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DEMONSTRATION OF SUB-NANOMOLAR AFFINITY OF BRYOSTATIN 1 FOR THE PHORBOL ESTER RECEPTOR IN RAT BRAIN

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Abstract—The effect of bryostatin 1 on [26-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding to a washed particulate preparation from rat brain was examined. Bryostatin 1 inhibited phorbol ester binding at concentrations considerably lower than previously reported. As would be expected for a ligand of high affinity, the apparent displacing potency of bryostatin 1 was dependent on the concentration of tissue/binding sites included in the assay. Decreasing the concentration of [^{3}H]PDBu binding sites to the picomolar detection limit resulted in apparent bryostatin displacing potencies in the picomolar range with these values representing an upper estimate of the true affinity. When included in saturation studies with [^{3}H]PDBu, bryostatin 1 displayed mixed competitive/non-competitive inhibition. Using either repetitive washing or dialysis of the membrane preparation, it was not possible to reverse the inhibition produced by bryostatin 1. The greater affinity of bryostatin 1 compared to other classes of agents that act directly on protein kinase C and the stability of its association may contribute to the unique biological properties of the bryostatins.

The bryostatins, natural products isolated from the marine bryozoan *Bugula neritina* [1], are macrocyclic lactones substituted with one or more ester side chains. The compounds were initially identified on the basis of antineoplastic activity against the murine P388 lymphocytic leukemia (PS system). Subsequently, they have attracted considerable attention because they mimic phorbol esters in some biological systems [2, 3] while displaying antagonist properties in others. Examples of such antagonism have been reported for HL-60 promyelocytic leukemia cells [4], Friend erythroleukemia cells [5], primary mouse epidermal cells [6] and GH_4C_5 rat pituitary cells [7]. Protein kinase C is the major receptor for the phorbol esters and plays a central role in the phosphatidylinositol signal transduction pathway [8–10]. Understanding the mechanism by which the bryostatins antagonize phorbol ester action may permit selective intervention in this pathway.

A direct action of bryostatin 1 on protein kinase C has been demonstrated by its ability to stimulate protein kinase C enzymatic activity [4]. In addition, bryostatin 1 has been reported to displace [26-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding with an IC_{50} of 5.3 nM in HL-60 cells [4] and 25–30 nM in Swiss 3T3 cells [3]. As yet, the mechanism by which bryostatin 1 exerts its complex pattern of stimulation and antagonism of protein kinase C mediated events is not understood. The following experiments were undertaken to describe more rigorously the interaction between bryostatin 1 and the phorbol ester binding site on protein kinase C. A well-washed particulate preparation of rat brain was chosen as the source of the enzyme. Such a preparation is more stable than reconstituted, purified enzyme and is amenable to a high volume filtration protocol for separation of the bound ligand ([^{3}H]PDBu), both features essential for the experiments described here.

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METHODS

Bryostatin 1 was isolated as described previously [1]. Phosphatidylserine (No. P8518, Sigma, St. Louis, MO) and dimyristoyl phosphatidylcholine (No. P6392, Sigma) were dissolved in chloroform, the solution was transferred to a glass tube, the solvent was removed under a stream of N_2, and the phospholipid was suspended by sonication into the binding assay buffer.

Rat brains (male Sprague–Dawley) were obtained frozen from Zivic-Miller (Zelienople, PA). The brains were homogenized for 20 sec at 4° in 20 vol. of 10 mM Tris–Cl (pH 7.4 at 25°) using a Polytron homogenizer at a setting of 5. The homogenate was centrifuged for 10 min at 14,000 rpm (16,000 *g*). The pellet was resuspended and recentrifuged three times, then suspended in 10 vol. of homogenization buffer and stored in aliquots at −70°. Tissue concentrations are expressed as mg wet weight of tissue/ml in the final incubation. These values may be converted to units of protein concentration using the value 0.027 mg protein/mg tissue obtained using a dye-binding protein assay (Bio-Rad Laboratories, Richmond, CA).

The binding assay was performed at 30° using a

buffer of 50 mM Tris–Cl (pH 7.4 at 25°) containing 2 mg/ml bovine serum albumin. Incubations were initiated by the addition of the brain particulate preparation (suspended by passing through a 23 gauge needle) to triplicate tubes containing the indicated concentrations of [^{3}H]PDBu (18.9 Ci/mmol, New England Nuclear, Boston, MA) and competing agent. Unless otherwise specified, incubations were allowed to continue for 100 min before filtration through glass-fiber filters (No. 7035, Skatron, Sterling, VA) pre-wetted with 2 mg/ml poly-L-lysine (No. P7890, Sigma). Filter disks were washed with 4 ml of buffer at room temperature, and bound radioactivity was assessed using liquid scintillation counting.

Binding data were analyzed using the collection of computer programs described by McPherson [11]. The concentration of non-radioactive ligand producing 50% inhibition (IC_{50}) of [^{3}H]PDBu binding and the Hill coefficient (slope) were provided by the iterative sigmoidal curve fitting program EBDA. The dissociation constant (K_D) and the total receptor concentration (B_{max}) were determined using the iterative non-linear curve fitting program LIGAND [12]. Errors are expressed as the standard errors of the mean provided by the computer analysis of all data points in a single experiment. Unless otherwise stated, non-specific [^{3}H]PDBu binding was a parameter determined by the computer analysis. Bryostatin 1 and PDBu defined a similar level of non-specific binding that represented less than 2% of total radioligand added.

RESULTS

The time course for [^{3}H]PDBu binding at 30° to the rat brain particulate preparation was determined in the absence and presence of bryostatin 1 (Fig. 1). An essentially constant level of [^{3}H]PDBu binding was found between 50 and 100 min. Based on this result, a standard incubation period of 100 min was employed in the experiments detailed below. The initial pattern of rise and fall in [^{3}H]PDBu binding

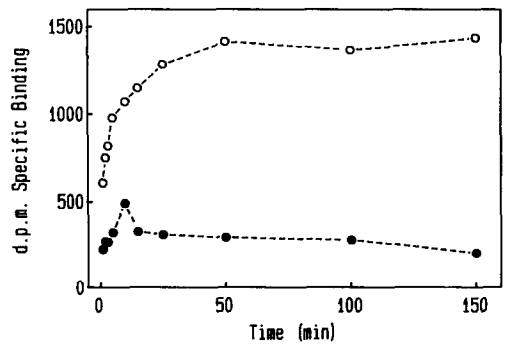

Fig. 1. Time course for association of [^{3}H]PDBu in the absence (○) and presence (●) of 1 nM bryostatin 1. Incubations were performed in triplicate at 30° in a volume of 0.75 ml with [^{3}H]PDBu at a concentration of 0.4 nM (13,000 dpm) and 0.56 mg tissue/ml. Non-specific binding was determined in triplicate using 1 μM PDBu at each time point. Two other experiments gave similar results.

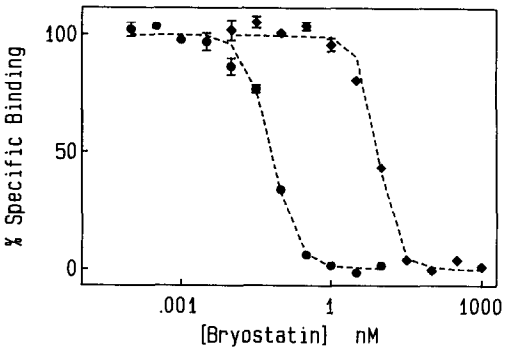

Fig. 2. Inhibition of [^{3}H]PDBu binding by bryostatin 1 at different concentrations of tissue. Key: (◆) 6.7 mg tissue/ml, incubation volume: 0.75 ml; and (●) 0.067 mg/ml tissue, incubation volume: 7.5 ml. Each data point with error bars represents the mean and standard error of the mean for triplicate determinations.

in the presence of bryostatin 1 was a reproducible feature (observed in each of three experiments). The kinetics are those predicted for a two ligand system in which the competing ligand equilibrates more slowly than does the radioactive ligand (see Discussion).

Representative curves for the inhibition of [^{3}H]PDBu binding as a function of bryostatin 1 concentration are presented in Fig. 2. The apparent potency of bryostatin 1 displayed a marked dependency on the amount of tissue employed. In the presence of 6.7 mg tissue/ml, bryostatin 1 displayed an IC_{50} of 8.6 nM ([^{3}H]PDBu = 140 nM, B_{max} = 6.0 nM, incubation volume 0.75 ml). In the presence of 0.067 mg/ml of tissue, bryostatin 1 had an IC_{50} of 0.065 nM ([^{3}H]PDBu = 0.61 nM, B_{max} = 0.038 nM, incubation volume 7.5 ml). In an effort to describe the extent of this relationship in further detail, a series of similar experiments were performed over a range of tissue concentrations from 0.007 to 7 mg/ml. To accommodate this range while providing significant numbers of bound counts, incubation volumes of 0.75, 7.5 and 22.5 ml were employed. Using this approach, IC_{50} values for bryostatin 1 were obtained over three orders of magnitude from nanomolar to picomolar (from 8.6 nM to 2.5 pM; Fig. 3). Competition studies between [^{3}H]PDBu and PDBu were performed in parallel at each tissue concentration so it was also possible to test for the relationship between the B_{max}, expressed in terms of concentration, and the IC_{50} for bryostatin 1. The log/log regression yielded a correlation coefficient of 0.932. The B_{max} and IC_{50} values were almost equivalent; the slope of the linear regression line was 1.006, and the predicted IC_{50} value was 1.23 nM at a B_{max} value of 1 nM.

Bryostatin 1 competition experiments (Figs. 2 and 3) were performed over a range of [^{3}H]PDBu concentrations to ensure that only a small proportion (less than 10%) of the binding sites were occupied by the radioactive ligand. Experiments in which the number of binding sites was held constant and the concentration of [^{3}H]PDBu was varied indicated little dependence of the IC_{50} for bryostatin 1 on

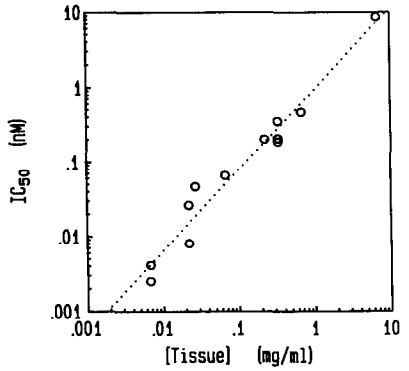

Fig. 3. Log–log plot of IC_{50} values obtained for bryostatin 1 as a function of tissue concentration. Data were obtained in experiments similar to that shown in Fig. 2 with [^{3}H]PDBu maintained at concentrations such that bound ligand represented less than 10% of total radiolabel. The linear regression line for the data is indicated, and the correlation coefficient was 0.980.

radioligand concentration and a consistently steep slope (Table 1, see also Fig. 2). In contrast, the IC_{50} for non-radioactive PDBu was dependent on the concentration of [^{3}H]PDBu.

To further characterize the inhibition of [^{3}H]PDBu binding by bryostatin 1, particulate preparations were exposed to bryostatin 1 before addition of the radioligand. The preincubation caused a decrease both in the affinity and in the number of [^{3}H]PDBu binding sites (Table 2). Extensive washing procedures did not reverse either of these effects of bryostatin 1 (Table 2). Another approach undertaken to reverse the inhibition of [^{3}H]PDBu binding produced by bryostatin 1 was to pretreat the tissue preparation with bryostatin 1 and then to incubate at 30° with phosphatidylserine or dimyristoyl phosphatidylcholine at concentrations of up to 100 μg/ml. Dramatically increasing the total lipid phase present in the assay with either of these phospholipids did not reverse the inhibition by bryostatin 1 (data not shown).

DISCUSSION

Previous investigations of the binding characteristics of [^{3}H]PDBu have found evidence for more than one binding site particularly at high ligand concentrations [13]. Binding to such low affinity site(s) is difficult to define and was effectively ignored by employing low concentrations of radiolabel and by analyzing saturation data with the program LIGAND assuming only a single site and allowing lower affinity interactions to be included in the program's estimate of non-specific binding.

The kinetic properties of the interaction of bryostatin 1 with the [^{3}H]PDBu binding site appear complex and may explain in part the anomalous activities of this class of compounds. The capacity of radioligand binding in the presence of a competitor to overshoot the equilibrium value, as noted in Fig. 1, has been described by Motulsky and Mahan [14] as being characteristic of a system in which the competitor dissociates more slowly than does the radioligand. Conceptually, at early time points both

Table 2. [^{3}H]PDBu binding in the absence, presence or after washout of 0.5 nM bryostatin 1

Group	Bryostatin 1	K_D (nM)	B_{max} (nM)
Coincubation	−	5.1 ± 0.7	0.51 ± 0.05
	+	6.6 ± 0.5	0.33 ± 0.02
Washout	−	4.3 ± 0.7	0.44 ± 0.05
	+	9.4 ± 1.5	0.31 ± 0.04
Dialysis	−	4.3 ± 0.5	0.43 ± 0.04
	+	7.9 ± 1.5	0.22 ± 0.04

Six aliquots of the particulate tissue preparation were suspended (20 mg of tissue in 30 ml) in assay buffer and incubated in the absence or presence of bryostatin 1 at 23° for 60 min. For the coincubation group, control and treated suspensions were then added directly into the binding assay system which contained 1.2 nM [^{3}H]PDBu and eleven concentrations of PDBu in triplicate ranging from 0.01 to 1000 nM such that the incubates were diluted only by 10% (0.675 ml in an assay volume of 0.75 ml). Before inclusion in the binding assay, the suspensions for the second group (washout) were centrifuged three times, with resuspension in fresh buffer (0.67 mg tissue/ml) and incubated for a further 60 min on each occasion. The suspensions for the dialysis group were centrifuged, resuspended in 0.5 ml of buffer, dialyzed against the assay buffer for 48 hr and diluted to the original volume of buffer before the [^{3}H]PDBu binding parameters were determined. Experiments under each set of conditions were repeated on at least one other occasion with similar results. Values are means ± SEM.

Table 1. Inhibition of [^{3}H]PDBu binding by nonradioactive PDBu or bryostatin 1 as a function of [^{3}H]PDBu concentration

[^{3}H]PDBu Concn (nM)	PDBu		Bryostatin 1	
	IC_{50} (nM)	Slope	IC_{50} (nM)	Slope
65	89 ± 21	0.80 ± 0.14	0.44 ± 0.10	2.16 ± 0.76
6.1	9.0 ± 1.7	0.79 ± 0.07	0.35 ± 0.02	1.30 ± 0.07
0.70	2.1 ± 0.1	0.73 ± 0.05	0.20 ± 0.01	1.89 ± 0.12

Incubations included 0.33 mg tissue/ml in a volume of 0.75 ml. Each dose–response curve employed eleven concentrations of the displacing agent with triplicate determinations at each concentration. Pooled analysis of the PDBu/[^{3}H]PDBu experiments indicated a B_{max} of 0.36 nM. Results (mean ± SEM) are representative of several experiments employing a range of radioligand concentrations.

labeled ligand and competitor are readily associating with the numerous unoccupied sites, whereas with time most sites become available only through dissociation of the radioligand. At the 100-min time point chosen for routine assays, the level of inhibition produced by bryostatin 1 appears to have attained a steady-state if not a true equilibrium. A radiolabeled bryostatin will be required to establish the association/dissociation kinetics in detail.

Determination of the true potency of bryostatin 1 at the phorbol ester receptor also remains a difficult problem. In our hands, the apparent potency of bryostatin 1 displayed an absolute dependence on the concentration of receptor sites available. When the concentration of [^{3}H]PDBu binding sites was reduced to picomolar levels, the IC_{50} was similarly reduced to picomolar levels. The lowest value obtained (2.5 pM) represents an upper limit of what the true affinity of bryostatin 1 is at this receptor. Receptor concentrations could not be reduced further due to the limited specific activities of the radiolabeled phorbol esters available. This characteristic of bryostatin 1, dependence of potency on receptor concentration, is one predicted for a ligand whose true dissociation constant is substantially less than the receptor concentrations employed. If a strictly stoichiometric relationship existed, it would be expected that the IC_{50} would always represent 50% of the B_{max}. Our observation of approximate equal measures of these two values may reflect some loss of bryostatin 1 to the lipid pool provided by the membranes or adsorption to the plasticware. For further discussion of the distortions in binding measurements under limiting receptor concentrations see Refs. 15–18. Since the bryostatins are lipophilic compounds, a further factor affecting apparent potency should be the concentration of the lipid phase. As for diacylglycerides and lipophilic phorbol esters [19, 20], the concentration recognized by the receptor would be predicted to be that in the lipid phase rather than the nominal molar concentration. The reported potencies (5–30 nM) of bryostatin 1 for displacing [^{3}H]PDBu from sites in cultured cells are considerably higher than those measured under limiting receptor concentrations. Although the reason for this discrepancy remains to be established, high receptor concentrations and non-specific loss of bryostatin 1 under culture conditions are probably contributing factors. The IC_{50} values for PS growth inhibition by a number of the bryostatins have been found in the range 10–20 pM [21–23], indicating that the affinity of some of the bryostatins is indeed subnanomolar.

The steep inhibition profiles obtained with bryostatin 1 may be a consequence of the depletion (titration) of ligand when the concentration of bryostatin 1 approaches that of the receptor. A theoretical analysis of this phenomenon is presented by Goldstein and Barrett [17]. The relative independence of the IC_{50} values obtained for bryostatin 1 from the concentration of radioligand also supports the proposition that bryostatin 1 possesses high affinity; it is not the affinity relative to [^{3}H]PDBu that is being measured but rather the concentration of binding sites that need to be occupied.

Saturation analysis of the [^{3}H]PDBu binding sites in the presence of bryostatin 1 indicates neither a clear competitive nor non-competitive interaction. The inability of the extensive measures taken to remove free bryostatin 1 and to reverse the inhibition suggests that, at least for biologically relevant periods, bryostatin 1 may in effect irreversibly occupy the phorbol ester binding site. PDBu appears to have some capacity to reverse the inhibition produced by bryostatin 1, indicating that the interaction is not truly irreversible. Elucidation of the precise parameters describing the interaction between the phorbol ester binding site and the bryostatins will require new tools, such as a radiolabeled phorbol ester of considerably higher activity or a radiolabeled bryostatin.

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