Kinetics and Subcellular Localization of Specific [3H]Phorbol 12,13-Dibutyrate Binding by Mouse Brain

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ABSTRACT

The specific binding of [3H]phorbol 12,13-dibutyrate ([3H]-PDBU) to particulate preparations from mouse brain has been further characterized. Kinetic analysis, using a filtration assay to measure binding, yielded a second-order rate constant at 23° of 3.75 × 10^7 M^−1 min^−1 and a first-order dissociation rate constant of 0.21 min^−1. The K_d of 5.6 nM calculated from the kinetic data agreed well with the value determined previously in equilibrium binding studies. The K_d for [3H]PDBU binding varied only slightly with temperature. From its temperature dependence, [3H]PDBU binding appeared to be associated with a small increase in enthalpy (ΔH^° = +0.4 kcal/mol) and a large increase in entropy (ΔS^° = +38 e.u.). Such values are characteristic for hydrophobic interactions. The dissociation rate constant for binding, in contrast to the K_d, varied dramatically with temperature. The half-time for release ranged from 1.75 min at 30° to 82 min at 4°. The K_d for binding was Ca^{2+} sensitive; chelation of Ca^{2+} by ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid increased the K_d 2.4-fold. Upon subcellular fractionation, the specific [3H]PDBU binding activity was exclusively particulate; no binding to cytosol was detectable. Binding clearly did not correlate with nuclear or mitochondrial markers. On the other hand, a broader distribution of binding activity was seen on sucrose density gradients than for either Na^+-K^+-adenosine triphosphatase activity or binding of quinuclidinyl benzilate (a muscarinic cholinergic antagonist). The localization of specific [3H]PDBU binding to the plasma membrane therefore remains uncertain.

INTRODUCTION

This laboratory has reported that the phorbol esters bind to particulate preparations from chicken embryo fibroblasts (11), mouse skin (5), and mouse brain (12, 29) in a specific, saturable, and reversible fashion. Binding was measured with [3H]PDBU, a phorbol derivative which, although less potent than PMA (35, 36), is substantially less lipophilic (25). In the case of chicken embryo fibroblasts, quantitative agreement was found between the binding affinities of a series of phorbol esters and their biological potencies for inducing fibronectin loss (8–11). Binding affinities to mouse skin and mouse brain preparations were quite similar (5, 12). Moreover, to the degree that the data permit comparison, general agreement was found between binding affinities of phorbol derivatives and their tumor-promoting potencies (12). In other studies, Estensen et al. (13) have reported saturable binding of [3H]PMA to lymphocytes at concentrations similar to those inducing mitogenesis. It would thus appear that many of the biological activities of the phorbol esters, including tumor promotion, may be mediated through a phorbol ester “receptor.”

Of the different mouse tissues examined, brain has proved to be of particular interest on account of its high specific binding activity, 7.5-fold that of skin (12, 29). As a consequence of this high activity, nonspecific binding typically accounts for only 5 to 10% of total binding, and quantitation is greatly facilitated. Using the brain system, we have been able to demonstrate that PDBU and PMA recognize the same receptors (12). In addition, we have shown that binding activity increases markedly during development and have studied its regional localization within the brain (29). The present report, which continues the characterization of phorbol ester binding activity in brain, analyzes the kinetics and temperature dependence of [3H]PDBU binding and release. It also describes subcellular localization of [3H]PDBU binding and its Ca^{2+} dependence.

MATERIALS AND METHODS

[3H]PDBU (1.38 Ci/mmol) was prepared as described (11). [14C]Tyramine (40 to 60 mCi/mmol) and [3H]QNB (40 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). PDBU, IDP, bovine serum albumin (A9647), diphenylamine, pyruvate, β-NADPH, ATP, and ouabain were obtained from Sigma Chemical Co. (St. Louis, Mo.).

The preparation of mouse brain particulate fraction and the centrifugation assay for specific [3H]PDBU binding were described previously (5, 11, 12). For some of the experiments in this paper, a filtration assay for specific [3H]PDBU binding was used. Brain particulate protein, [3H]PDBU, and, in some cases, excess nonradioactive PDBU (30 μM) were incubated in 50 mM Tris-Cl (pH 7.4) containing bovine serum albumin (4 mg/ml) (incubation buffer). At the end of the incubation, either 0.2- or 1-ml aliquots were removed from the incubation mixture and rapidly filtered on Whatman GF/F glass fiber filters (Fisher Scientific Co., Medford, Mass.) which had been presoaked in ice-cold incubation buffer and dried. Bound [3H]PDBU was determined by immersing the filter in Scintiverse (Fisher Scientific Co., Medford, Mass.) which had been presoaked in incubation buffer. After sample filtration, each filter was washed once with either 3 or 5 ml (depending on volume filtered) of ice-cold incubation buffer and dried. Bound [3H]PDBU was determined by immersing the filter in Scintiverse (Fisher) and counting in a scintillation counter. In each assay, 0.1-ml aliquots were removed from the incubation mixture and counted for determination of total [3H]PDBU. Free [3H]PDBU represents...
the difference between total and bound [3H]PDBU. Nonspecific [3H]PDBU binding was determined in the presence of 30 μM nonradioactive PDBU as before (5, 11, 12). As part of the analysis of the kinetics of specific [3H]PDBU binding, the time course for nonspecific [3H]PDBU binding was examined in parallel. Since nonspecific binding was found to reach equilibrium by the earliest time point examined (0.2 min), nonspecific binding was routinely measured only at a single time point, normally the maximum incubation time for the experiment.

Subcellular fractionation of mouse brain was carried out as follows. Whole brains were removed from 16 to 18 female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and homogenized in 4 volumes of ice-cold 0.32 M sucrose with 8 to 10 strokes at 500 rpm in a Potter-Elvehjem homogenizer. The homogenate was diluted 2.5-fold with 0.32 M sucrose, and a crude nuclear fraction (P₁) was prepared by centrifuging at 900 x g for 10 min and washing once with 0.32 M sucrose. Centrifugation of the combined supernatants at 17,500 x g for 20 min yielded a crude mitochondrial fraction (P₂). The 17,500 x g supernatant was separated into microsomes (P₃) and a cytosol fraction (S₃) by centrifugation at 100,000 x g for 60 min.

The crude mitochondrial fraction was osmotically lysed according to the procedure of Cotman and Matthews (4) and then centrifuged at 10,000 x g for 20 min. The pellet was resuspended in 0.32 M sucrose and then subfractionated as described by DeRobertis et al. (6) on discontinuous sucrose gradients consisting of 7.5-ml layers of 1.2, 1.0, 0.9, and 0.8 M sucrose. The gradients were centrifuged at 25,000 rpm in a SW 27 rotor (Beckman Instruments, Irvine, Calif.) for 2 hr. After centrifugation, Fractions A to E were collected as described (6), diluted in 50 mM Tris-Cl (pH 7.4), pelleted at 100,000 x g for 60 min, and subsequently resuspended in 50 mM Tris-Cl (pH 7.4).

Monoamine oxidase was measured by the method of Wurtman and Axelrod (40) with [14C]tyramine as substrate. Specific [3H]QNB binding was determined according to Yamamura and Snyder (41), except that nonspecific binding was measured in the presence of 1 μM atropine. Lactate dehydrogenase and DNA were assayed by the methods of Kornberg (23) and Giles and Myers (18), respectively. IDPase was determined by the method of Novikoff and Heus (30). Ouabain-sensitive Na⁺-K⁺-ATPase was measured according to the method of Schimmel et al. (34). All assays were linear with enzyme concentration. The gel filtration method of Hummel and Dreyer (20) was used for measurement of [3H]PDBU and [3H]QNB binding of cytosol fractions. The method of Lowry et al. (26) was used for protein determination.

RESULTS

The centrifugation assay which we used in the initial studies to measure specific [3H]PDBU binding was unsuitable for rapid kinetic measurements. A filtration assay was therefore developed capable of permitting measurements on a time scale of sec (see “Materials and Methods”). Under equilibrium conditions, the filtration and centrifugation assays yielded the same values for Kₐ and specific binding activity of particulate brain preparations.

The rate of binding of [3H]PDBU to the mouse brain particulate fraction was measured at 23° and a [3H]PDBU concentra-
The second-order rate constant ($k_1$) is given by the relation (see Ref. 27 for derivation):
\[
k_1 = \frac{1}{t} \left[ \frac{B_{so} - B_{so} B_{lo}}{B_{so}} \right] \ln \frac{B_{so} - B_i}{B_{lo}} + B_{so} \]

The derivation of this expression takes into account the decrease in free ligand (under these conditions, 15%) which occurs as the binding reaction proceeds. $B_0$ and $L_0$ are the initial concentrations of target and ligand, respectively. $B_t$ is the concentration of the target-ligand complex at time $t$, and $B_{so}$ is its concentration at equilibrium. The value of $k_1$ is determined from the slope of the line obtained when In$(B_{so} - B_i)/(B_{lo} - B_{so} B_i)$ is plotted as a function of time.

The semilogarithmic plot of the association data showed biphasic kinetics (Chart 1B). The initial slope, which represented the binding occurring in the first 3 min and which accounted for 70% of the total binding at equilibrium, corresponded to a second-order association rate constant $k_1$ of 3.75 ± 0.17 (S.E.) x 10$^7$ M$^{-1}$ min$^{-1}$ (n = 4 experiments).

The basis for the biphasic kinetics is not known. It could reflect 2 classes of targets, either different receptors per se or a single target able to exist in 2 states, whether through a time-dependent conformational change (3, 17) or through interaction with a modulatory molecule. On the other hand, equilibrium binding studies with brain preparations (12) provide no evidence for more than one [3H]PDBU target. Alternatively, differential accessibility of [3H]PDBU to its binding sites in the particulate preparation might explain the results.

As with the rate of binding, the rate of dissociation of [3H]PDBU at 23° was rapid (Chart 2). For the first 3 min, dissociation could be described as a first-order process. The first-order dissociation rate constant ($k_d$) was 0.209 ± 0.006 min$^{-1}$, which corresponds to a half-life of 3.32 min. Again, at later times (>3 min), deviation from this rate was observed. With the $k_1$ and $k_d$ values above, $K_d$ of 5.6 nM was calculated. This value matches the value of 7.4 nM which was determined previously in equilibrium binding studies (12). Because neither the association nor dissociation of [3H]PDBU binding obeyed a single exponential rate throughout the entire time course, the kinetic parameters presented here should be regarded as estimates. Since the explanation for the slower phases of binding is not known, more detailed analysis of the kinetic data (which would involve corrections of the faster rates for the slower rates) is probably not justified at present.

Nonetheless, to provide a measure of the uncertainty in the rate constants as a result of the choice of model, the data were also analyzed assuming a model of 2 independent sites. A good fit of the data was obtained. The second-order association rate constants were 9.1 ± 1.5 (range) and 1.1 ± 0.2 (range) x 10$^7$ M$^{-1}$ min$^{-1}$ (2 experiments). The first-order dissociation rate constants were 0.59 ± 0.08 (range) and 0.085 ± 0.009 (range) min$^{-1}$ (2 experiments). The association and dissociation rate constants for the faster component assuming a 2-site model were thus 2.4- and 2.8-fold, respectively, that for the one-component model. Because the rates shifted in parallel on the assumption of a 2-site model, calculated equilibrium constants for the putative first and second components remained similar, 6.5 and 7.8 nM.

The thermodynamics of binding can provide insight into the nature of the interaction between a ligand and its binding component. We therefore examined the temperature dependence of the [3H]PDBU binding affinity. We observed that the binding affinity varied by less than 12% over the temperature range of 4-37°. The standard enthalpy change ($\Delta H^o$) of binding was calculated from the Van’t Hoff plot of the dependence of the association constant ($K_a = 1/K_d$) of [3H]PDBU binding on temperature (Chart 3). The standard entropy change ($\Delta S^o$) of binding was calculated from the equation $\Delta G^o = \Delta H^o - T \Delta S^o$, where $\Delta G^o$ (the standard free energy change) = $\Delta H^o - T \Delta S^o$; $\Delta S^o$ (the standard free energy change) = $-RT \ln K_a$.

The values obtained from the thermodynamic parameters were: $\Delta G^o = -11.0$ kcal/mol; $\Delta H^o = +0.4$ kcal/mol; and $\Delta S^o = +38.4$ e.u. Thus, binding occurs with a small unfavorable increase in enthalpy and a very large favorable increase in entropy.
In contrast to the $[^{3}H]$PDBU binding affinity, the dissociation rate constant of binding showed a dramatic dependence on temperature (Chart 4). The half-time for release decreased from 62 min at 4° to 1.75 min at 30°. The rate of release at 37° was too fast for convenient measurement. The value obtained by extrapolation was 0.69 min. The association rate constant of binding also changed markedly with temperature (not shown). This result was expected, since the $K_d$ ($k_2/k_1$) of $[^{3}H]$PDBU binding was not altered greatly by temperature.

The pH dependence of $[^{3}H]$PDBU binding was examined at a subsaturating ligand concentration in order to detect changes in either the affinity or extent of $[^{3}H]$PDBU binding (Chart 5).

Binding activity was lost below pH 6 or above pH 10. Similar results were obtained at a ligand concentration substantially above the $K_d$ (33 nM $[^{3}H]$PDBU; data not shown), thus indicating that the decrease was in the extent of binding. The loss of binding activity at low pH was irreversible. Following incubation of the brain particulate fraction at pH 4.0 for 30 min at 37°, pelleting of the membranes and resuspension in Tris-CI, pH 7.4, failed to restore activity.

Addition of Ca$^{2+}$ to brain particulate preparations had been found previously not to affect $[^{3}H]$PDBU binding (29). In order to determine whether $[^{3}H]$PDBU binding might be affected by chelation of Ca$^{2+}$ potentially retained by the preparations, the saturation curve for $[^{3}H]$PDBU was measured in the presence of 1 mM EGTA. Scatchard analysis indicated a 2.4 ± 0.4 (S.E.; 4 experiments) -fold increase in the $K_d$; controls were assayed in the presence of EGTA plus an excess of Ca$^{2+}$ (Chart 6). Absolute binding affinities in these 4 experiments were 27.9
± 2.8 and 9.6 ± 0.7 nM for membranes assayed in the presence of EGTA or EGTA plus Ca²⁺, respectively. Specific binding at saturation was unaltered.

The concentration of calcium able to enhance the binding affinity of [³H]PDBU in EGTA-treated membrane preparations was determined using an EGTA-Ca²⁺ buffer system (Chart 7). The free Ca²⁺ concentration was calculated as described by Potter and Gergely (32). The ED₅₀ for Ca²⁺ was 3.5 ± 0.5 (S.E.) x 10⁻⁷ M (3 experiments). This value is similar to the values determined by Potter et al. of 2 x 10⁻⁷ M for binding of Ca²⁺ to the Ca²⁺-specific binding sites on purified troponin (32) and of 3 x 10⁻⁷ M for calmodulin-activated phosphodiesterase (31). Typically, the concentration of Ca²⁺ in the cytosol is 10⁻⁸ to 10⁻⁶ M, whereas the concentration in extracellular fluid is 10⁻³ M (24).

We examined the subcellular distribution of [³H]PDBU binding in the hope that it might provide further information regarding the character of the phorbol ester binding component. The fractionation scheme, which used a combination of differential and sucrose density gradient centrifugation, was essentially that described by DeRobertis et al. (6). The results are summarized in Tables 1 and 2. The crude nuclear fraction, which contained cell debris as well as nuclei, contained 100% of the recovered DNA of the brain homogenate but only 17% of the [³H]PDBU binding activity. While this result does not rigorously exclude the presence of some phorbol ester binding sites in the nucleus, it argues against an exclusive nuclear localization. The finding is consistent with the observation that enucleated cells still can respond to the phorbol esters (28). Specific [³H]PDBU binding to cytosol was assayed by the gel filtration technique of Hummel and Dreyer (20). Within the detection limits of the assay (<1 pmol/mg), no specific [³H]PDBU binding to cytosol was observed. Soluble [³H]PDBU binding sites could
therefore account for no more than 1% of the binding activity of the homogenate.

Over one-half of the \(^{3}H\)PDBU binding activity was present in the crude mitochondrial fraction, which contained mitochondria, pinched-off nerve endings (synaptosomes), membranes, and myelin. Results of further subfractionation of this fraction on discontinuous sucrose gradients are given in Table 2. Because synaptosomes contain in their interior other cell components (cytosol, mitochondria, etc.), osmotic lysis prior to density gradient centrifugation is required for effective fractionation of the crude mitochondrial fraction (7). Osmotic lysis was therefore carried out according to Cotman and Matthews (4); the effectiveness of this procedure was demonstrated by the release of 85% of the lactate dehydrogenase (cytosol marker) present in the crude mitochondrial fraction.

The distribution of \(^{3}H\)PDBU binding activity through the sucrose gradient fractions clearly did not parallel that for monoamine oxidase (an enzyme located in the outer membrane of the mitochondrion). Fraction E had the highest specific activity for monoamine oxidase, whereas it had the lowest specific \(^{3}H\)PDBU binding activity. The distribution of \(\text{Na}^{+}\text{-K}^{+}\)-ATPase, binding of \([H]\)QNB (an antagonist which binds to the muscarinic acetylcholine receptor), and the microsomal marker IDPase were also examined for comparison with \(^{3}H\)PDBU binding. The patterns of \(\text{Na}^{+}\text{-K}^{+}\)-ATPase activity and \([H]\)QNB-binding was similar to that for \(^{3}H\)PDBU binding. However, \(^{3}H\)PDBU binding was more diffusely distributed throughout the gradient than these 2 markers. Within the sucrose gradients, the endoplasmic reticulum marker IDPase did not separate well from the 2 plasma membrane markers, although their profiles differed in the differential centrifugation fractions.

**DISCUSSION**

The kinetics and temperature dependence of \(^{3}H\)PDBU binding have several implications. First, a number of biological responses to the phorbol esters have been reported to occur very rapidly. Membrane depolarization of human granulocytes in response to 1 \(\mu\)M PMA was detectable within 10 sec (39). Enhanced levels of cyclic GMP were reported to be observed within 15 sec after exposure of BALB/c 3T3 mouse cells to 160 nM PMA (14). Aggregation of human platelets was evident within 45 sec after addition of 160 nM PMA (16). The second-order association rate constant for PDBU predicts a half-time for binding at 23° of 75 sec at its \(K_d\) and of 10 sec at a PDBU concentration of 100 nM. At 37°, the extrapolated half-time would be <2 sec. The rate of binding is therefore fast enough to account for the fastest biological responses observed.

The marked temperature dependence of the binding kinetics is of considerable experimental utility. Because the half-time for \(^{3}H\)PDBU release at 4° is greater than 1 hr, assays can be carried out in which the preparations are rinsed in the cold to remove unbound ligand after equilibration has been achieved. It is thus possible to measure binding either with a filtration assay such as that described here or with living cells adhering to culture dishes (21). On the other hand, the rapid rate of PDBU release at 37° makes it possible to quickly and efficiently remove PDBU from cells in biological experiments when desired. We have therefore been able to measure down modulation of \(^{3}H\)PDBU receptors in the GH4C1, rat pituitary cell line

**Table 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Na(^+)-K(^+)-ATPase</th>
<th>IDPase</th>
<th>Protein</th>
<th>SRA* relative specific activity (percentage of recovered activity) (protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>Fraction A (0.2-0.9 M)</td>
<td>10.7</td>
<td>7.5</td>
<td>7.5</td>
<td>21.7</td>
</tr>
<tr>
<td>Fraction B (0.6-1.0 M)</td>
<td>10.7</td>
<td>7.5</td>
<td>7.5</td>
<td>21.7</td>
</tr>
<tr>
<td>Fraction C (0.8-1.8 M)</td>
<td>30.7</td>
<td>21.5</td>
<td>21.5</td>
<td>53.0</td>
</tr>
<tr>
<td>Fraction E (&gt;1.2 M)</td>
<td>45.4</td>
<td>31.7</td>
<td>31.7</td>
<td>69.0</td>
</tr>
</tbody>
</table>

* SRA, relative specific activity = (percentage of recovered activity) (protein).
by incubating cells with nonradioactive PDBU, removing the [3H]PDBU by washing, and then measuring receptor levels with [3H]PDBU (21).

The thermodynamic measurements of binding indicate a small unfavorable increase in enthalpy, +0.4 kcal/mol, and a very large increase in entropy, 38.4 e.u. Such values are typical for hydrophobic interactions, which result in the displacement of water molecules ordered around a ligand and its binding site (22). This finding supports the conclusions from in vivo structure-activity studies, which emphasized the important role of the hydrophobic side chains in determining phorbol ester potency (19, 25).

A number of the biological activities of the phorbol esters are interrelated with those of Ca2+. PMA overcame the inhibition of proliferation of low-passage BALB/c 3T3 cells by low calcium levels in the medium although not the inhibition by calcium-free medium (1). PMA and the calcium ionophore A-23187 were synergistic for mitogenesis in human lymphocytes, and the presence of the ionophore shifted the ED50 for PMA by 30-fold (37). In rat thymic lymphocytes, PMA shifted the dose-response curve for calcium (38). In polymorphonuclear leukocytes, synergy between A-23187 and PMA for induction of chemotaxis was reported (15). In cultured chicken myoblasts, PMA decreased both Ca2+ influx and efflux, although the absolute effects reported were relatively small (33). Modulation of phorbol ester binding affinity by calcium may provide feedback control in these and other systems.

The subcellular fractionation studies demonstrated no specific [3H]PDBU binding in the cytosol. The use of particulate preparations for the analysis of [3H]PDBU binding by cells and tissues would therefore appear to be inappropriate. Within the particulate fraction, the data rule out either the nucleus or the mitochondria as the principal site of [3H]PDBU binding activity. Although the peak of [3H]PDBU binding activity in the sucrose density gradients coincided with that for the 2 plasma membrane markers examined, its distribution was more diffuse. This broad distribution of [3H]PDBU binding may reflect the heterogeneity of the plasma membranes within single nerve cells and among the numerous cell types in the brain. In support of this view, differential centrifugation in Fact fraction caused enrichment in Fraction P2 (microsomal) of one plasma membrane marker, [3H]QNB binding, relative to the other marker, Na+-K+-ATPase (Table 1). Similarly, DeRobertis et al. (6) found that acetylcholinesterase activity banded on sucrose density gradients at a lower density than did Na+-K+-ATPase. On the other hand, the data could indicate that [3H]PDBU binds specifically to cell membranes in addition to the plasma membrane. Further studies will be required to distinguish these possibilities.

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