Variation with Embryonic Development and Regional Localization of Specific [³H]Phorbol 12,13-Dibutyrate Binding to Brain

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ABSTRACT

We have previously characterized specific binding of the phorbol ester tumor promoter [20-³H]phorbol 12,13-dibutyrate to several tissues, including mouse skin and brain. We report here that specific binding activity in chicken brain increases dramatically during development. In whole embryonic chicken brain, binding increased from 2.0 to 6.8 to 10.6 pmol/mg of protein at 7, 14, and 20 days, respectively. Adult chicken brain bound 15 pmol/mg of protein. In contrast to specific binding activity, binding affinity remained constant. Substantial regional localization of binding activity within the brain was found. For calf brain, representative values ranged from 4.6 pmol/mg for medulla to 38.1 pmol/mg for frontal lobe. Binding affinity did not vary. Inhibition of binding by postulated neurotransmitters and antagonists was examined. d-Propranolol and quinidine, membrane-stabilizing drugs, inhibited binding competitively, although at mM concentrations.

INTRODUCTION

The phorbol esters are the most potent class of tumor promoters and have dramatic effects on cells both in vivo and in vitro (6, 7, 11, 38). Recently, this laboratory was able to demonstrate specific binding of phorbol esters to chick embryo fibroblasts, using as a ligand [³H]PDBU (13, 14). [³H]PDBU was used because, although it is somewhat less potent than PMA (12, 33, 40), it is much less lipophilic (23), and therefore shows substantially less nonspecific binding. Specific binding of [³H]PDBU was saturable, reversible, and compatible by nonradioactive phorbol esters. K₅ values determined for inhibition of [³H]PDBU binding by phorbol esters agreed quantitatively with the biological potencies of these derivatives for inducing fibronectin loss in this cell type. Closely similar characteristics were observed for the specific binding of [³H]PDBU to mouse skin preparations (10).

A survey of specific binding activity of various mouse tissues for [³H]PDBU revealed considerably higher specific binding activity in brain than in any other tissue, e.g., 7.5-fold greater than in skin. The binding activity in mouse brain homogenates has been characterized (15). Except for the higher specific activity, it is generally similar to the binding activities in chick embryo fibroblasts and mouse skin (10, 13, 14). A major technical advantage of the brain preparation was that the higher specific binding activity reduced nonspecific binding to a very small fraction of the total. As a consequence, equilibrium binding of [20-³H]PMA could be measured directly. It was thus possible to confirm that PMA and PDBU in fact bound to the same site; each compound inhibited binding of the other greater than 95%.

There is some indication that responses to phorbol esters may be developmentally regulated. Newborn mouse skin has been reported to be less susceptible to PMA-induced hyperplasia relative to adult mouse skin (5, 37). This may or may not reflect a difference in receptor binding activity which is developmentally regulated. Because of the high specific binding activity of brain, we used this tissue to examine whether phorbol ester binding activity changed during development. In addition, we have examined the regional localization of the phorbol ester receptors in brain in the hope that specific localization may suggest or eliminate possible functions of the receptor. Lastly, we have screened several known neuroactive agents for inhibition of phorbol ester binding.

A critical issue in the tumor promotion field is to identify the second messenger for the phorbol esters, i.e., the biochemical pathway directly modulated by interaction of the phorbol esters with their receptor. This pathway should be the same in different tissues, although more distal responses in the biochemical cascades might well differ. The high level of receptors in brain and the detailed knowledge of brain biochemistry offer unique opportunities for identifying this process.

MATERIALS AND METHODS

Fertile eggs were obtained from Spafas (Norwich, Conn.) and fresh calf brains were from Trelegan and Co. (Cambridge, Mass.). [³H]PDBU (1.38 Ci/mmol) was prepared as described (1). PDBU, serotonin, γ-aminobutyric acid, tryptophanol, melatonin, valyltryptophan, ergonovine, bradykinin, Substance P, methionine enkephalin, carbamylcholine, dl-isoproterenol, and methylamine were from Sigma Chemical Co. (St. Louis, Mo.). d-Propranolol and l-propranolol were the generous gifts of Ayerst Laboratories (New York, N. Y.).

Binding assays with [³H]PDBU were carried out on particulate preparations from tissues as described by Delclos et al. (10). Briefly, whole brain or brain fractions were suspended in 0.05 M Tris-Cl (pH 7.4) and homogenized in a Potter-Elvehjem homogenizer. The particulate preparations were obtained by centrifuging the homogenates at 100,000 × g for 60 min. Binding to the particulate preparation was carried out for 30 min at 39° in the presence of 0.05 M Tris (pH 7.4), 40 to 120 nM [³H]PDBU (as indicated), bovine serum albumin (4 mg/ml; A9647, Sigma), 0.15% dimethyl sulfoxide, and other ligands.

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4 Recipient of a Research Career Development Award from the NIH. To whom requests for reprints should be addressed.
5 The abbreviations used are: [³H]PDBU, [20-³H]phorbol 12,13-dibutyrate; PDBU, phorbol 12-myristate 13-acetate; PMA, phorbol 12,13-dibutyrate; EGF, epidermal growth factor.
6 K. B. Delclos and P. M. Blumberg, manuscript in preparation.

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where indicated. The particulate material was then spun down, and the amounts of free and bound [$^3$H]PDBU were determined. Specific binding of [$^3$H]PDBU represents the difference between total binding and that occurring in the presence of 30 μm nonradioactive PDBU. Specific binding was normalized to protein. The percentage of protein as a function of brain wet weight has been reported to increase during chick brain development (47) and to be 1.4-fold greater in gray than in white matter (30). Such differences should be taken into account in interpretation of the data. Analysis of [$^3$H]PDBU binding to brain after subcellular fractionation\(^7\) indicates that >99% of total binding is in the particulate fraction.

**RESULTS**

We chose to study the developmental changes in specific binding of [$^3$H]PDBU in chicken brains because of the ease in obtaining embryos. Binding rose linearly with time between 7 and 18 days of development, reflecting a 6-fold increase in specific activity (Chart 1). A further small increase in activity was found in chicks 2 days posthatching or in the adult. Binding affinities for brains from 10- and 11-day-old embryos were compared with that from adults. No difference was observed (data not shown). Likewise, mixing experiments with brain preparations from 8-day-old embryos and adults provided no evidence for the presence of an excess of inhibitor of binding in the embryonic brain. The developmental changes would therefore appear to reflect an increase in the number of binding sites.

Regional localization of phorbol ester binding was determined in calf brain because of its suitability for dissection. Large variations in binding activity were observed (Chart 2). Specific binding by frontal cortex was 38.1 pmol/mg protein. In contrast, binding activity of medulla was only 4.6 pmol/mg, just slightly greater than that previously found for mouse skin (2). Complete saturation curves were compared for binding to medulla and to a fraction from the limbic system (which contains the amygdala and hippocampus). As was the case with the embryonic chicken brain, the differences in binding activities reflected differences in the total number of binding sites rather than in binding affinity (Chart 3).

The high levels of binding activity in brain prompted us to explore possible inhibition of [$^3$H]PDBU binding by postulated neurotransmitters and antagonists (Table 1). High concentrations were used to detect low-affinity interactions. Of particular potential interest had been catecholamines. PMA has been reported to inhibit induction of cyclic adenosine 3′:5′-monophosphate by the β-adrenergic agonist isoproterenol (2, 18, 29), although this inhibition is thought to reflect uncoupling of the receptor from the adenylate cyclase rather than competition for binding to the β-adrenergic receptor.

[$^3$H]PDBU binding was markedly inhibited by D- and L-propranolol. Inhibition of [$^3$H]PDBU binding was apparently competitive (Chart 4), with a calculated Kᵢ of 0.36 ± 0.09 μM (n = 3). Pharmacologically, propranolol has 2 activities. At lower concentrations, the L-isomer acts as a β-adrenergic antagonist. At higher concentrations, both the D- and L-isomers possess quinidine-like activity, depressing cardiac excitability. The inhibitory activities of D- and L-propranolol on [$^3$H]PDBU binding were therefore compared. Both isomers caused a similar degree of inhibition (Table 1). Likewise, the activity of quinidine was examined. It too was inhibitory, acting in a competitive manner.

A second class of agents examined for inhibition of [$^3$H]PDBU binding were commercially available indole derivatives. Fujiki et al. (17) reported that dihydroteleocidin B, a cyclic valytryptophan derivative, possessed several of the same biological activities, e.g., induction of ornithine decarboxylase in mouse skin, induction of adhesion in HL-60 cells, and inhibition of differentiation in Friend erythroleukemia cells, as did PMA. Since dihydroteleocidin B was not readily available, the possible activity of structurally related compounds was examined. None blocked binding.

Binding was not affected by the indicated changes in ionic composition. In particular, no inhibition was observed for methylamine, which had been reported to block clustering and endocytosis of EGF receptors and to potentiate the mitogenic activity of EGF, possibly through interaction with transgluta-

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\(^7\) W. G. Dunphy, R. J. Kochenburger, M. Castagna, and P. M. Blumberg, manuscript in preparation.
specific binding of phorbol esters to brain

chart 3. comparison of [3h]pdbu binding to calf brain regions of high and low binding capacity. specific and nonspecific binding were determined as described in "materials and methods." a, saturation curves for specific [3h]pdbu binding; the curves were calculated from the parameters derived from the scatchard plots. ○, limbic system; ●, medulla. nonspecific [3h]pdbu binding: △, limbic system; ▲, medulla. b, scatchard plots of specific [3h]pdbu binding. ○, limbic system; ●, medulla.

chart 4. inhibition by dl-propranolol of binding of [3h]pdbu to particulate preparations from whole mouse brain. ○, control; ▲, 0.5 mM dl-propranolol; ■, 1.0 mM dl-propranolol.

β-hydroxylase and choline acetyltransferase activities (39). These activities are thought to be markers of synaptogenesis. The membrane markers phosphodiesterase and 5′-nucleotidase increase in a parallel fashion (39). In contrast, structural proteins such as actin (31, 32) or tubulin (1) show little developmental change, as do metabolic enzymes such as lactate dehydrogenase or citrate-cleaving enzyme (39). The enzymes involved in DNA synthesis decrease markedly over this time period (21).

the developmental pattern of appearance of binding activity and the high level of [3h]pdbu binding in brain both support a role for the phorbol ester receptors in brain activity. the high level of binding which is observed, up to 38.1 pmol/mg protein, implies that the phorbol ester receptor is a major constituent. Were it to have a molecular weight of 100,000, the phorbol ester receptor would comprise 0.4% of the total particulate protein (0.3% of the total cellular protein). Receptors for neurotransmitters, in contrast, are present at 1/20 to 1/1000 this level. The high level of pdbu receptors and their presence in nonnervous tissue imply that the phorbol ester receptor serves a functional rather than exclusively an information-transducing role in the cell. The receptor may thus be more analogous to the Na+-K+-ATPase than to the opiate receptor.

discussion

comparison of the pattern of increase of specific [3h]pdbu binding in chick embryo brain during development resembles that observed for α-bungarotoxin binding, as well as dopamine

minase (26). PMA had been hypothesized to induce the clustering of EGF receptors (25). High concentrations of Tris, which have been reported to solubilize clathrin (22), a protein present in high concentrations in brain and thought to be involved in receptor-mediated endocytosis, did not diminish the amount of pelletable pdbu binding activity.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Binding activity (% of control)</th>
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<td>Dopamine</td>
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<tr>
<td>Ca2+</td>
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</table>

Table 1 binding of [3h]pdbu to mouse brain preparations in the presence of potential inhibitors

compounds were dissolved in H2O or DMSO to give the indicated final concentrations. Binding was measured with [3h]pdbu (40 to 50 nM) as described in "materials and methods."
REFERENCES


33. Yamamura, H. I., Kuhar, M. J., Greenberg, D., and Snyder, S. H. Muscarinic
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