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SPECIFIC BINDING OF PHORBOL ESTERS TO NUCLEI OF HUMAN PROMYELOCYTIC LEUKEMIA CELLS

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SUMMARY: In this report, we demonstrate that HL-60 nuclei isolated in calcium but not EGTA containing buffers specifically bind PE and express approximately 37,000 receptor sites/nucleus. Nuclear phorbol ester binding is lost by isolation in the absence of calcium, but can be repleted by the addition of partially purified protein kinase C and calcium. When HL-60 cells are treated with bryostatin 1, a compound which activates protein kinase C in a similar fashion to phorbol esters but does not induce differentiation of HL-60 cells, and nuclei are isolated in the presence of EGTA, these nuclei continue to bind phorbol esters. These experiments suggest that HL-60 nuclei bind PE *in vitro*, and that compounds that activate protein kinase C may increase nuclear binding of PE *in situ*.

The induction of differentiation of human promyelocytic leukemia cells (HL-60) to macrophages by phorbol esters is a complex process which is currently thought to involve the activation of a calcium, phospholipid dependent protein kinase, protein kinase C (1). This enzyme is thought to be the cellular phorbol ester receptor (2). The importance of protein kinase C to differentiation of HL-60 cells is demonstrated by the observation that compounds which block the activity of this enzyme inhibit the ability of phorbol esters to induce HL-60 differentiation (3,4). Differentiation of HL-60 cells is accompanied by morphologic and biochemical changes within the nucleus, including inhibition of DNA synthesis (5), increase in the synthesis of specific genes, i.e. c-*fos*, c-*fms*, and c-*src* (6,7,8), and the decrease of others, i.e. c-*myc* and c-*myb* (9,10). How activation of protein kinase C

ABBREVIATIONS: phorbol ester, PE; calcium phospholipid-dependent protein kinase, protein kinase C; phorbol dibutyrate, PDBμ.

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leads to changes in nuclear RNA and DNA synthesis is uncertain.

Treatment of HL-60 cells with phorbol ester causes a tight association of protein kinase C with the plasma membrane (11) and phosphorylation of specific membrane proteins (12,13), suggesting that an early signal for differentiation may originate at the level of the plasma membrane. Recently, however, phorbol ester treatment of HL-60 cells has been shown to cause phosphorylation of specific nuclear proteins (14). In addition, immunofluorescence techniques demonstrate that protein kinase C is located near but not in the nucleus (15). These results suggest that phorbol esters might directly bind to the nucleus. Because the nuclear membrane shares many biochemical properties, i.e. phospholipid content and turnover (16) with the plasma membrane, it seems possible that phorbol ester treatment of HL-60 cells could cause the association of protein kinase C with the nucleus. In order to evaluate whether protein kinase C associates with the nucleus, we have examined the ability of phorbol esters to specifically bind to isolated nuclei.

MATERIALS AND METHODS

Bryostatin 1 was a gift of Drs. G.R. Pettit, C.L. Herald, J.E. Leet, and Y. Kamano at the Arizona State University, Temple, AZ. 20-[^{3}H](N) phorbol dibutyrate (12.5 Ci/mol) ([^{3}H]-PDBμ) was purchased from New England Nuclear. Non-radioactive phorbol esters were from P.L. Biochemicals.

HL-60 were grown as described elsewhere (17). For nuclear isolation, cells were washed in PBS, suspended in 50 ml of buffer A (20 mM Tris-HCl, pH 7.5, 0.33 M sucrose, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 25 mM KCL) and subjected to nitrogen cavitation at 850 psi for 5 minutes. Nuclei were pelleted at 500 x g for 10 minutes, washed twice with buffer A and then twice with buffer B (20 mM Hepes, pH 7.0, 0.14 M NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl). These nuclei were layered over a buffer A containing 2.1 M sucrose and ultracentrifuged for 2 hours at 100,000 x g. The pellet used as purified nuclei was free of cytoplasmic and membrane contamination as measured by phase contrast microscopy, lack of lactate dehydrogenase (18), 5' nucleotidase, a plasma membrane marker (19). For nuclear isolation in calcium free buffers, 2 mM EGTA replaced calcium in the above buffers.

Protein kinase C was partially purified from 2.5 x 10^8 HL-60 cells as previously described for other homogenates (20). Protein kinase C was extracted from 2.5 x 10^8 nuclei purified, as above, with 1% NP-40 (20) for 30 minutes.

Phorbol ester binding to isolated nuclei was measured using nuclei pelleted by ultracentrifugation and washed twice with buffer B. These nuclei were then added to tubes containing 4 mg/ml bovine serum albumin, buffer B, and [^{3}H]-PDBμ. Tubes were gently shaken for 20 minutes at 30°C unless otherwise indicated. The reaction was stopped by filtration over Whatman GF/C glass filters. The filters were washed with 10 ml of buffer B, dried, placed in Scinti Verse E, and counted. All experiments were performed in triplicate (S.E.M. of values obtained was less than 8%). Non-specific binding was determined by adding non-radioactive PDBu in

1,000-fold excess to parallel reaction. Specific binding was obtained by subtracting binding in the presence of excess non-radioactive phorbol ester from that obtained when only [^{3}H]-PDBu was present.

RESULTS

In order to examine whether nuclei contained specific phorbol ester binding, HL-60 nuclei were isolated in calcium containing buffers, incubated with varied concentration of [^{3}H]-PDBu, with or without 1,000-fold excess of non-radioactive PDBu for 20 minutes at 30°C. Figure 1 demonstrates that [^{3}H]-PDBu binds to nuclei in a saturable fashion, and that each nucleus possesses 3.7×10^4 receptors with a Kd of 7.4×10^{-9} as determined by Scatchard analysis (see insert, Fig. 1). When the binding studies were performed at 30°C, maximal specific binding of [^{3}H]-PDBu to the nuclei occurs within 1 minute (Fig. 2a). In contrast at 4°C, binding occurs much more slowly with maximal binding delayed until approximately 80 minutes. We next examined the ability of PE analogs to compete with [^{3}H]-PDBu for binding to the nuclei. Phorbol myristate acetate (PMA) inhibits [^{3}H]-PDBu binding to

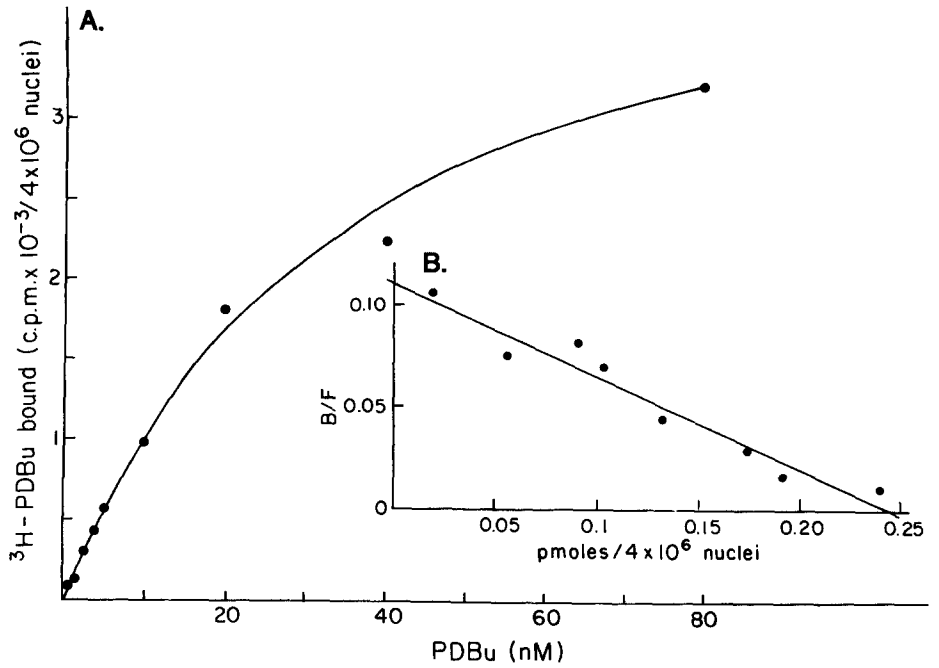

Fig. 1. Phorbol ester binding to isolated nuclei. A) Nuclei were added to tubes containing varying concentrations of [^{3}H]-PDBu, and a binding assay performed as described in Methods. B) Scatchard analysis of [^{3}H]-PDBu nuclear binding gave a correlation coefficient by least-squares analysis of 0.95. B/F is the ratio of bound to unbound phorbol ester.

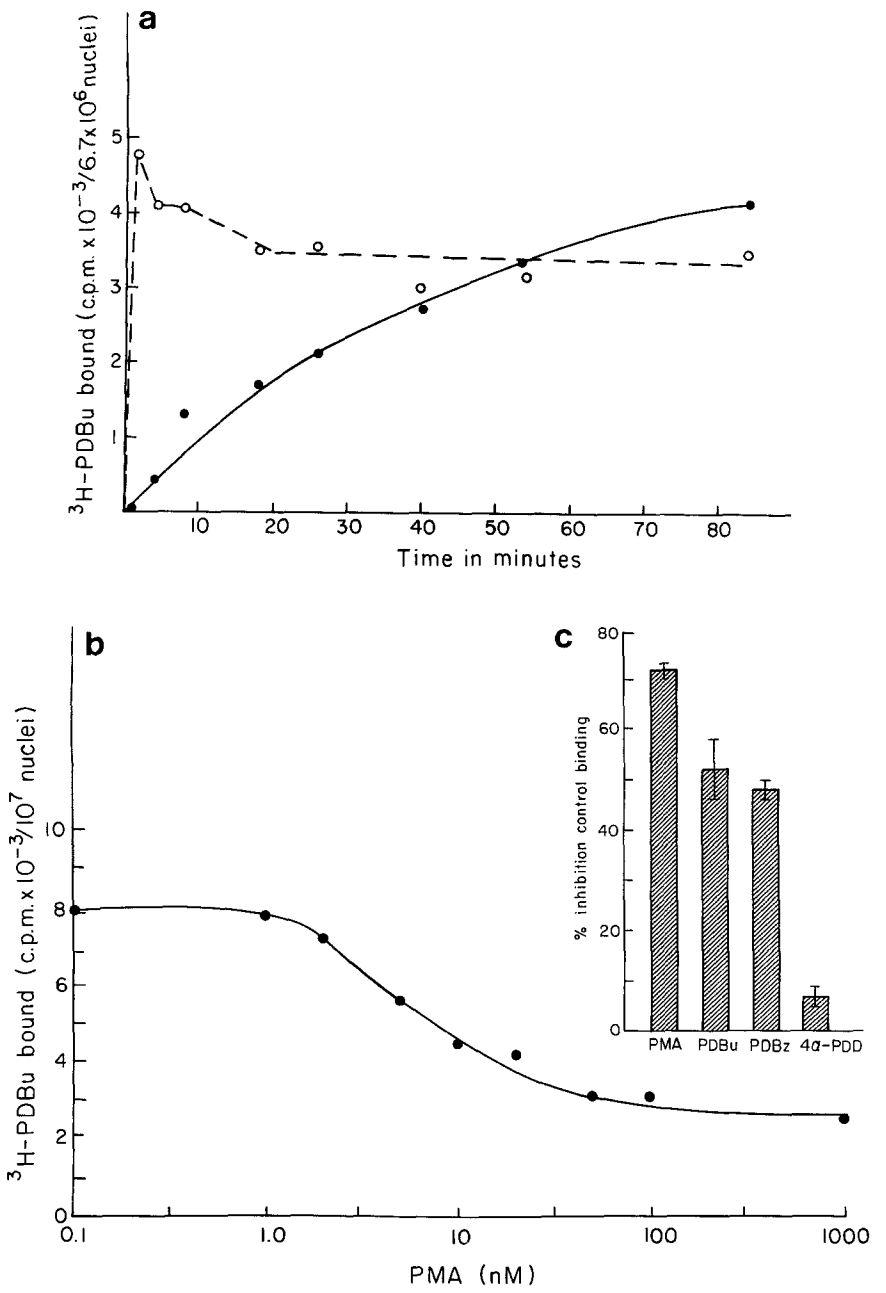

Fig. 2a. Temperature dependence of phorbol ester binding. Nuclei were prepared and binding measured as outlined in Methods. Each reaction tube contained 40 nM [^{3}H]-PDBu, while 10 uM PDBu was added to half the tubes to determine non-specific binding.

Fig. 2b. Phorbol myristate acetate inhibition of [^{3}H]-PBDu binding. The binding reaction was done as in Methods with tubes containing 40 nM [^{3}H]-PDBu and the stated concentration of PMA.

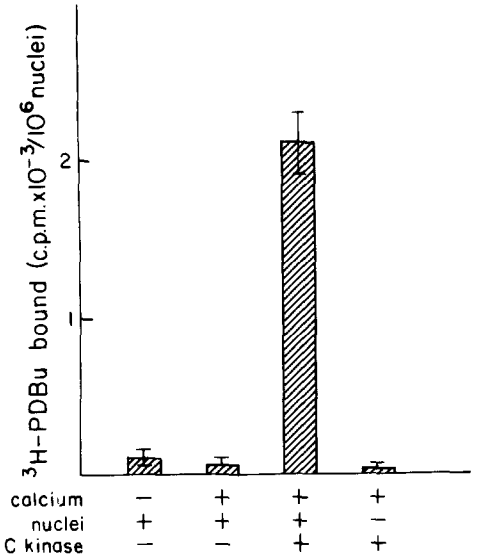

Fig. 3. Reconstitution of phorbol ester binding. Nuclei were isolated in the absence of calcium (see Methods), suspended in buffer B and incubated for 20 minutes at 30°C in the presence of 40 nM [^{3}H]-PDBu either with or without 2 mM $CaCl_2$ and/or partially purified C-kinase isolated from HL-60 cells (see Methods). After a 20 minute incubation, the nuclei were centrifuged for 10 minutes at 500 x g; the reaction mixture was removed by suction; and nuclei were resuspended in 2 ml of buffer B and filtered on glass fiber filters. The data presented are the mean ± S.E.M. of triplicate determinations. Non-specific binding has been subtracted.

nuclei in a concentration-dependent fashion (Fig. 2b), and at a concentration of 50 nmol/L, the ability of PE analogs to inhibit binding of [^{3}H]-PDBu to the nuclei directly parallels their ability to act as a tumor promoter (Fig. 2c) (21).

In order to examine the role of calcium in nuclear binding, nuclei were prepared in buffers containing EGTA. Nuclei isolated in this fashion were unable to bind [^{3}H]-PDBu (Fig. 3, Col. 1). Addition of calcium alone (Fig. 3, Col. 2) was unable to reconstitute normal binding. Partially purified protein kinase C added to

Fig. 2c. Inhibition of [^{3}H]-PDBu binding by various phorbol ester analogs. Various phorbol ester analogs (PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13 dibutyrate, PDBz, phorbol 12,13 dibenzoate; 4α-PDD, 4α-phorbol 12,13 didecanoate) were added at a concentration of 50 nM to the binding reaction. Results are expressed as the percentage of specific binding of [^{3}H]-PDBu to nuclei incubated with PE compared with those incubated with vehicle alone. Non-specific binding is determined as in Methods.

nuclei in the absence of calcium did not induce phorbol ester binding (data not shown). However, the addition of calcium and protein kinase C reconstituted binding to these nuclei (Fig. 3, Col. 3). This result suggested that nuclei might bind protein kinase C in the presence of calcium.

Since nuclear membranes contain a similar membrane phospholipid content (16) to that found in the plasma membrane to which protein kinase C is known to bind (22,23), it seems likely that the addition of calcium to the homogenizing buffer caused the association of protein kinase C with the nucleus. We therefore prepared 2.5 x 10^{+8} nuclei in calcium containing buffers, extracted them with 1% NP-40, centrifuged the extract and chromatographed the supernatant on a molecular sizing column. The results of a Sephacryl S-200 (Fig. 4) column demonstrate that calcium phospholipid-dependent protein kinase activity can be extracted from HL-60 nuclei isolated in the presence of calcium.

We next examined whether nuclei isolated after treatment with bryostatin 1, a non-phorbol ester macrocyclic lactone, which activates protein kinase C, and stimulates translocation to the

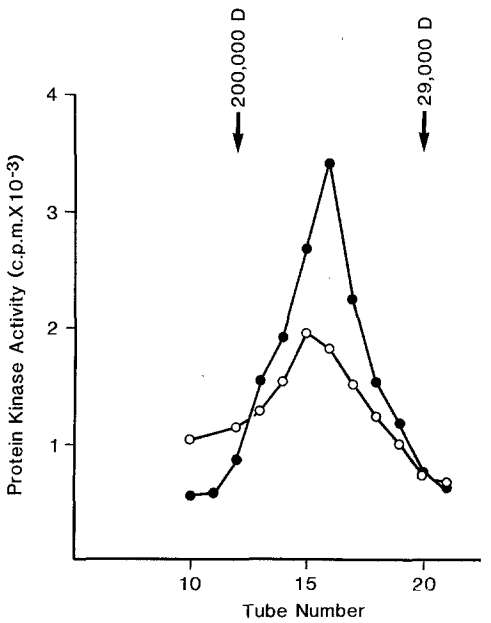

Fig. 4. Extraction of protein kinase C from isolated HL-60 nuclei. Nuclei (2.5 x 10^{8}) were isolated in the presence of calcium, extracted with 1% NP-40, and the extract was run over a Sephacryl S-200 column (1.5 x 30 cm). Fractions (2 ml) were assayed for protein kinase C activity with phospholipids (●—●) or without (○—○) as previously described (11).

Table I

The Effect of Bryostatin 1 on Nuclear Phorbol Ester Binding

	Incubation time (hrs)	control	Bryostatin (10 nM)
Exp. 1 (a)	0.5	0	1,743 (c)
Exp. 2 (b)	72	10,149	144

(a) Nuclei were isolated in calcium free buffers plus 2 mM EGTA.
(b) Nuclei isolated in buffers containing 2 mM calcium.
(c) Results are expressed as c.p.m. of [^{3}H]-PDBu bound/4 x 10^6 nuclei and are average of triplicate experiments with S.D. ± 15%.

particulate fraction but does not cause differentiation of HL-60 cells (17), effects nuclear PE binding. Short treatments with bryostatin 1 cause protein kinase C to bind to membranes, while prolonged exposure (72 hours) causes the loss of all cellular protein kinase C activity from HL-60 cells (24). Nuclei isolated in EGTA from HL-60 cells treated for 0.5 hours with bryostatin 1 were found to specifically bind [^{3}H]-PDBu, whereas control nuclei demonstrated no binding (Table 1). As another control HL-60 cells were treated for 72 hours with bryostatin 1 and nuclei from these cells were isolated in the presence of calcium. As predicted from our data on protein kinase C activity in these cells, after prolonged bryostatin 1 treatment, no [^{3}H]-PDBu binding was found on these nuclei (Table I).

DISCUSSION

It is unknown how the addition of phorbol esters to undifferentiated HL-60 cells induces specific changes in nuclear RNA transcription. One possibility is that phorbol esters stimulate protein kinase C to phosphorylate either a plasma membrane or cytoplasmic protein, which can induce nuclear changes by functioning as a second messenger. A second possibility is that phorbol esters cause the association of protein kinase C with the plasma membrane where it is cleaved to a calcium, phospholipid independent form, which then leaves the membrane and translocates to the nucleus. The third possibility, which we have explored in this communication, is that stimuli which cause the association of protein kinase C with the plasma membrane also cause the association of protein kinase C with the nucleus.

Because protein kinase C is thought to be the phorbol ester binding receptor (2), we have examined HL-60 nuclei isolated in the presence of calcium for specific phorbol ester binding. Our nuclei, which showed no evidence of plasma membrane contamination

either by phase contrast microscopy or by measurement of 5' nucleotidase (a plasma membrane marker), demonstrated approximately 37,000 receptors/nucleus. These findings are consistent with the 18,000 receptors/nucleus found in murine epidermal cell nuclei (25). In our studies, phorbol ester binding was removed by isolating nuclei in calcium-free EGTA containing buffers and replaced by the addition of partially purified protein kinase C and calcium. In addition, protein kinase C could be isolated from nuclei prepared in the presence of calcium. These data suggest that in vitro protein kinase C can bind directly to HL-60 nuclei, and that calcium is required for this binding. We next evaluated whether treatment of HL-60 cells with compounds that activate protein kinase C and cause a tight association of this enzyme with the plasma membrane would cause a similar association with the nucleus. Table I demonstrates that bryostatin 1 treatment induces an increase in [^{3}]H-PDBu binding to nuclei isolated in the presence of EGTA. This suggests that treatment of HL-60 cells with compounds which activate protein kinase C causes this enzyme to tightly associate with the nucleus under in situ conditions.

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