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Binding of [26-^{3}H]Bryostatin 1 and Analogs to Calcium-dependent and Calcium-independent Protein Kinase C Isozymes

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SUMMARY

In this study we explored the pattern of protein kinase C (PKC) isozyme selectivity of the bryostatins, a unique class of PKC activators that induce only a subset of the typical phorbol ester responses and antagonize those phorbol ester-mediated responses that they themselves fail to induce. The binding properties of individual recombinant PKC isozymes that had been expressed in insect cells, isolated, and reconstituted in Triton X-100/phosphatidylserine mixed micelles were determined. [^{3}H]Bryostatin 1 showed lower affinity for PKC-β_1 and -γ, compared with PKC-α, -δ, -ϵ, and -η. This pattern contrasts with that observed for other PKC ligands. These latter assays were conducted with isozymes reconstituted in phosphatidylserine, conditions that unfortunately do not permit quantitation of bryostatin 1 binding under equilibrium conditions. Using $\Delta^{19,20}$-bryostatin 10 and $\Delta^{19,20}$-isobryostatin 10, we could distinguish the respective roles of ligand and lipid in the pattern of selectivity. When isozymes were reconstituted in phosphatidylserine vesicles, $\Delta^{19,20}$-bryostatin 10 and $\Delta^{19,20}$-isobryostatin 10 showed similar affinities for PKC-α and -γ, similarly to the phorbol esters. However, in the mixed micellar system, PKC-γ showed a significantly lower binding affinity, as had been observed for bryostatin 1. These results suggest that the unique pattern of biological responses to the bryostatins does not represent a unique pattern of isotype recognition. Furthermore, the lipid environment of PKC plays an important role in determining the binding selectivity for individual isozymes.

The bryostatins (Fig. 1) are macrocyclic lactones isolated from the marine bryozoan *Bugula neritina* and related organisms on the basis of their antileukemic activity (1, 2). Initial attempts to characterize biological activity suggested that the bryostatins functioned as PKC activators. Similarly to phorbol esters, the bryostatins activate PKC enzymatic activity; in addition, they inhibit phorbol ester binding to the enzyme (3). The bryostatins mimic many of the phorbol ester-mediated responses, such as induction of proliferation in Swiss 3T3 cells (4) and activation of neutrophils (5). However, subsequent studies have shown that the biology of these compounds is rather complex. In many cellular systems, the bryostatins not only fail to induce some phorbol ester-like responses but also antagonize those effects mediated by phorbol esters (6–8).

PKC represents a family of calcium-dependent (α, β_1, β_2, and γ) and calcium-independent (δ, ϵ, η, ζ, and θ) isozymes. With the exception of PKC-ζ, all of the PKC isozymes bind phorbol esters with high affinity in the presence of phospholipids. We have previously shown that phorbol esters and related compounds differ in their patterns of recognition for the different PKC isozymes (9).

A common assumption used to explain the heterogeneity of the biological responses to PKC activators, including bryostatins, is that they might differ in their recognition of the different PKC isozymes. In an effort to understand the mechanistic differences between the phorbol esters and bryostatins, we have examined the properties of the binding of bryostatins to recombinant PKC isozymes expressed in the baculovirus-insect cell expression system. We conclude that the bryostatins do not show unique isotype selectivity. Selectivity is appreciable affected, however, by the lipid environment in which PKC is reconstituted.

Experimental Procedures

Materials. [^{3}H]PDBu (17.5 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). [26-^{3}H]Bryostatin 1 (4.8 Ci/mmol) was prepared as previously described for [26-^{3}H]bryostatin 4 (10). Phosphatidylserine and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). PDBu was purchased from LC Services Corp. (Woburn, MA). Synthesis of $\Delta^{19,20}$-bryostatin 10 and $\Delta^{19,20}$-iso-

ABBREVIATIONS: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

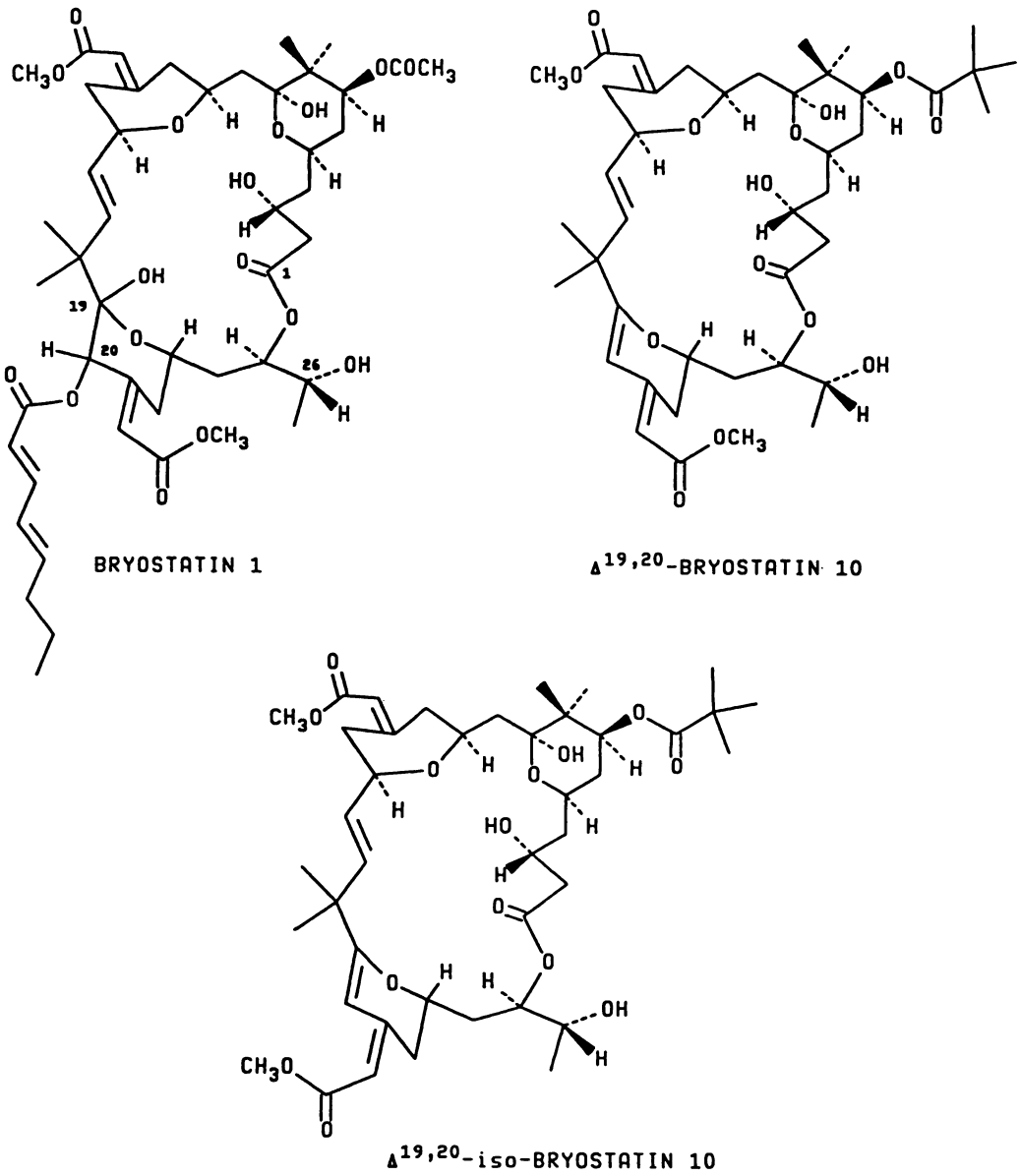

Fig. 1. Structure of bryostatin 1 and derivatives.

bryostatin 10 is described elsewhere.[1] Cell culture reagents and media were purchased from GIBCO (Gaithersburg, MD).

Cell culture. Culture of Sf9 insect cells was done at 27° without CO_2 in spinner flasks, using Grace's insect medium containing 10% fetal bovine serum, 3.3 g/liter yeast extract, 3.3 g/liter lactalbumin hydrolysate, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone.

PKC isozymes. Recombinant PKC isozymes were expressed in Sf9 cells using the baculovirus expression system. Construction of recombinant baculovirus transfer vectors and expression and purification of the individual recombinant PKC isozymes are described elsewhere (9).

Binding of [26-^{3}H]bryostatin 1. Binding of [26-^{3}H]bryostatin 1 was measured by a variant of the filtration assay previously developed in our laboratory for [26-^{3}H]epibryostatin 4. Briefly, partially purified PKC isozymes were incubated (5 min, 37°) with [26-^{3}H]bryostatin 1 in the presence of 20 mM Tris·HCl, pH 7.4, 1 mg/ml IgG, 1.5 mg/ml Triton X-100, 300 μg/ml phosphatidylserine, with either 0.1 mM $CaCl_2$ or 1 mM EGTA, in a total volume of 250 μl. After incubation, the samples were chilled on ice for 5 min, and two aliquots of 50 μl were removed for determination of total radioactivity. Two additional 50-μl samples were applied to ion exchange Whatman paper disks (DE-81), and adsorption was allowed to proceed for 30 sec. The disks were then washed with an ice-cold solution containing 55% (v/v) methanol and 20 mM Tris·HCl, pH 7.4. Aquasol (3 ml) was added both to the 50-μl aliquots removed for determination of total radioactivity and to the filters (for determination of bound ligand), and radioactivity was measured in an LKB 1218 scintillation counter. Nonspecific binding was determined under the same conditions in the absence of added PKC isozymes. Free [26-^{3}H]bryostatin 1 was determined by subtracting the amount of ligand bound to the filters from total ligand. For competition assays, six to eight concentrations of competing ligands and a fixed concentration of [26-^{3}H]bryostatin 1 (3 nM) were used. K_i values were calculated as described for [^{3}H]PDBu binding (see below). When the assays were performed in the presence of 1 mM EGTA, calcium levels were below the detection limit (5 nM), as measured by atomic absorption.

Binding of [^{3}H]PDBu. [^{3}H]PDBu binding to PKC isozymes was measured using the polyethylene glycol precipitation assay developed in our laboratory (11), using 100 μg/ml phosphatidylserine vesicles and

[1] G. R. Pettit, P. M. Blumberg, and F. Gao. Synthesis of 26-oxo-bryostatin 1 and $\Delta^{19,20}$-bryostatin 10. Manuscript in preparation.

either 0.1 mM $CaCl_2$ or 1 mM EGTA, as described previously (9). To measure competition by bryostatins with [^{3}H]PDBu binding, a fixed concentration of [^{3}H]PDBu (3 nM) and six to eight increasing concentrations of the competing ligand were used. The K_i values for the competing ligands were calculated from the ID_{50} values by using the relationship $K_i = ID_{50}/(1 + L/K_d)$, where L is the concentration of free [^{3}H]PDBu at the ID_{50} and K_d is the dissociation constant for [^{3}H]PDBu under the specific assay conditions for the corresponding PKC isozyme (see Ref. 9). The ID_{50} values were determined from the competition curve. Assays were performed in triplicate, and determinations generally differed by <10%.

PKC assay. PKC activity was measured as described previously (9), using 100 μM PDBu as a measure of 100% of activation.

Results

To evaluate whether the unusual pattern of cellular responses to the bryostatins could be explained by differential recognition of specific isozymes, we performed Scatchard analysis of [26-^{3}H]bryostatin 1 binding to PKC isozymes α, β_1, γ, δ, ε, η, and ζ expressed in the baculovirus-Sf9 cell system. This system is highly efficient for producing fully active PKC isozymes, as described by several laboratories including our own (9, 12, 13). Contamination between isozymes is avoided because each expressed protein comes from a defined cDNA, and lack of contamination was confirmed with specific antibodies (Ref. 9 and data not shown). [26-^{3}H]Bryostatin 1 binding was quantitated using conditions previously developed for [26-^{3}H]bryostatin 4. Although bryostatins 1 and 4 are similar, bryostatin 1 is of particular interest because it is currently undergoing clinical trials and is more readily available. The assay conditions for [26-^{3}H]bryostatin 1 used PKC reconstituted in Triton X-100/phosphatidylserine mixed micelles, in contrast to our usual assay conditions for phorbol esters. Under the latter conditions, the ultra-high affinity of the bryostatins and the slow off-rate make determination of equilibrium dissociation problematic (see Ref. 10).

Scatchard analysis of [26-^{3}H]bryostatin 1 binding to the different PKC isozymes in the presence of Triton X-100/phosphatidylserine mixed micelles and calcium gave linear plots with affinities in the low nanomolar range, thus suggesting that under these binding conditions only high affinity sites could be detected (Fig. 2). Dissociation constants (K_d) are listed in Table 1. PKC-β_1 and -γ, two of the calcium-dependent isozymes, showed relatively weaker affinity, compared with the other calcium-dependent isozyme (PKC-α) or the calcium-independent isozymes (PKC-δ, -ε, and -η). Interestingly, the affinity of [26-^{3}H]bryostatin 1 for PKC-γ in the absence of calcium was even lower. The binding affinity for the other PKC isozymes remained unchanged in the presence or absence of calcium. For comparison, we also listed in Table 2 the K_d values for [^{3}H]PDBu as determined in our laboratory (9). The recognition pattern of [^{3}H]PDBu for the PKC isozymes differed from that of [26-^{3}H]bryostatin 1, showing a weaker relative affinity for the calcium-independent isozymes PKC-δ, -ε, and -η. The K_d of [^{3}H]PDBu/K_d of [26-^{3}H]bryostatin 1 ratio suggests a relative preference of the calcium-dependent PKC isozymes for [^{3}H]PDBu. As was also the case for [^{3}H]PDBu, [26-^{3}H]bryostatin 1 did not bind to PKC-ζ. Binding of [26-^{3}H]bryostatin 1 to PKC-ζ was also undetectable when PKC-ζ was reconstituted in phospatidylserine vesicles at high receptor concentrations, suggesting that in this isozyme not even a low affinity site is present (14).

A major complication in the direct comparison of the binding properties of phorbol esters and bryostatins was that the lipid environments used in the assays were different in each case. [26-^{3}H]Bryostatin 1 binding assays were performed using phosphatidylserine/Triton X-100 mixed micelles and binding of [^{3}H]PDBu was done with 100% phosphatidylserine vesicles. Determination of the binding affinity of [26-^{3}H]bryostatin 1 using the latter lipid conditions has not been possible, reflecting its very high affinity under those conditions (15). To compare the affinity of bryostatins in the two lipid systems, we took advantage of two bryostatin derivatives, $\Delta^{19,20}$-bryostatin 10 and $\Delta^{19,20}$-isobryostatin 10 (see Fig. 1). Computer modeling had suggested that the oxygen in position C-19 of the bryostatins plays a role in the interaction with the receptor (16). We were able to confirm this prediction using these compounds. The reduction in affinity proved to be such that these bryostatin analogs could be assayed under either set of lipid conditions.

We measured the inhibition by $\Delta^{19,20}$-bryostatin 10 of [^{3}H]PDBu binding, using 100% phosphatidylserine vesicles, either in the presence or in the absence of calcium. As shown in Table 2, this bryostatin derivative showed similar potencies for all of the calcium-dependent and calcium-independent PKC isozymes, independently of the presence or absence of the divalent cation. It thus reflected a pattern of isotype recognition similar to that of the indole alkaloids and diacylglycerol (9), not to that of bryostatin 1 assayed in Triton X-100/phosphatidylserine.

Unfortunately, this difference might reflect either the influence of the lipid environment or a difference between $\Delta^{19,20}$-bryostatin 10 and bryostatin 1. To distinguish between these possibilities, we compared binding potencies using mixed micelles and phosphatidylserine vesicles for the two isozymes that show the greatest difference in K_d, i.e., PKC-α and PKC-γ. Because of the extremely limited amounts of compound available, we used $\Delta^{19,20}$-isobryostatin 10. Competition experiments using $\Delta^{19,20}$-isobryostatin 10 as a competing ligand showed that, as with $\Delta^{19,20}$-bryostatin 10, the K_i values were very similar for PKC-α and PKC-γ using phosphatidylserine vesicles. However, in the mixed micellar assay the competing ligand showed greater potency for binding to PKC-α than to PKC-γ (Fig. 3). Results were similar in the presence and in the absence of calcium (data not shown). Thus, it appears that the unique pattern of isozyme selectivity in fact reflects the lipid environment in the usual bryostatin binding assays, rather than unique selectivity of the ligand.

A distinct mechanism for bryostatin selectivity would be involved if the PKC isozymes differed in their ability to be activated upon bryostatin binding. We therefore compared the levels of enzymatic activity induced by PDBu and bryostatin 1 for the isozymes α, β_1, γ, δ, and ε, measuring phosphorylation using a peptide substrate based on the pseudosubstrate sequence of PKC-α (9). Similar activities were induced by the two ligands (Fig. 4). These findings confirm and extend earlier reports, using undefined mixtures of PKC isozymes, that bryostatin 1 fully activated PKC (3).

Discussion

The bryostatins represent a family of natural products showing an unusual pattern of biological responses, compared with the typical PKC ligands, i.e., the phorbol esters. For a range of responses, the bryostatins are the only known selective antagonists of the PKC pathway. A variety of possible mechanisms

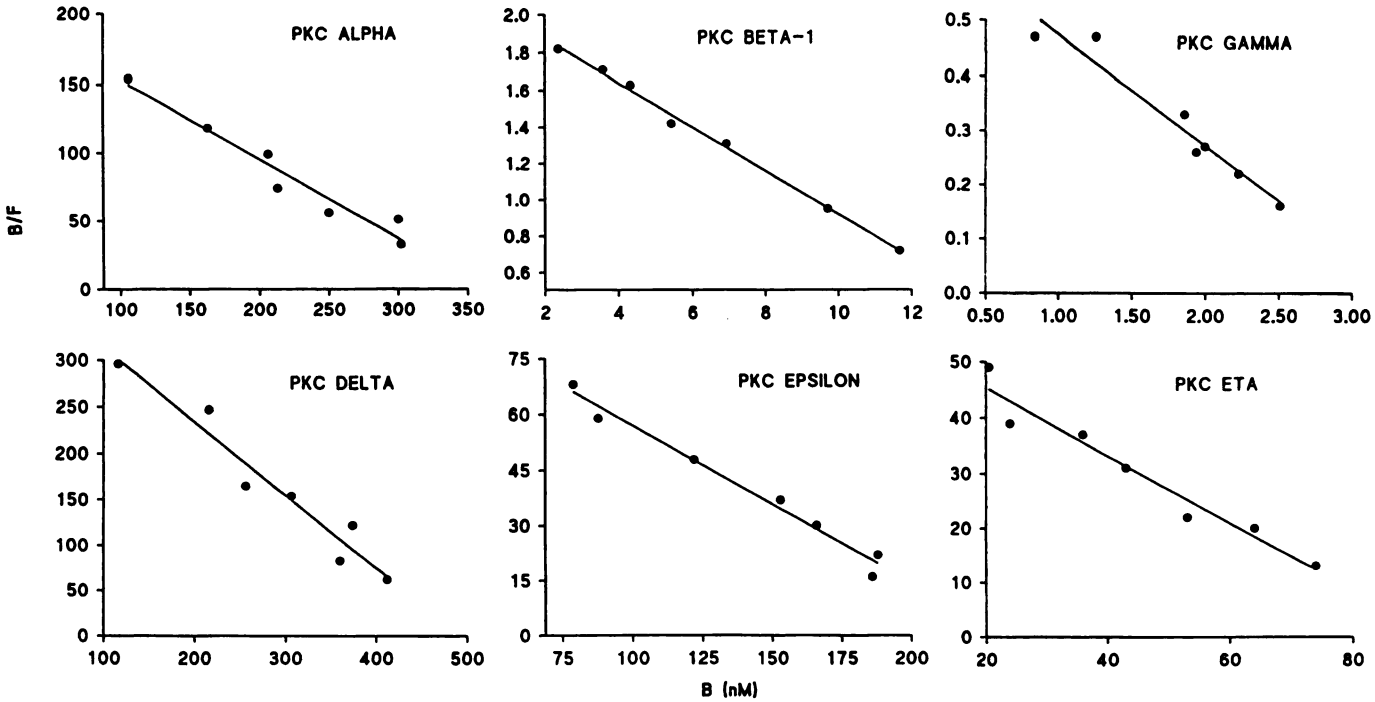

Fig. 2. Scatchard plots for [26-^{3}H]bryostatin 1 binding to PKC isozymes. Increasing concentrations of [26-^{3}H]bryostatin 1 (0.5–5 nM) were incubated with the different PKC isozymes, using phosphatidylserine/Triton X-100 mixed micelles and 0.1 mM $CaCl_2$, and binding was measured as described in Experimental Procedures. Each experiment was done three or four times, and for each PKC isozyme a representative experiment is shown.

TABLE 1

Dissociation constants (K_d) for [26-^{3}H]bryostatin 1 for the different PKC isozymes

Experiments were performed using phosphatidylserine/Triton X-100 micelles and either 0.1 mM $CaCl_2$ or 1 mM EGTA. Each K_d value represents the mean ± standard error of the number of experiments in parentheses. Binding affinities for [^{3}H]PDBu (from Ref. 9) are included for comparison.

PKC isozyme	K_d				Ratio of K_d for PDBu/K_d for bryostatin 1	
	[26-^{3}H]Bryostatin 1		[^{3}H]PDBu			
	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}
	nM					
α	1.7 ± 0.1 (3)	1.8 ± 0.2 (3)	0.20 ± 0.03	0.15 ± 0.02	0.11	0.08
β_1	5.6 ± 1.1 (4)	6.9 ± 0.7 (3)	0.20 ± 0.02	0.14 ± 0.01	0.04	0.02
γ	5.3 ± 0.3 (3)	16.0 ± 2.6 (4)	0.33 ± 0.03	0.37 ± 0.03	0.06	0.02
δ	1.2 ± 0.1 (4)	1.4 ± 0.1 (3)	0.94 ± 0.09	0.71 ± 0.10	0.78	0.51
ε	1.7 ± 0.2 (4)	2.0 ± 0.1 (3)	0.81 ± 0.09	0.63 ± 0.07	0.59	0.32
η	1.8 ± 0.1 (3)	2.2 ± 0.6 (3)	0.87 ± 0.15	0.58 ± 0.11	0.48	0.26
ζ	No binding (3)	No binding (2)	No binding	No binding		

TABLE 2

Binding affinity of $\Delta^{19,20}$-bryostatin 10 in phosphatidylserine vesicles

Increasing concentrations of $\Delta^{19,20}$-bryostatin 10 were used to compete with [^{3}H] PDBu binding, using 100% phosphatidylserine vesicles and either $CaCl_2$ or 1 mM EGTA. Binding was measured using the polyethylene glycol precipitation assay. K_i values were calculated from the ED_{50} values as described in Experimental Procedures. Values are expressed as mean ± standard error. The numbers of experiments are shown in parentheses. PKC-η could not be assayed because of limited amounts of the compound.

PKC isozyme	K_i	
	+Ca^{2+}	−Ca^{2+}
	nM	
α	0.96 ± 0.20 (3)	0.75 ± 0.08 (3)
β_1	0.58 ± 0.10 (3)	0.54 ± 0.07 (3)
γ	0.76 ± 0.09 (3)	0.78 ± 0.12 (3)
δ	0.77 ± 0.06 (3)	0.98 ± 0.19 (3)
ε	0.69 ± 0.35 (3)	1.00 ± 0.10 (3)

might account for their atypical biological responses. First, the high binding affinity of the bryostatins, compared with less potent activators like phorbol esters, may represent one mechanism (15). Because the concentrations of bryostatins used in cellular systems greatly exceed their K_d values, these compounds might interact with secondary low affinity receptors, such as receptors distinct from PKC, divergent PKC isozymes, or PKC reconstituted into an unfavorable lipid environment. A second mechanism to explain the unique pattern of biological responses to the bryostatins is the slow rate of release of bound bryostatin from PKC. In this case, PKC cannot be redistributed within the cell because the receptor is sequestered by the bryostatin at the first site at which PKC binds the ligand. The access of PKC isozymes to different subcellular sites where some of their targets might be located would be thus constrained.

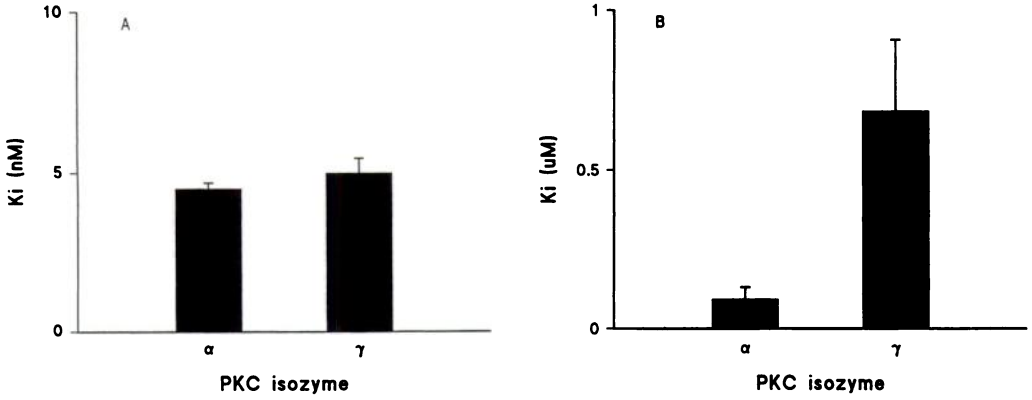

Fig. 3. Binding of $\Delta^{19,20}$-isobryostatin 10 to PKC-α and PKC-γ. The K_i for $\Delta^{19,20}$-isobryostatin 10 was determined by competition with [^{3}H]PDBu binding in phospholipid vesicles (A) or by competition with [26-^{3}H]bryostatin 1 binding in phosphatidylserine/Triton X-100 vesicles (B). ID_{50} values were obtained from inhibition curves, and K_i values were calculated from the ID_{50} values as described in Experimental Procedures.

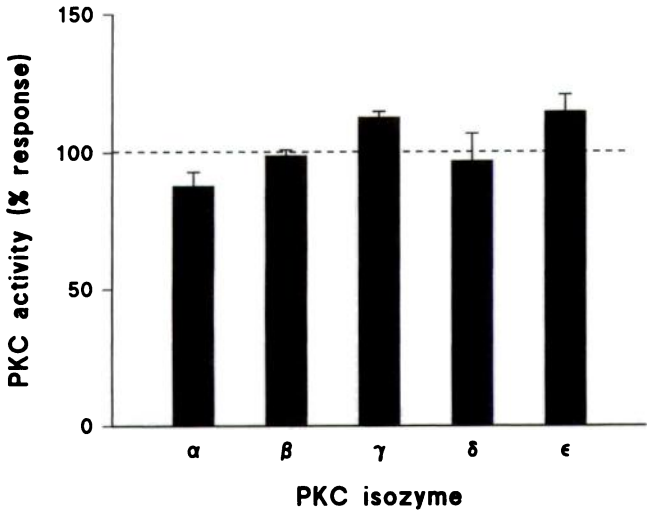

Fig. 4. PKC activation by bryostatin 1. Bryostatin 1 (10 μM) was used to activate PKC isozymes. Values are expressed as the percentage of the maximum activity generated by using 100 μM PDBu and represent the mean ± standard error of three to five experiments.

A logical hypothesis that still needed to be tested was that of a differential pattern of isozyme selectivity for the bryostatins. In fact, this is the first study that compares the binding of bryostatins to both calcium-dependent and calcium-independent PKC isozymes. It is interesting that, using our standard mixed micellar system, [26-^{3}H]bryostatin 1 showed relatively weak binding to calcium-dependent isozymes PKC-β_1 and -γ. In a previous report we have likewise shown a relative higher potency of the related [^{3}H]bryostatin 4 for PKC-α, compared with PKC-β and -γ (10). Our results are in contrast to those of Kraft *et al.* (3), who reported lower affinity for PKC-β, relative to PKC-α and -γ. Those authors did not consider the unusually slow kinetics of the bryostatin, its high affinity, and its instability in aqueous solution, and therefore the assays were probably done under nonequilibrium conditions. Although the differences in potencies that we detected between isozymes are significant, we do not favor the idea of a difference in isozyme selectivity as an explanation for the unique pattern of responses to the bryostatins. PKC-γ, the most divergent PKC isozyme in terms of affinity for bryostatins, is selectively located in brain, and most of the atypical responses to these class of ligands were reported in cellular systems (epidermal cells and fibroblasts) where this isozyme is not present (17).

The development of a phosphatidylserine/Triton X-100 micellar assay for binding of radiolabeled bryostatins overcame many of the methodological problems associated with these highly potent ligands, because under these conditions the binding affinity was reduced from the picomolar to the nanomolar range. At picomolar affinities, the measured K_i simply reflects receptor titration, rather than equilibrium binding (15). On the other hand, binding of [^{3}H]PDBu to PKC in mixed micelles gave very weak affinities, and experiments showed a high degree of variability and lack of reproducibility. Under these circumstances, a comparison of [^{3}H]PDBu and [26-^{3}H]bryostatin 1 using the micellar system was unreliable. For this reason, the technical approach we adopted involved the use of modified bryostatins with lower affinity that could be measured under both lipid conditions.

Computer analysis of the phorbol ester pharmacophore suggests that the critical residues in phorbol are the hydroxyl groups at positions C-9 and C-20, together with the C-4 hydroxyl group or the C-3 ketone (18, 19). By modeling the bryostatins to the phorbol ester pharmacophore, it was demonstrated that those residues in the phorbol esters show an excellent spatial correlation with the C-1 carbonyl and the C-19 and C-26 hydroxyl groups in the bryostatins. We previously showed that changes at position C-26 dramatically reduced the potency of the bryostatins (20). Our results with $\Delta^{19,20}$-bryostatin 10 confirm that the C-19 hydroxyl group of the bryostatins forms one of the key units of the pharmacophore and is essential for binding, as predicted by computer modeling.

The $\Delta^{19,20}$-isobryostatin 10 derivative showed measurable binding affinity under either of the lipid conditions tested. In Triton X-100 micelles a weaker affinity for PKC-γ, compared with PKC-α, was observed for this derivative. Nevertheless, potencies of the $\Delta^{19,20}$-bryostatin derivatives for the different PKC isozymes were relatively similar in phosphatidylserine vesicles. Under these assay conditions, and unlike phorbol esters and mezerein derivatives, bryostatins resembled the diacylglycerols and indole alkaloids as PKC ligands, because they had similar binding affinities for calcium-dependent and calcium-independent PKC isozymes (9).

Our results clearly indicate that lipid composition may play an important role in determining the affinity of the binding of

the different ligands to PKC isozymes. Although we do not have evidence for such regulatory mechanisms at the intracellular level, the binding affinities of phorbol esters in *in vitro* experiments depend dramatically on the phospholipid composition (21). Moreover, the binding affinity of [^{3}H]PDBu in intact cells is often orders of magnitude weaker than that observed with purified PKC reconstituted with phosphatidylserine (22). Because PKC can be translocated from the cytosol to the membrane, cytoskeleton, and nucleus, different lipid microenvironments might dictate the binding affinity of the ligands and contribute to the subcellular distribution of different PKC isozymes. It is noteworthy that different PKC isozymes show different subcellular distributions.

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