Both the high potency and the slow rate of release of the bryostatins may contribute to their unique pattern of biological activity. By reconstituting PKC in a mixture of 1.5% Triton X-100:0.3% PS, we were able to establish reversible conditions for [^{3}H]bryostatin 4 binding. Under these latter conditions, binding of [^{3}H]bryostatin 4 was competitively inhibited by PDBu, consistent with both the bryostatin and phorbol esters binding to PKC in a qualitatively similar fashion. Binding affinities to PKC isozymes α, β, and γ were compared and little difference was found, suggesting that differential recognition by these isozymes does not account for the unique biological activity of the bryostatins.

The bryostatins are a family of macrocyclic lactones isolated from marine bryozoans (Fig. 1). These natural products have potent antileukemic activity [1] and are currently undergoing Phase 1 clinical trials. Studies with the bryostatins demonstrate that the bryostatins have a unique spectrum of biological activity. In a number of systems, bryostatin 1 acts similarly to the phorbol esters. For example, bryostatin 1 stimulates mitogenesis in Swiss 3T3 cells [2], induces oxidative activity in neutrophils [3], and inhibits the binding of phorbol 12,13-dibutyrate to these cells and activates protein kinase C (PKC¶) [2, 4]. In addition, the bryostatins show excellent spatial correlation with the phorbol ester pharmacophore derived from computer modeling of activators of PKC [5, 6]. These results argue that the bryostatins are a structurally distinct class of PKC activators in analogy with the indole alkaloids and the polyacetates. On the other hand, the bryostatins only display phorbol ester-like activity for some responses and systems; in those cases in which the bryostatins fail to induce phorbol ester like effects, the bryostatins block, in an apparently noncompetitive manner, the induction of these responses by the phorbol esters. Examples include:



	R_1	R_2
BRYOSTATIN 1	$COCH_3$	OCO⟋⟍⟋⟍⟋
BRYOSTATIN 4	$COCH_2CH(CH_3)_2$	$OCOCH_2CH_2CH_3$

Fig. 1. Structures of bryostatin 1 and 4.

differentiation in HL-60 promyelocytic leukemia cells [4], in Friend erythroleukemia cells [7] and in mouse primary keratinocytes [8]; induction of arachidonic acid release in C3H10T1/2 cells [9]; and tumor promotion in mouse skin [10, 11]. An understanding of the mechanistic basis of the unique

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¶ Abbreviations: PKC, protein kinase C; [^{3}H]PDBu, [20-^{3}H]phorbol 12,13-dibutyrate; PS, phosphatidylserine; and DMSO, dimethyl sulfoxide.

spectrum of activity of the bryostatins may both allow the dissection of subpathways of PKC control and suggest novel strategies for selective therapeutic intervention in PKC-mediated responses.

PKC represents one arm of the phosphatidylinositol signal transduction pathway which transmits signals for a number of hormones, growth factors, and neurotransmitters. Accordingly, PKC has been found to play a role in the action of several oncogenes, in multidrug resistance, and in carcinogenesis [12]. The endogenous activator of PKC is *sn*-1,2-diacylglycerol [12], which binds to PKC competitively with the phorbol esters [13, 14]. PKC is thus thought to also transduce signals arising from other pathways which generate *sn*-1,2-diacylglycerol, e.g. from the hydrolysis of phosphatidylcholine by phospholipase D [15]. Molecular cloning of PKC has shown that this kinase exists as a family of at least eight isozymes which differ in their tissue distributions [16], subcellular localizations [17], substrate specificities [16], and activation requirements [16, 18–20]. It would thus appear that the PKC isozymes are functionally different, although unique physiological roles for individual isozymes remain, in general, to be established.

In the present studies we have characterized the interaction of bryostatin 4 with PKC. Bryostatin 4 was labeled with ^3H, and its direct binding to a mixture of the PKC isozymes isolated from mouse brain was well as to three of the individual PKC isozymes was determined. Bryostatin 4 was found to bind to PKC in a fashion qualitatively similar to that of the phorbol esters. Quantitatively bryostatin 4 differed dramatically, however, in its high binding affinity and its slow rate of release. It did not show substantial selectivity among PKC isozymes α, β, or γ.

METHODS

Bryostatin 1 and bryostatin 4 were isolated as described [21, 22]. [26-^3H]Bryostatin 4 was prepared as reported earlier [23] by oxidation of bryostatin 4 to 26-oxo-bryostatin 4 and subsequent reduction with [^3H]borohydride. It was separated from its epimer by HPLC chromatography, and its identity was confirmed by isoelution with an authentic standard. The specific activity of the [26-^3H]-bryostatin 4 produced for these studies was 6.33 Ci/mmol (1 Ci = 37 GBq). Bryostatin 4 was only available in very limited amounts, and all of the [^3H]-byrostatin 4 was used in previous studies and in the studies described below.

PKC was partially purified from mouse brain using diethylaminoethyl cellulose chromatography (DE-52, Whatman, Clifton, NJ) as reported earlier [24]. PKC isozymes from COS cells transfected with the cDNA for either PKCα, PKCβ, or PKCγ (provided by Dr. John Knopf, Genetics Institute) were also partially purified by DEAE chromatography. Control cells were transfected with vector alone. PKCα and PKCβ were purified from rat brain by consecutive resolution of activity over DE-52, phenyl-Sepharose, polylysine-agarose, and hydroxyapatite columns [25].

DEAE-dextran transfection was used to introduce the cDNA for the PKC isozymes into COS cells [26]. In brief, 1 hr prior to transfection, 10-cm dishes of cells were washed twice with phosphate-buffered saline and refed with fresh medium containing 10% NuSerum. Four to twenty micrograms of highly purified plasmid DNA in pellet form was resuspended in Tris-buffered saline. This solution was then added to an equal volume of warm (~ 37°) DEAE-dextran (10 mg/mL) in Tris-buffered saline. The DEAE-dextran/DNA solution (80 μL) was added dropwise to each dish, which was agitated gently for uniform distribution. The dish was then incubated for 4 hr at 37°, followed by a change of medium after rinsing twice with phosphate-buffered saline. At this point the cells were shocked with dimethyl sulfoxide (DMSO) by a 1-min treatment with 5 mL of 10% DMSO in phosphate-buffered saline at room temperature. Then the plates were washed twice in phosphate-buffered saline and put in fresh medium. At 24–30 hr following this treatment the cells were harvested. The cDNAs for PKCα, PKCβ, and PKCγ were from rat brain [27].

Specific binding of [26-^3H]bryostatin 4 was routinely measured using a filtration assay [23]. Briefly, 0.1 mM $CaCl_2$, 0.02 M Tris-Cl, pH 7.4 (at 21°), immunoglobin G (Sigma Chemical Co., St. Louis, MO) at 1 mg/mL, Triton X-100 at 1.5 mg/mL, PS (Sigma) at 100 μg/mL, PKC preparations and [26-^3H]bryostatin 4 were incubated for 5 min at 37° in a volume of 250 μL. Following incubation, the samples were placed on ice, and two 50-μL aliquots were removed for determination of total radioactivity. Two additional 50-μL aliquots were applied to ion exchange paper discs (Whatman DE-81; Clifton, NJ). Following an absorption period of 15–30 sec, the discs were washed with 20 mL of cold 55% methanol in 20 mM Tris-Cl, pH 7.4 (at 4°),

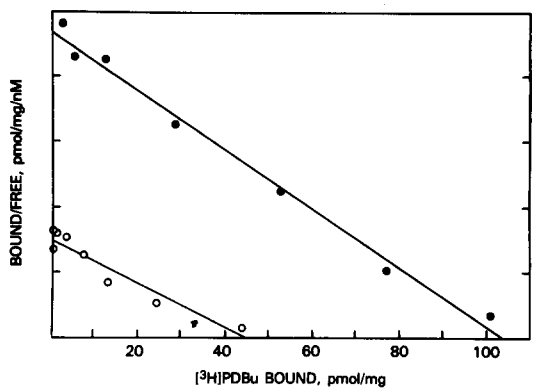

Fig. 2. Scatchard analysis of the binding of [^3H]PDBu in the presence (○) and absence (●) of 6 nM bryostatin 1. PKC was reconstituted with 100 μg/mL PS and incubated for 15 min in the presence or absence of bryostatin 1. [^3H]-PDBu was added and the incubation continued for 5 min at 37°. Specific binding was then determined. Points are the average of triplicate determinations; the lines were derived by linear regression analysis. Qualitatively similar results were observed in seven other experiments using a range of preincubation times (0–60 min) and bryostatin concentrations (2–10 nM).

with 0.1 mM $CaCl_2$. Various percentages of methanol were examined for this wash; ultimately a 55% (v/v) solution was chosen as most suitable. The radioactivity remaining on the discs was quantified by scintillation counting. Nonspecific binding was determined under the same conditions in the absence of added PKC. Free ligand was derived from the difference between total ligand bound to the filter. In some experiments, binding was assayed in the presence of 100 μg/mL PS in lieu of the Triton X-100/PS mixture described above.

RESULTS

Although the previous studies by Kraft *et al.* [28] had demonstrated that bryostatin 1 inhibits [20-^{3}H]-phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding to PKC, the nature of this inhibition had not been further characterized. To confirm that the inhibition was indeed competitive, we compared the Scatchard plots for [^{3}H]PDBu binding in the absence and presence of bryostatin 1 (Fig. 2). [^{3}H]PDBu binding was assayed as described previously [29], using PKC reconstituted in the presence of PS at 100 μg/mL and an incubation time of 5 min at 37°. We have shown previously that [^{3}H]PDBu rapidly equilibrates with its receptor at 37° [30]. Byrostatin 1 caused an apparent decrease in the B_{max} for [^{3}H]PDBu binding with little effect on the binding affinity. These surprising results suggested a non-competitive mechanism of binding inhibition by bryostatin 1.

To analyze the binding of bryostatin to PKC in greater detail, we prepared radiolabeled bryostatin. The bryostatin was labeled by oxidation of the C-26 hydroxyl group to the ketone followed by reduction with [^{3}H]borohydride back to the hydroxyl [23]. The standard approach used for labeling of the phorbol esters is analogous, except that the hydroxyl group in the case of the bryostatins is secondary rather than primary. As a consequence, the reduction of the 26-oxo-bryostatin not only regenerates the starting conformation at the C-26 carbon but also produces its epimer. These two products were resolved by HPLC (Fig. 3). For the labeling, bryostatin 4 was used rather than bryostatin 1 because the initial oxidation reaction proceeded more cleanly [31]. Bryostatin 4 acts similarly to bryostatin 1 both in its biological spectrum [9] and its binding [5]. Bryostatin 4 differs chemically in possessing a C-20 butyrate ester in place of the C-20 octadienoate, which has the minor additional advantage of making bryostatin 4 somewhat less lipophilic.

The bryostatins are much more lipophilic compounds than are the phorbol esters. Monitoring radioactivity using [^{3}H]bryostatin 4, we found that the concentrations in aqueous solutions tended to be unstable, presumably because of partitioning to the air/water interface and binding to surfaces. Unlike in the case of the phorbol esters, the concentrations of bryostatins were not adequately stabilized by the addition of proteins such as bovine serum albumin. We routinely therefore make bryostatin solutions in organic solvents such as DMSO or ethanol and add these directly to our assays. Because of this physical behavior of the bryostatins, ED_{50} values in biological systems should be viewed as upper limits, especially when such precautions have not been taken; small quantitative differences in potency are probably not interpretable.

For the development of a direct binding assay, the high lipophilicity of the bryostatins posed a formidable technical problem. Under our typical assay conditions for phorbol ester binding [13], 5% of the total PDBu added to the assay partitions into the lipid phase; in contrast, fully 80% of the bryostatin 4 was found to be lipid associated under these conditions (data not shown). This lipid-associated bryostatin constitutes nonspecific binding. A possible approach for reducing this high nonspecific binding was suggested by earlier studies of Ashendel and Boutwell [32], who had shown that the nonspecific binding associated with the direct measurement of binding of phorbol 12-myristate 13-acetate, a considerably more lipophilic phorbol ester than PDBu, could be reduced by washing with acetone and that binding to PKC was measurable under these conditions. As a variant of this approach we explored the use of mixtures of methanol in 20 mM Tris-Cl, pH 7.4 (at 4°), 0.1 M $CaCl_2$ to reduce nonspecifically bound bryostatin 4.

We first needed to determine the sensitivity of PKC to methanol in the wash solution. Since we could measure [^{3}H]PDBu binding to PKC in the absence of methanol, this provided a good test for the stability of the binding activity. We observed that the binding of [^{3}H]PDBu to PKC was essentially unchanged over the range of 0–40% methanol in the wash solution, gradually decreasing to approximately 30% of control values as the methanol in the wash solution was increased to 80% (Fig. 4 top panel). In the case of [^{3}H]bryostatin 4, specific binding was measurable at 40% methanol and changed little over the range of 40–70% methanol (Fig. 4 center panel). The binding was thus somewhat more stable than in the case of PDBu. The partition coefficient dropped dramatically above 40% methanol. A wash with 55% methanol was chosen as the standard condition for the binding assays, reflecting a compromise between the stability of [^{3}H]bryostatin binding at even higher concentrations, e.g. 65%, which further reduced nonspecific binding, and the gradual decrease in the binding of [^{3}H]PDBu above 40% methanol.

Using the methanol wash procedure, we were in a position to characterize the binding of bryostatin 4 to partially purified PKC assayed in the presence of PS (100 μg/mL). Under these conditions we obtained markedly convex Scatchard plots (data not shown). This behaviour was consistent with receptor titration and was also consistent with the competition studies we had conducted previously that had suggested a picomolar dissociation constant for bryostatin 1 [33]. Since binding analysis is problematic unless the receptor concentration is maintained below the dissociation constant, direct measurement of the binding of bryostatin 4 to PKC under these conditions would require bryostatin 4 of a very high specific activity.

Although the high affinity of bryostatin 4 for PKC reconstituted in PS precluded the quantitation of equilibrium binding, the kinetics of release of

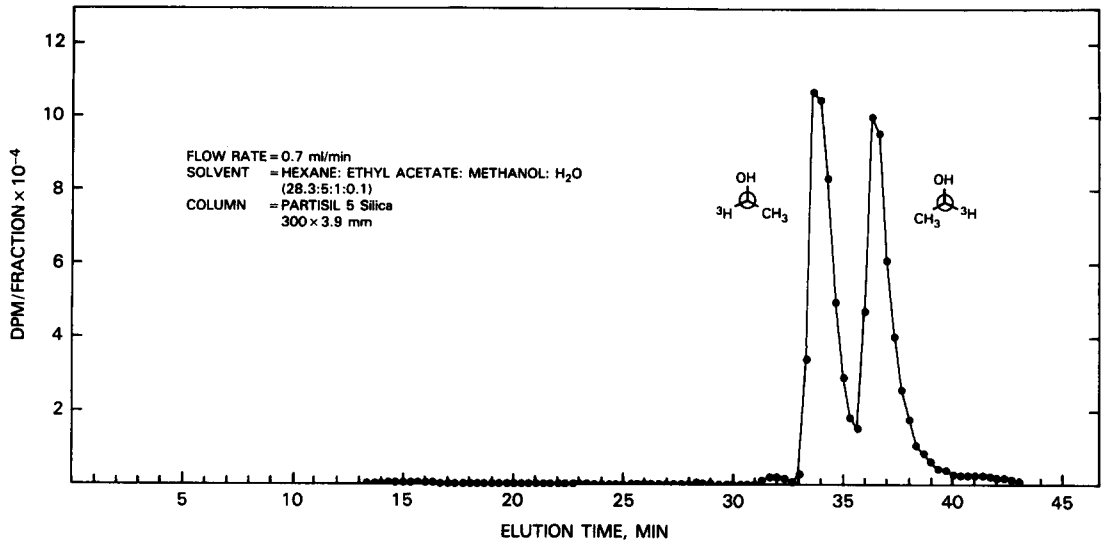

Fig. 3. Separation of [26-^{3}H]bryostatin 4 and its epimer by HPLC. The identity of the peaks was verified by coelution with an authentic sample. The peak with the slower elution time is the epimer.

bryostatin 4 could still be determined under these conditions. As shown in Fig. 5, the levels of bound [^{3}H]bryostatin 4 decreased with a biphasic time course following addition of a large excess of PDBu. The half-life for the first phase was approximately 10 min and that of the second phase was approximately 2–3 hr. Earlier studies in our laboratory had likewise demonstrated biphasic release of PDBu from its receptor [30]. The basis for these biphasic kinetics has not been determined. The slow half-life of release for bryostatin 4 adds an additional complication in the examination of the equilibrium kinetics of binding. The period required for equilibration should be greater than 3.5 × the rate constant of release [34], which would exceed the receptor stability in the assays. A consequence of the pseudoirreversible kinetics arising from the slow off-rate of bryostatin 4 is the apparent noncompetitive inhibition of PDBu binding which we observed.

Since the high affinity and slow off-rate under our usual assay conditions prevented us from observing competition between the bryostatins and phorbol esters or evaluating bryostatin structure–activity relations, we sought to develop alternative conditions which would allow measurement of equilibrium binding. Since PS is required for PKC binding activity, our strategy was to use suboptimal levels of PS; we diluted the PS with Triton X-100, generating a mixed micellar system of the type pioneered by Hannun and Bell [35] for PKC enzymatic analysis. The binding of [^{3}H]bryostatin 4 to PKC in the presence of 1.5 mg/mL Triton X-100 alone was undetectable. With increasing amounts of PS, binding was restored and 17.5% PS sufficed to give near maximal binding (Fig. 6). We chose 20% PS for our standard assay conditions. A 55% methanol wash remained satisfactory under these lipid conditions (Fig. 4, bottom panel). Under these conditions, the binding of [^{3}H]bryostatin 4 was rapid and reversible.

Using Triton X-100/PS to reconstitute PKC, we were able under these conditions to determine the equilibrium binding of [^{3}H]bryostatin 4 to PKC (Fig. 7). The K_d of [^{3}H]bryostatin 4 was 0.7 ± 0.1 nM (mean ± SEM, 7 experiments). As expected, the affinity of PDBu was also reduced. Its K_d was 12 ± 1 nM (mean ± SEM, 12 experiments) compared to a value of 1.4 ± 0.2 (mean ± SEM, 8 experiments) under our usual conditions of 100 μg/mL PS in the absence of Triton X-100. In the presence of Triton X-100/PS, PDBu competitively inhibited the binding of bryostatin 4 (Fig. 7). The K_i for PDBu, calculated from the Scatchard plots, was 11 nM, in excellent agreement with the direct measurements of [^{3}H]-PDBu binding.

The binding of [^{3}H]bryostatin 4 to the three PKC isozymes α, β, and γ was examined to evaluate if the unusual pattern of cellular responses to the bryostatins could be explained by differential activation of specific isozymes. PKC isozymes from two sources were utilized. PKCα, PKCβ, and PKCγ were expressed and partially purified from COS cells, and PKCα and PKCβ were purified from rat brain. Scatchard analysis gave linear plots in all cases; the K_d values are listed in Table 1. No major differences were found in the binding affinities between the different PKC isozymes. PKCβ and PKCγ exhibited approximately equal K_d values; PKCα had an affinity approximately three times higher. The dissociation constants for [^{3}H]bryostatin 4 binding to PKCα and PKCβ from both the COS transfected cells and rat brain were approximately equivalent, indicating that post-transcriptional processing in the COS cells either did not affect binding or was carried out effectively.

One possible explanation for the biphasic release curves for bryostatin 4 could be a difference in the

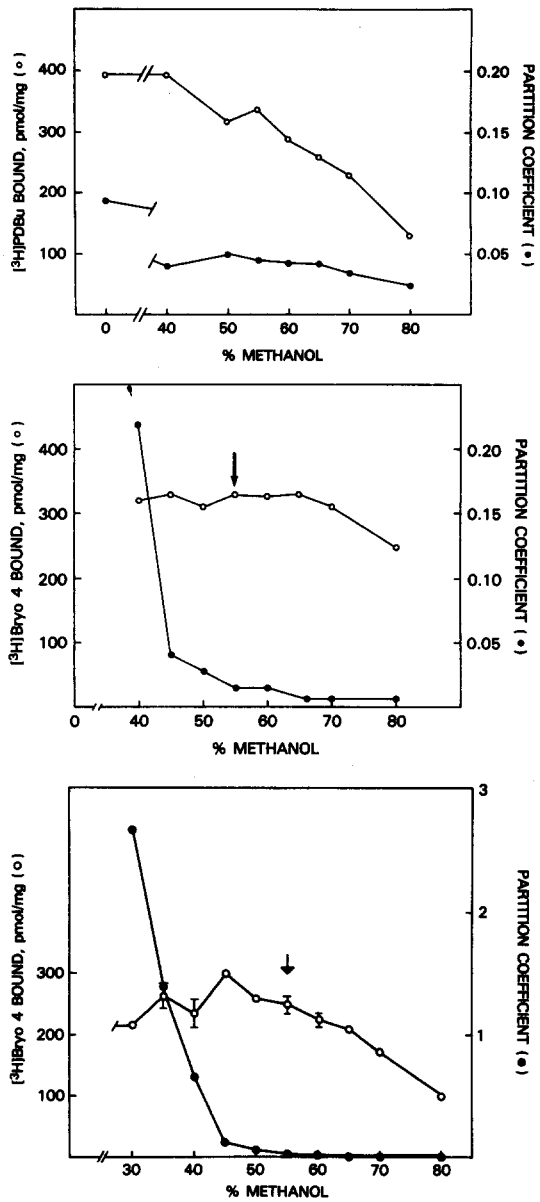

Fig. 4. Binding of [^{3}H]PDBu and [^{3}H]bryostatin 4 to protein kinase C as a function of the percent of methanol in the filter wash solution. The partition coefficient represents the ratio of the label retained on the filter to free label, both determined in the absence of receptor. Top panel: [^{3}H]PDBu (4 nM) binding in the presence of 100 μg/mL PS. Center panel: [^{3}H]bryostatin 4 (6 nM) binding in the presence of 100 μg/mL PS. Bottom panel: [^{3}H]bryostatin 4 (3 nM) binding in the presence of Triton X-100/PS. Key: (○) specific binding, and (●) partition coefficient. The arrow indicates the concentration of methanol subsequently used in our bryostatin binding assay.

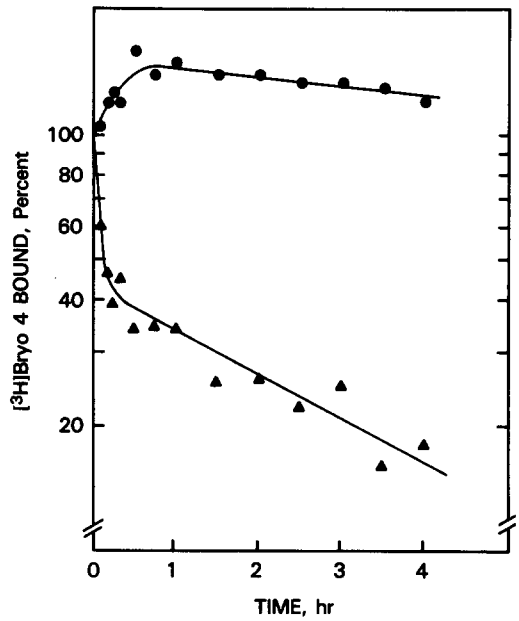

Fig. 5. Time course of release of [^{3}H]bryostatin 4 from PKC. [^{3}H]Bryostatin 4 (7.5 nM) was incubated for 10 min with PKC reconstituted in PS. PDBu (20 mM, ▲) or carrier (●) was added, and sequential samples were taken for the determination of specific binding. A second experiment gave comparable results.

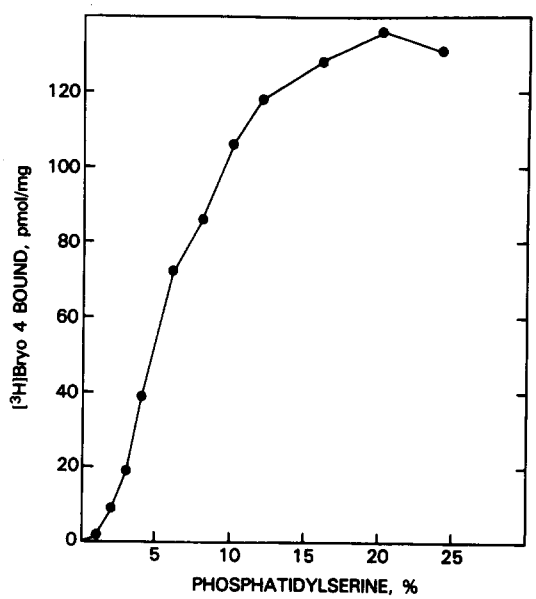

Fig. 6. Binding of [^{3}H]bryostatin 4 to PKC as a function of the percentage of PS in Triton X-100/PS micelles. [^{3}H]-Bryostatin 4 (5 nM) was incubated for 5 min at 37° in the presence of the indicated Triton X-100/PS mixtures and specific binding then determined. The Triton X-100 concentration was held constant at 1.5 mg/mL. A second experiment gave similar results.

rate of release from the PKC isozymes. Therefore, experiments were conducted to determine if the PKC isozymes exhibited different off-rates of binding for bryostatin 4. In all cases release was biphasic with an initial rapid phase followed by a slower

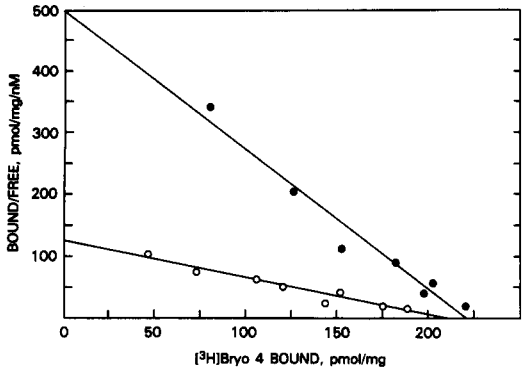

Fig. 7. Scatchard analysis of the binding of [^{3}H]bryostatin 4 with Triton X-100/PS in the presence (○) and absence (●) of 30 nM PDBu. Incubation was for 5 min at 37°. A second experiment gave similar results.

Table 1. Dissociation constants for the binding of [^{3}H]-bryostatin 4 to the isozymes of PKC from transfected COS cells and rat brain

Isozyme, Source	K_d* (nM)
PKCα, COS cells	0.74 ± 0.18 (3)
PKCβ, COS cells	1.15 ± 0.47 (3)
PKCγ, COS cells	1.78 ± 0.23 (3)
PKCα, rat brain	0.42 ± 0.04 (6)
PKCβ, rat brain	0.98 ± 0.24 (5)

* Values are means ± SEM, with the number of experiments given in parentheses.

terminal phase. The PKCα and PKCβ isozymes exhibited similar disappearance curves; the initial half-life was 3–4 min and terminal half-life was 3–4 hr. The PKCγ isozyme had a slower initial phase (half-life = 20 min) and a more rapid terminal disappearance phase (half-life = 70 min) (data not shown). Similar rates of loss were also observed with the PKCα and PKCβ isozymes purified from rat brain. In the absence of competing ligand, binding changed little over the course of the experiment.

DISCUSSION

The bryostatins aptly illustrate the technical difficulties which are associated with the analysis of the binding of very high affinity ligands. Meaningful quantitation is greatly complicated if the receptor concentration is not below the K_d for binding. This requires ligand labeled to very high specific activity. For example, one tritium per molecule corresponds to a specific activity of only 29 Ci/mmol and under our assay conditions represents 3.2 dpm at 1 pM (before consideration of lower specific activity, actual counting efficiency, etc.). Matters are further complicated by the slow off rates often associated with high affinity binding. Equilibrium binding should be conducted over a period of at least 3.5 times the rate constant for release of ligand [34] which, in the case of bryostatin 4 binding to PKC in the presence of PS, is many hours; this time exceeds receptor stability in our assay system. In addition to the problems associated with the high affinity of the bryostatins, the lipophilicity of the bryostatins also presented a technical challenge. These compounds transfer readily to the air/water interface, bind to plasticware, and are taken up avidly into the lipid phase of the assay mixture.

To surmount the complex problems associated with binding of bryostatin 4 to PKC, several modifications of the usual binding approach were required. First, the high binding affinity had to be reduced in order to be able to examine equilibrium binding. PKC can be viewed as an aporeceptor requiring phospholipid for both binding and kinase activity. A decrease in the phospholipid to suboptimal levels by the addition of Triton X-100 to the lipid phase of the assay system was sufficient to increase the off rate so that reversible binding could be measured. Second, the lipophilicity of bryostatin 4 led to a high level of nonspecific ligand binding. This was reduced by organic solvent washes as had been used for earlier studies examining the direct binding of PMA [32]. Binding activity was found to be stable to washes with a Tris-buffered solution containing 55% methanol. Structural analysis of PKC has identified the binding site as the zinc finger regions. Their highly ordered structure should render them relatively resistant to denaturation and may explain the stability of the binding to methanol or acetone [32] washes. A high resistance of phorbol ester binding to proteolytic degradation has also been noted [36].

Using an appropriate hydrophobic environment, we demonstrated that bryostatin 4 interacts with PKC in a manner similar to the phorbol esters. Both compounds compete for the same site as predicted by the fit of the chemical structures to the model of the phorbol ester pharmacophore [5]. The bryostatins differ, however, in their far higher affinity for PKC. This high affinity may suggest that the bryostatins define an extra element of the pharmacophore not seen with the phorbol esters.

The high binding affinity of the bryostatins may represent one mechanism for the difference in biological response relative to the typical, less potent PKC activators. Because of non-specific uptake and receptor titration, the concentrations of bryostatin employed in biological systems typically greatly exceed its K_d. An implication is that bryostatin might therefore interact not only with its primary receptors but also with secondary, lower affinity receptors. These might reflect divergent PKC isozymes, PKC reconstituted into an unfavorable lipid environment, or receptors distinct from PKC. In support of this concept, we found that the "unique" pattern of phosphorylation induced by treatment with nanomolar bryostatin 1 in HL-60 cells could be obtained at micromolar concentrations of PDBu [37]. Likewise, micromolar concentrations of PDBu could partially suppress release of arachidonic acid metabolites in C3H10T1/2 cells (unpublished observations), another characteristic response to the bryostatins.

The slow rate of release of bound bryostatin represents a second possible mechanism for the unique biological pattern of response to the bryostatins. Because phorbol esters both bind rapidly to PKC and are released rapidly, PKC can potentially redistribute within the cell from the sites of initial activation. In contrast, the slow off rate of the bryostatins predicts that PKC will be anchored by the bryostatin at the first site at which PKC comes in contact with the bryostatin. This mechanism might thereby sequester PKC, diminishing its access to a subclass of sites within the cell.

A further consequence of the sustained activation of PKC might be increased down-regulation of the enzyme. The loss of PKC has been demonstrated to be increased by phorbol ester binding and membrane association [38]. It has been suggested that this acts as a feedback mechanism preventing prolonged activation of an important signal transduction pathway [39]. Nonetheless, studies to date have only demonstrated increased down-regulation of PKCα by the bryostatins; this is not true for all isozymes (unpublished data). Accelerated down-regulation is an attractive mechanism for the anomalous behaviour of the bryostatins, since the bryostatins block in a dominant fashion those responses which they do not induce.

Our studies did not find appreciable differences in the binding affinity of bryostatin 4 to the α, β and γ isozymes of PKC. These results are in contrast to the results of Kraft and coworkers who reported lower affinity binding to PKCβ [28] as determined by competition with nonradioactive ligand. Based on the behavior observed with the radiolabeled bryostatin 4, that approach for analyzing binding is not satisfactory. Differential binding to PKC isozymes is an appealing hypothesis for the differences in action between the bryostatins and the phorbol esters but was not observed for PKCα, PKCβ and PKCγ. Differential binding to the noncalcium-dependent isozymes remains a possible mechanism, and these studies are currently in progress in our laboratory.

Differential binding to PKC isozymes also represented a potential mechanism to explain the biphasic release of the phorbol esters and the bryostatins from PKC. However, since this behavior was also observed with preparations of purified isozymes, other mechanisms must be involved.

Finally, the bryostatins and the phorbol esters show different dependence on the hydrophobic phase for binding. Inclusion of Triton X-100 in our assays decreased the affinity of PDBu for PKC approximately 10-fold whereas the change for bryostatin 4 was of a much greater magnitude. Since microenvironments of different lipid composition exist within cells, different dependence of lipid composition on binding predicts different patterns of activation and consequent differences in the patterns of response.

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