Induction of Protein Kinase C in Mouse Melanoma Cells by Retinoic Acid

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ABSTRACT

Retinoic acid inhibits the proliferation of B16 mouse melanoma cells. It also eliminates the ability of these cells to grow in soft agar. These biological actions of retinoic acid have been shown to be accompanied by an increase in the amount of cyclic AMP-dependent protein kinase and an induction of a new isozyme form (RII β). In this report we demonstrated that retinoic acid-treated B16 melanoma cells had large increases in protein kinase C activity.

This increased enzyme activity was accompanied by increases in both the number of phorbol dibutyrate binding sites and the amount of immunoreactive protein kinase C. Other treatments (melanocyte-stimulating hormone, serum deprivation) which inhibited the growth of these cells did not increase protein kinase C activity. When B16 melanoma cells were treated for a prolonged time (72 h) with phorbol dibutyrate, protein kinase C activity was barely detectable. Under these conditions, melanin production was inhibited and cell growth was accelerated. When retinoic acid was added together with phorbol dibutyrate, it prevented the growth stimulatory effect of the phorbol ester and increased protein kinase C activity. However, the absolute activity of the enzyme was still below that found in control cells and very much lower than in cells treated with retinoic acid alone. Taken together with previous findings, we propose that the increase in protein kinase C might be part of a differentiation program induced by retinoic acid.

INTRODUCTION

PKC, a phospholipid- and Ca2+-dependent kinase that phosphorylates substrate proteins on serine and threonine residues, is found in almost all tissues, with the highest activity being observed in brain (1). PKC is activated by several growth factors, hormones, and neurotransmitters. It is also the major if not the only receptor for phorbol ester tumor promoters (2-4). Phorbol esters can stimulate proliferation of several cell types. In addition, PKC has been shown to phosphorylate the epidermal growth factor receptor (5), the insulin receptor (6), and the protein product of the ras oncogene (p21) (7). These data suggest that protein kinase C plays an important role in regulating cell proliferation.

RA has been shown to induce differentiation and/or inhibit the growth of many different types of tumor cells (8-12). In B16 mouse melanoma cells, RA specifically blocks progression through G1, as well as eliminating growth in soft agar (13). We have previously found that, in B16 melanoma, RA stimulates the activity of PKA and increases the amount of the R1 cyclic AMP-binding subunit (13, 14). A mutant of B16 melanoma cells having defective PKA did not have its growth inhibited by RA, implying that PKA may play a critical role in the pathway leading to RA-induced growth inhibition (13). In this report we demonstrate that RA treatment of B16 mouse melanoma cells leads to increases in both the activity and amount of PKC, a kinase which is of critical importance in control of growth.

MATERIALS AND METHODS

Cell Culture. B16 F1 cells were obtained from Dr. Fidler (M. D. Anderson Hospital and Tumor Clinic, Houston, TX) and were maintained as stock in Dulbecco’s minimal essential medium that was supplemented with 2 mm l-glutamine, 2 mm sodium pyruvate, 50 units/ml penicillin G, 50 μg/ml streptomycin sulfate, and 10% newborn supplemented calf serum (Sterile Systems, Logan, UT). Every 2 months new cultures were initiated from frozen stock, in order to minimize the changes in cell phenotype that can occur with prolonged in vitro culture.

Treatment of Cells with Test Agents. B16 F1 cells were routinely seeded onto 100-mm tissue culture dishes (Falcon). The following day, cells were refed with growth medium (see above) with or without the following agents: MSH (Sigma), 5 μg/ml; all-trans-retinoic acid (Eastman Kodak), 10 μM; or PDB (Sigma), 1 μM. At the end of the incubation, replicate dishes were taken for determination of cell number by use of a hemocytometer. The remainder of the dishes were harvested for the various assays discussed below.

Protein Kinase C Assay. At selected time points, cells were harvested by aspirating off the growth medium, washing twice with cold phosphate-buffered saline, and scraping the cells into a small volume of PBS. The cell suspension was sonicated at power setting 3.5 for 20 s using the microtip of a Bronson sonifier. A solubilized fraction was prepared by centrifuging the sonicated cells at 100,000 x g for 1 h. The supernatant was then put into a 1-ml DEAE-cellulose column which had been preequilibrated in column buffer (20 mm Tris, pH 7.5, 2 mm EDTA, 0.5 mm [ethylenebis(oxethyl)enilenitri]tetraacetate, 5% Trition X-100, 1 mm dithiothreitol, 10 μg/ml aprotenin, and 5 μg phenylmethylsulfonyl fluoride). The cell suspension was sonicated at power setting 3.5 for 20 s using the microtip of a Bronson sonifier. A solubilized fraction was prepared by centrifuging the sonicated cells at 100,000 x g for 1 h. The supernatant was then put into a 1-ml DEAE-cellulose column which had been preequilibrated in column buffer (20 mm Tris, pH 7.5, 2 mm EDTA, 0.5 mm [ethylenebis(oxethyl)enilenitri]tetraacetate, 1 mm dithiothreitol). The column was then washed with 15 ml of column buffer and eluted with 2 ml of column buffer that contained 100 mm NaCl (preliminary experiments showed that the majority of PKC eluted at this salt concentration). An aliquot of this fraction was then taken for protein analysis using the Bradford dye-binding assay (15) while another aliquot was taken for PKC assay. The standard reaction mixture for PKC analysis consisted of 20 mm Tris-HCl, pH 7.5, 10 mm MgCl2, 2 mm dithiothreitol, 1 mm CaCl2, 400 μg/ml histone type HIIS (Sigma), 50 μM ATP, with or without 16.5, 5 μg phosphatidylserine, 0.2 μg phorbol, 2 μg [γ-32P]ATP (2-10 Ci/mmol; New England Nuclear), and 20 μg of sample protein in a total volume of 100 μl. The reaction was incubated at 30°C for 10 min, and then the entire reaction mixture was spotted onto Whatman P81 filter paper discs, dried, and then washed extensively in 0.2% phosphoric acid. After drying, the radioactive histone was quantitated by liquid scintillation counting. PKC specific activity is defined as the activity in the presence of phospholipid minus the activity in the absence of phospholipid per mg of protein.

Phorbol Dibutyrate Binding Assay. B16 cells were seeded onto 60-mm tissue culture dishes in growth medium and the following day were re-fed with or without 10 μM RA. At various time points during the incubation with RA, the medium was aspirated and the cells were washed with Eagle’s basal medium plus 25 mm 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.2. They were incubated with 4 nm [3H]phorbol dibutyrate (0.1 μCi/ml; New England Nuclear) in the above medium (1.5 ml) with or without 5 μM unlabeled phorbol dibutyrate (Sigma). After a 4-h incubation, the medium was removed, the cells were washed twice with cold PBS and scraped into 0.5 ml of PBS and the radioactivity was determined.

Depletion of Protein Kinase C. Cells were seeded at 1.25 x 104/100-mm tissue culture dish in growth medium. The following day, one half...
of the dishes were re-fed with medium containing 1 µM phorbol dibutyrate, while the remaining dishes (control) were re-fed with medium containing the solubilization vehicle (DMSO). After a 24-h incubation, replicate dishes were harvested for determination of cell number and protein kinase C activity, while the remainder were re-fed with medium containing the following agents: DMSO (control), phorbol dibutyrate (1 µM), retinoic acid (10 µM), or phorbol dibutyrate plus retinoic acid. These agents were incubated with the cells for 48 h, and then all plates were harvested for determination of cell number and protein kinase C activity by the methods outlined above.

Western Blot Analysis of Protein Kinase C. B16 cells treated with or without 10 µM retinoic acid for 48 h were harvested as described above for assay of protein kinase C enzyme activity. The 100,000 × g supernatant fractions were applied to a DEAE-cellulose column and the runoff plus a 150 mM NaCl eluate in column buffer were collected. These fractions were concentrated and desalted by filtration through Minicon (Amicon, Bedford, MA; M, 10,000 cutoff) units. Protein content was determined by the Bradford assay (15), and 100 µg of protein from each fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were then electrophoretically transferred (300 mA, 4 h) onto nitrocellulose paper (Hybond C, Amersham). Two different sources of protein kinase C antibodies were used in this study. Using the monoclonal antibody MC-5 from Amersham, the blots were preincubated with 3% bovine serum albumin in PBS at 4°C overnight. They were then incubated with a 1:28 titer of the antibody in 0.1% bovine serum albumin/PBS overnight at 4°C. The following day, the antibody solution was removed and the blots were washed 8 times for 5 min with 0.1% Tween 20 in PBS. Antibody binding was visualized by incubating the blots with 3.5 µCi 125I-Protein A in 0.1% Tween 20/PBS overnight at 4°C. The blots were then washed with PBS/Tween until the radioactivity in the washes reached background levels. The second antibody used in this study was chicken anti-PKC, generously provided by Dr. Curtis Ashendel (Purdue University, West Lafayette, IN). Using this antibody, the blots were preincubated with TNS containing 5% Carnation nonfat dry milk, overnight, at 4°C. They were then incubated with a 1:500 titer of the antibody for 2 h at room temperature. The antibody solution was then removed and the blots washed 5 times for 5 min in TNS containing 1 ml/liter Tween 20. The blots were then treated for 15 min with TNS containing 5% Carnation nonfat dry milk and then incubated for 2 h at room temperature with rabbit anti-IgG (kindly provided by Dr. Ashendel) at a titer of 1:500. Following this incubation, the blots were washed 5 times for 5 min with TNS/Tween and treated for 15 min with TNS containing 5% Carnation nonfat dry milk, and then antibody binding was visualized by incubation with 125I-Protein A (3.5 µCi) overnight at 4°C. The blots were then washed with TNS/Tween until the radioactivity in the washes was reduced to background levels. Blots were wrapped in plastic wrap and exposed to X-ray film (Kodak X-AR) at —70°C for 48-72 h. For quantitation of the level of immunoreactive protein kinase C, the autoradiograms were scanned with an LKB laser densitometer (model Ultrascan XL) and the integrated areas under the peaks for each sample were compared.

RESULTS

Stimulation of Protein Kinase C Activity by Retinoic Acid. We have previously determined that MSH and RA arrest growth of B16 melanoma cells in G₁ (12). Since protein kinase C has been implicated in regulating the growth of various cell types (16), we measured its activity in cells treated with the above agents and also in cells growth arrested by serum deprivation. Table 1 shows that all of the agents inhibited growth relative to the control cells; however, only RA stimulated PKC activity. The degree of stimulation varied between experiments but was generally in the range of 4-8-fold. The enzyme activity from both control and treated cultures was proportional to protein concentration and was predominantly (>95%) in the 100,000 × g soluble fraction (data not shown).

The relationship between the time of RA treatment and stimulation of PKC activity was examined. Fig. 1 shows that, by 24 h of treatment with 10 µM RA, B16 melanoma cells have a 4-fold higher specific activity of PKC, compared to untreated cells. The absolute specific activity of PKC from RA-treated cells increased 3-fold during the second 24 h of treatment; however, since the untreated cells also had higher PKC activity at this time point, the relative difference was still about 4-fold. It was technically difficult to measure time points beyond 48 h of treatment because the large amount of melanin produced by these cells became cytotoxic.

Phorbol Dibutyrate Binding Studies. In order to determine if the RA-induced increase in PKC activity was due to an increase in the number of PKC molecules, we performed two kinds of experiments. In the first experiment, we measured the specific binding of PDB to intact B16 mouse melanoma cells. Fig. 2A shows that, with increasing time of RA treatment there is a corresponding increase in the specific binding of PDB. These data correlate with the time-dependent RA-induced increase in PKC activity. At 48 h of RA treatment, we compared the relative affinity of PDB binding to its receptor in control and treated cells (Fig. 2B). This Scatchard plot revealed both high and low affinity binding sites, similar to previous findings (17). However, we believe that the low affinity site may be due to nonspecific binding. The Kᵣ for the high affinity receptor (PKC) in control and treated cells is approximately the same (20–35 nM); however, the number of receptor sites is 10² in control cells versus 3–4 × 10⁵ in RA-treated cells.

Western Blot Analysis of Protein Kinase C Levels. The increase in the amount of PKC was verified by a second approach, in which we measured the steady state levels of PKC by the Western blot technique. B16 cells were incubated with and without 10 µM RA for 48 h. Equal protein aliquots from the crude 100,000 × g supernatant fractions and a 150 mM NaCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell no. (x 10⁶)</th>
<th>Specific activity/cell/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.1</td>
<td>9.5 ± 1.2</td>
</tr>
<tr>
<td>10 µM RA</td>
<td>1.1</td>
<td>35.1 ± 3.5</td>
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<tr>
<td>2 µg/ml MSH</td>
<td>1.6</td>
<td>7.2 ± 0.2</td>
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<td>0.2% serum</td>
<td>1.2</td>
<td>11.8 ± 0.6</td>
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<tr>
<td>100 µM RA</td>
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<td>589 ± 59</td>
</tr>
<tr>
<td>10 µM RA</td>
<td>1.0</td>
<td>95 ± 3</td>
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<tr>
<td>10 µM RA</td>
<td>1.0</td>
<td>115 ± 6</td>
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![Fig. 1. Time-dependent increase in protein kinase C activity in retinoic acid-treated B16 mouse melanoma cells. B16 cells were treated with or without 10 µM retinoic acid. At the indicated time points, cells were harvested and assayed for protein kinase C activity as described in the text. ◊, Control; □, +10 µM retinoic acid. Bars, SE (three experiments).](image-url)
with l-UM phorbol dibutyrate had less than one-half the PKC eluate of a DEAE-cellulose column were separated by sodium (Table 2). PDB did not alter the growth of the cells during the activity of control cells, while an additional 48 h incubation produced complete loss of PKC activity (18, 19). B16 cells treated for 24 h with phorbol esters results in a nearly complete depletion of PKC. Treatment of cells with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly complete depletion of PKC activity (18, 19). B16 cells treated for 24 h with phorbol dibutyrate results in a nearly...
ditions, RA still stimulated PKC activity relative to cells treated
with PDB alone; however, the absolute activity was considerably
less in control cells or cells treated with RA alone.

DISCUSSION

In this report we have shown that retinoic acid can induce a
substantial increase in protein kinase C in B16 melanoma cells.
This effect of retinoic acid is specific and not simply due to the
growth state of the cells, since other agents which inhibited
proliferation did not increase the activity of protein kinase C.
The amount of time required before the increase in protein
kinase C activity could be detected, together with the increases
in the number of phorbol dibutyrate binding sites and the
amount of immunoreactive protein kinase C (as deduced by
Western blots), suggests that retinoic acid is increasing the
number of protein kinase C molecules. Furthermore, retinoic
acid treatment of the cells did not alter the affinity of protein
kinase C for phorbol dibutyrate binding (Fig. 2B) or its affinity
for calcium or phospholipid (data not shown). There is some
discrepancy between the degree of increased protein kinase C
measured by either enzyme activity or phorbol ester binding
(4–8-fold) versus that quantitated by Western blots (20–25-
fold). This could be explained if we were overestimating the
PKC enzyme activity or phorbol ester binding activity in control
cells. The amount of these two activities is very low in control
cells, and a slight error could be magnified during the subse-
quent calculations. An alternative explanation is that not all of
the immunoreactive PKC is enzymatically active. It does not
appear to be degraded, since the M, of the immunoreactive
material is 80,000, the same as authentic rat brain PKC. Further
experiments will be needed to resolve this question.

It was previously shown that retinoic acid-induced differen-
tiation of the human promyelocytic cell line HL-60 was accom-
panied by increased protein kinase C activity (20). Other agents,
such as DMSO and vitamin D,( which induced granulocytic
differentiation of HL-60 also increased protein kinase C activity
(21). In the case of vitamin D,( induced differentiation of HL-
60, there was also an increase in phorbol ester receptors (22).
More recently, Makowske et al. (23), using antipeptide anti-
bodies that specifically recognized the a, ß, and y isozymes of
protein kinase C, showed that all three isozymes increased
about 3-fold in abundance after a 96-h treatment of HL-60 cells
with 1 $mu;m$ retinoic acid. DMSO, which also induced differen-
tiation, also increased the amount of all three isozymes, al-
though there was a relatively greater increase in the a and ß
forms of protein kinase C. In addition to HL-60, the human
monoblastoid cell line U937 also exhibited increased protein
kinase C activity when induced to differentiate by treatment
with vitamin D,( (24). It is not clear from these studies whether
the increase in protein kinase C is a result of differentiation or
the inhibition of cell proliferation which accompanies this proc-
ess. We have previously shown (12) that both MSH and retinoic
acid arrest B16 cell growth in the G, phase of the cell cycle.
However, as shown in this report, only retinoic acid increases
the relative amount of protein kinase C. Therefore, in these
melanoma cells, the increase in protein kinase C cannot simply
be a consequence of arrest in G,.

In order to determine what role the increased amount of
protein kinase C might play in the biological effects of retinoic
acid, we took advantage of the fact that prolonged treatment
of cells with phorbol esters results in depletion of protein kinase
C (18, 19). Using phorbol dibutyrate, we found that a 24-h
treatment markedly diminished protein kinase C activity, and
a subsequent additional 48-h incubation with phorbol dibuty-
rate almost completely eliminated the activity of this enzyme.
The B16 cells responded to prolonged phorbol dibutyrate treat-
ment by an increased cell proliferation and a decrease in melan-
in production. Mufson et al. (25) also found that TPA inhib-
ited melanin production in the C3 clone of B16 melanoma.
However, they found that the phorbol ester did not alter growth.
This may have been due to the fact that the concentration of
TPA which they used ($10^{-4}$ to $10^{-3}$ M) was not sufficient
to completely deplete the cell of protein kinase C. From our
results, we cannot deduce whether the enhanced cell growth is
directly due to the depletion of protein kinase C or a conse-
quence of depressed melanin synthesis, the byproducts of which
are cytotoxic. When retinoic acid was added to B16 cells in the
continued presence of phorbol dibutyrate, most of the growth
stimulation induced by the phorbol ester was eliminated. How-
ever, the ability of retinoic acid to inhibit growth relative to
untreated controls was also negated. Thus, these two com-
ounds have almost equal and opposing effects on B16 melano-
ma cell growth. Retinoic acid was also able to induce a 5-
fold increase in PKC activity even in the continued presence of
PDB. However, the absolute enzyme activity was still signifi-
cantly below the untreated control level.

Phorbol esters have been shown to support the growth of
normal melanocytes in culture by permitting preferential at-
tachment of melanocytes from epidermal cell suspensions and
then stimulating the attached melanocytes to grow (26). Since
the phorbol esters were present continuously in the culture
medium, it is likely that protein kinase C was depleted, a
situation analogous to our treatment of B16 melanoma with
phorbol dibutyrate. Also, a recent report has shown that, in
C3H 10T½ mouse fibroblasts transfected with the human Ha-
ras oncogene, PKC is also down-modulated (27). Thus it may
be that, in certain cell types, high levels of protein kinase C are
incompatible with enhanced cell proliferation. In contrast to
these results, it has been recently reported (28, 29) that NIH-
3T3 cells or rat fibroblasts transfected with protein kinase C
plasmids under the control of strong viral promoter/enhancer
elements resulted in high expression of protein kinase C and
disordered growth control. The transfected cells grew to a
higher saturation density and formed small colonies is soft agar
in the absence of TPA. These conflicting results suggest either
that different cell types respond differently to fluctuations in
protein kinase C levels or that, at least in the case of retinoic
acid, multiple biochemical pathways interact to result in growth
inhibition.

It is not clear how retinoic acid increases protein kinase C
levels. B16 cells contain retinoic acid-binding proteins,4 but we
have not determined whether this corresponds to the well
known cellular retinoic acid-binding protein (30) or the newly
discovered retinoic acid receptor (31, 32). Since the retinoic
acid receptor has the characteristics of a DNA-binding protein
and belongs to the family of steroid receptor genes (31), it
provides a potential pathway by which retinoic acid could
directly influence the transcription of protein kinase C.

Finally, we are left with the question of what is the role of
the retinoic acid-induced increase in protein kinase C. Since
retinoic acid was still able to increase protein kinase C activity
in melanoma cells which had been depleted of PKC by phorbol
ester treatment, these experiments did not definitely settle
whether this enzyme is involved in the retinoic acid-mediated
growth inhibition. However, we would like to propose an alter-

* Niles and Traish, unpublished results.
nitive explanation for the increase in protein kinase C, i.e., that it is part of a differentiation program induced by retinoic acid. Melanocytes are derived from the neural crest during development and share many characteristics of nerve cells (e.g., presence of nerve growth factor receptors). Since the brain has the highest amount of protein kinase C of any organ, it is quite likely that mature melanocytes are also rich in this enzyme. Therefore, the retinoic acid-induced increase in protein kinase C in these melanoma cells could be indicative of differentiation to a more mature state. Examination of other melanocyte-specific markers in retinoic acid-treated B16 melanoma cells will be required to provide further evidence for this hypothesis.

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REFERENCES

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