Characterization of Retinoic Acid-induced AP-1 Activity in B16 Mouse Melanoma Cells*  

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Retinoic acid (RA), a biologically active metabolite of vitamin A, has been shown to play an essential role in maintaining the differentiated phenotype in a variety of tissues (1, 2). This retinoid also induced differentiation in a number of tumor cell lines in culture (3–7). In B16 mouse melanoma cells, RA inhibits both anchorage-dependent and -independent growth, stimulates melanin production, and increases nerve growth factor receptors on the cell surface (8).

Several groups, including our own, have shown that the RA-induced differentiation of certain tumor cells is accompanied by an increase in PKC expression (5, 9–12). In B16 mouse melanoma cells RA induces a 5–8-fold increase in PKCa protein (9, 10), which is accompanied by a selective enrichment of nuclear PKCa. We report here that RA also increases AP-1 activity in these cells. Transient transfection of B16 cells with luciferase reporter gene constructs indicated that RA induced a concentration-dependent increase in AP-1 activity. Acute treatment (2 h) of B16 cells with phorbol dibutyrate (PDB) increased AP-1 activity by 10-fold. RA treatment did not change the expression of Jun family members; however, it decreased the expression of c-Fos. In contrast acute PDB treatment induced c-Fos expression, while having little effect on c-Jun. Five DNA-protein complexes were formed with nuclear extracts from B16 cells and an oligonucleotide containing an AP-1 consensus sequence. Several complexes were decreased in cells treated with RA. Conversely, certain complexes were increased in cells acutely treated with PDB. The slowest migrating complexes were shown to contain Fos family members. Down-regulation of PKCa inhibited both the acute PDB-induced and the RA-induced increase in AP-1 activity. The selective PKC enzyme inhibitor, bisindolylmaleimide, reduced PDB-stimulated AP-1 activity, but enhanced RA-induced AP-1 activity. These results together with our previous studies suggest the intriguing possibility that PKC protein, but not enzyme activity, may be required for RA-induced AP-1 activity.

ExPERIMENTAL PROCEDURES

Cells and Culture Conditions—B16 mouse melanoma cells were grown in a humidified atmosphere of 7% CO2, 95% air at 37 °C in Dulbeco’s modified Eagle’s medium. This medium contained 1 g/liter glucose and was supplemented with 10% heat-inactivated bovine calf serum (Sterile Systems, Logan, UT), 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate.

Retinoic Acid—All-trans-RA was obtained from Fluka Chemical Co. (New York). All experiments involving the use of RA were conducted in subdued light to prevent photo-oxidation of the retinoid. A concentrated stock solution of RA (10 mM) was prepared in ethanol. This stock solution was diluted to the desired final concentration in tissue culture medium and was sterile filtered before adding to the cells.

Northern Blotting—RNA was isolated by a single-step method as described previously (24). The RNA was then fractionated on 1% agarose containing formaldehyde and transferred to Hybond N nylon membranes (Amersham Corp.) by downward alkaline blotting (25). The transferred RNA was cross-linked to the membrane by UV light. The membrane was prehybridized for 1 h in 6 × SSC and 2% SDS. 32P-Labeled cDNA probes (1 × 106 dpm/ml) were then incubated with the membranes in fresh hybridization solution for 20 h. Blots were washed three times for 15 min each in 1 × SSC + 0.1% SDS, 0.5 × SSC + 0.1% SDS, and 0.2 × SSC + 0.1% SDS, respectively. The blots were exposed to Kodak XAR film in cassettes at −70 °C for 2–5 days. All the cDNA probes were labeled using the “prime-a-gene” labeling system from Promega (Madison, WI) + 0.25 mM dNTP. The relative amount of the different RNA species was quantitated by imaging the autoradiogram with a Molecular Dynamics laser densitometer, making sure that the signals were within the linear range of the instrument. The data are expressed as the ratio of the specific mRNA to the internal control, glyceraldehyde-3-phosphate dehydrogenase.

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1 The abbreviations used are: RA, retinoic acid; PKC, protein kinase C; PDB, phorbol dibutyrate; GADPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.
Proteins (50 μg) were fractionated using SDS-polyacrylamide gel electrophoresis with 10% separating and 5% stacking gels. Proteins were then transferred to Hybond-C membranes (Amersham Corp.) by using a semidry transfer cell. The membrane was incubated in blocking solution (Tris-buffered saline containing 0.2% Tween 20 and 5% nonfat dry milk, TBST) overnight. Blots were then incubated with 1 μg/ml polyclonal anti-c-Jun antibody (Ab-2, Oncogene Science, Mineola, NY), or with 1 μg/ml polyclonal anti-c-Fos antibody (Oncogene Science) for 1 h. They were then washed three times in TBST and incubated with 1:3000 dilution of secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG, Amersham Corp.) for 1 h. The membranes were washed three to five times in TBST, and signals were visualized by use of the enzyme-substrate kit from Amersham Corp. Reverse Transcription-PCR—The RNA PCR core kit provided by Perkin-Elmer was used for these assays. 1 μg of RNA from control or RA-treated B16 cells was converted to cDNA using Moloney murine leukemia virus reverse transcriptase with oligo(dT) (1.5 mM) as primers. The 30-μl reaction contained 1 × PCR buffer II, 1.5 mM MgCl₂, 0.5 μM each of dATP, dTTP, dCTP, and dGTP, and 30 units of RNAse. RNA was denatured at 70 °C for 3 min and cooled on ice before adding the above reagents. Samples were incubated for 45 min at 42 °C and then 10 min at 95 °C.

PCR amplification of the cDNAs was performed using 50-μl reactions containing 1 × PCR buffer II, 1.5 mM MgCl₂, 0.2 μM each of dATP, dCTP, dGTP, and dTTP, plus 2.0 units of Taq polymerase, 1.0 mM of each primer, and 10% of the cDNA synthesized in the RT reaction. Primer sequences for c-jun were (5'-CCAGGATCTTTGCGGCTCCTA-3'), junB (5'-AAACCCACCTTGCGGCTCA-3'), and junD (5'-CCGGATCTTGGGCTCCTCA-3') (sequence information kindly provided by Dr. Steven Estus, Sanders-Brown Center on Aging, University of Kentucky). All reactions were covered with a drop of mineral oil and subjected to 17–25 PCR cycles. The typical reaction conditions were 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The amplified cDNA products were separated by electrophoresis on 7.5% polyacrylamide gels, stained with ethidium bromide, and visualized by UV light. c-jun, junB, and junD cDNAs were used as positive controls. Primers specific for β-actin were used to normalize the RNA in each RT reaction.

Electrophoretic Mobility Shift Assay—An oligonucleotide containing a consensus AP-1 sequence (5'-CCAGGATCTTTGCGGCTCCTA-3') was radiolabeled with 32P and used as a probe. Nuclear extract control and transfected cells (10 μg of protein) were incubated with 30,000 dpm of probe in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 40 mM NaCl, 12% (v/v) glycerol, and 0.5 μg of poly(dI-dC) in a total reaction volume of 25 μl. Where indicated, 1–2 μg of anti-c-Jun or c-Fos antibodies (Santa Cruz Biotech, Santa Cruz, CA) were added to the reaction mixture, which was then incubated at room temperature for an additional 45 min. Samples were separated on 5% nondenaturing polyacrylamide gels.

Transfections—B16 cells were transfected with 4 μg of pGL-2-AP-1 DNA or the pGL-2 vector alone + 1 μg of SV40-β-galactosidase DNA to correct for transfection efficiency using the calcium phosphate precipitation method (29). After an overnight incubation, the transfection medium was removed, and the cells were incubated with the various compounds as indicated under “Results.” Cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase activity using kits from Promega. All transfections were performed in triplicate dishes, and the experiments were repeated three to five times.

PKC Enzyme Assay—Cells were lysed on ice in extraction buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM diithiothreitol, 5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Cell homogenates were further ensured by the con- secutive 10-s sonications with a Tekmar sonic disruptor at power set- ting 60. The total lysate was centrifuged at 12,000 × g for 15 min. The supernatant was loaded onto a DEAE-cellulose anion exchange column (Cellex-D, Bio-Rad), previously equilibrated with column buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM diithiothreitol). The column was washed with 5 volumes of column buffer. The PKC fraction was eluted with 2.5 column volumes of buffer containing 100 mM NaCl and concentrated with a Centricon-10 microconcentrator (Amicon, Bedford, MA). Protein concentrations of samples were determined by the Pierce BCA reagent. Samples were diluted to equal protein concentrations (20 μg) and assayed with a commercially available PKC assay system (Amersham Corp.) + 0.2 μCi of [γ-32P]ATP in the presence and absence of 12-O-tetradecanoylphorbol-13-acetate and phosphatidylserine. The system utilizes synthetic, PKC-specific, substrate peptides, which become phosphorylated with the radiolabeled phosphate group from ATP. At the end of the reaction, the radiolabeled peptide was separated from the unincorporated 32P by the use of affinity paper for the peptide. The degree of phosphorylation was determined by liquid scintillation counting. Enzyme activity was calculated from counts/min taken into account the specific activity of the radioisotope and reaction time. Specific enzyme activity was obtained by subtracting the counts/min obtained in the absence of the lipid mixture from that obtained in the presence of the lipid mixture.

Data Presentation—All experiments were repeated a minimum of three times. The data in most figures are from a representative experiment, which was qualitatively similar in the repeat experiments. Data from all transfection experiments are expressed as the mean ± S.E. of the mean of triplicate dishes of transfected cells for each treatment group. Since transfection experiments can have a higher degree of variability, these experiments were repeated a minimum of four to five times with similar qualitative results.

RESULTS

AP-1 Transcriptional Activity—Phorbol ester-activated PKC stimulates AP-1 activity (15). In light of the 6–8-fold increase of PKCs protein in RA-treated B16 melanoma cells (10) and a selective enrichment of nuclear-associated PKCα (13), we determined whether RA also increased AP-1 activity. B16 melanoma cells were transiently transfected with a pGL-2 plasmid containing four tandem AP-1 consensus sequences inserted 5′ to an SV40 promoter driving the expression of luciferase. Transfected cells were treated for 24 h with or without various concentrations of all-trans-retinoic acid. As a positive control, one group of transfected cells was treated with 1 μM PDB for the last 2 h of incubation. At the end of the incubation period (48 h from the start of transfection), all cells were harvested and assayed for luciferase and β-galactosidase activity. These experiments (Fig. 1) revealed that B16 cells have endogenous
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AP-1 activity (pGL-2 without AP-1 sequences gave only background levels of luciferase activity) and that this activity is stimulated 9-fold by PDB. RA increased AP-1 activity 2-fold at the lowest concentration (10 nM), and the stimulation increased to 4-fold at the highest RA concentration (10 μM).

Effect of RA and PDB on Expression of Fos and Jun Family Members—The AP-1 transcription complex is most commonly composed of c-Jun homodimers or c-Jun/c-Fos heterodimers. We examined the possibility that the RA and/or PDB-induced increase in AP-1 activity could be due to an increase in the expression of one of these transcription factors. The amount of c-jun and c-fos mRNA in control, PDB-treated, or RA-treated B16 cells was examined by Northern blotting. Two messages (3.2 and 2.6 kb) for c-jun were found in B16 cells (Fig. 2A). The 2.6-kb mRNA was the predominant form in these cells. c-jun mRNA was not consistently altered by RA treatment. The small increases depicted in this blot were not always seen in replicate experiments. In contrast, a 1-h treatment with PDB increased the amount of c-jun mRNA by 2-fold. B16 cells also express a 2.1-kb c-fos mRNA (Fig. 2B). Treatment of the cells with RA led to a consistent decrease in this c-fos message, while a 1-h treatment with PDB induced a 5-fold increase in the amount of c-fos mRNA. We were not able to detect junB or junD mRNA by Northern blotting. Semiquantitative reverse transcription-PCR was employed to examine the expression of these jun family members. We found that these genes were expressed in B16 cells, but after ensuring a linear signal (by varying the number of PCR cycles and correcting by β-actin levels), we found no change in junB or junD mRNA levels (data not shown).

To determine if the changes in mRNA were reflected in the amount of c-Jun or c-Fos protein, we treated B16 cells for different times with RA and measured the amount of the appropriate protein by Western blotting. B16 cells express a 39-kDa immunoreactive c-Jun protein (Fig. 3A). There was no consistent increase in the amount of this protein in RA-treated cells compared with untreated cells. The antibody used to detect c-Fos protein also recognizes Fos B as well as fra-1 and fra-2 (Fig. 3B, NIH 3T3 cell extract used as a control). In control cells no signal was detected at 6 h, but these cells had increasing amounts of c-Fos protein at 24 and 48 h of incubation. The expression of other Fos family members was barely detectable in nuclear extracts from B16 cells. Treatment of these cells for 48 h with RA markedly decreased (60% of control) the amount of c-Fos protein compared with control cells at the same time point (Fig. 3B).

Since PDB induced a large increase in c-fos mRNA, we examined the time course for this induction at both the RNA and protein level. A 1-h PDB treatment increased c-fos mRNA by 4-fold (Fig. 4A). This increase was transient and returned to unstimulated levels by 12 h despite the continued presence of PDB. PDB also increased the level of c-Fos protein within 2 h of

FIG. 2. The effect of retinoic acid or phorbol dibutyrate on c-jun and c-fos mRNA expression in B16 cells. Cells were treated with 10 μM RA for 24 and 48 h. Phorbol dibutyrate (1 μM) was incubated with the cells for 1 h. RNA (25 μg) from each treatment group was isolated as described under ‘‘Experimental Procedures’’ and analyzed for the c-jun (A) and c-fos (B) mRNA level by Northern blotting. Hybridization of the blots with the cDNA for GAPDH was used to correct for equal loading of the RNA and for the specificity of any changes in the level of c-jun and c-fos mRNA. The relative amount of RNA was determined by computerized densitometric analysis of the autoradiograms after normalizing for the amount of GAPDH.

FIG. 3. Effect of RA on c-Jun and c-Fos protein level. B16 cells were treated with 10 μM RA for different times and then processed to obtain nuclear extracts. 50 μg of nuclear extracts were analyzed by Western blotting for the amount of c-Jun protein (A) using anti-Jun (polyclonal, Oncogene Science) and c-Fos protein (B) using anti-Fos antibody (polyclonal, Oncogene Science), respectively. Antibody-protein complexes were detected by enhanced chemiluminescence.
Fig. 4. Temporal induction of c-Fos expression by phorbol dibutyrate. Cells were treated \(\pm 1 \mu M\) PDB for different periods of time and then harvested and RNA extracted (A). Northern analysis (20 \(\mu g\)/sample) was used to determine the amount of c-fos mRNA at each time point. The amount of GAPDH mRNA in each sample was used as an internal control. An autoradiogram in the linear signal range was scanned by densitometry and normalized to the GAPDH signal to obtain the relative amount of c-fos mRNA. Nuclear protein extracts (B) were analyzed by Western blotting and the relative amount of c-Fos protein at each time point determined by densitometry. This experiment was repeated several additional times with similar results.

Effect of RA and PDB on AP-1 Binding Activity—Since RA did not increase the amount of any of the AP-1 family members, we determined whether an increase in binding activity might account for the RA-induced increase in AP-1 transcriptional activity. Using electrophoretic mobility shift assays with a radiolabeled 15-base pair oligonucleotide containing one AP-1 consensus site, five protein-DNA complexes were observed (Fig. 5A). Complex 5 was absent when nuclear extracts from 24- or 48-h RA-treated cells were analyzed. Nuclear extracts from 48-h RA-treated cells were also missing complex 1, while

Fig. 5. Effect of retinoic acid and phorbol dibutyrate on nuclear protein binding to a consensus AP-1 oligonucleotide. A, nuclear protein (10 \(\mu g\)) from B16 cells which had been treated with 10 \(\mu M\) RA for either 24 or 48 h or with PDB for 2–24 h was incubated with radioactive oligonucleotides as described under “Experimental Procedures.” Some samples were incubated for an additional 45 min with 2 \(\mu g\) of anti-Jun (lane 7) or anti-Fos (lane 8) antibodies (Santa Cruz Biotech, Santa Cruz, CA). B, reactions were prepared as described above and were incubated with 10-, 20-, or 50-fold excess of nonradioactive wild type or mutated (5'-CGCATGAGTCAGACA-3') AP-1 oligonucleotides.
the binding activity of complexes 2 and 4 were reduced compared with control cells (Fig. 5A). In contrast, nuclear extracts from B16 cells treated for 2 h with PDB had markedly increased binding activity of complex 2. As the time of PDB treatment was increased, binding activity of complexes 1 and 2 decreased, but the activity of complexes 4 and 5 increased dramatically. Incubation of nuclear extracts with antibodies to c-Jun did not change the intensity of any bands, nor were any bands supershifted. In contrast, incubation of the nuclear extracts with c-Fos antiserum decreased the binding activity of complexes 1 and 2 and also induced the formation of a supershifted complex. Competition experiments with nonradioactive wild type and mutant AP-1 oligonucleotides indicated the following sensitivity: complex 1 > complex 4 > complex 2 = complex 3 > complex 5. All five complexes were still present at a 50-fold excess of the mutant oligonucleotide (Fig. 5B).

Role of PKC in RA-induced AP-1 Activity—RA-induced differentiation of B16 cells is accompanied by a 6–8-fold increase in AP-1 mRNA and protein. PDB, via its activation of PKC, is known to increase AP-1 activity. Therefore we investigated whether PKC was required for the RA-induced increase in AP-1 activity in B16 melanoma cells. We down-regulated PKC protein in cells transfected with the AP-1-luciferase reporter gene by chronic PDB treatment and measured their ability to respond to RA. We also treated the transfected cells with the selective PKC inhibitor bisindolylmaleimide and measured the ability of RA or PDB to increase AP-1 activity. Fig. 6A shows that down-regulation of PKC inhibited both the acute PDB-induced (lane 2 versus 5) and the RA-induced (lane 3 versus 6) increase in AP-1 activity. Western blot analysis (Fig. 6B) shows that RA induced PKCa, while chronic PDB treatment depleted the cells of PKCa. RA + PDB-treated cells have PKCa levels slightly lower than control cells, but higher than cells treated with PDB for 24 h. In separate experiments we tested the effect of inhibition of PKC enzyme activity on the PDB and RA-induced AP-1 transcriptional activity. We found that PKC enzyme activity from B16 cells was reduced by 90% at 0.1 μM bisindolylmaleimide and was not detectable at 1.0 μM concentration of this inhibitor (Fig. 7A). When this inhibitor was added to the transfected cells, it reduced the PDB-induced AP-1 activity from 11-fold down to 4-fold. In contrast, the inhibitor enhanced RA-induced AP-1 activity from 5-fold to 8-fold. To determine if changes in the level of PKCa might explain these unanticipated results, Western blot analysis was performed on cells treated/untreated with 10 μM RA, 2 μM bisindolylmaleimide, or a combination of these two compounds. We found an increase in PKCa protein in inhibitor-treated cells compared with the controls. PKCa protein was also increased in RA + inhibitor-treated cells compared with RA-treated cells (Fig. 7C).

**DISCUSSION**

We have demonstrated that RA increases AP-1 transcriptional activity in a dose-dependent manner. This is in marked contrast to several studies in which RA inhibited AP-1 transcriptional activity (19, 20). RA-induced F9 teratocarcinoma differentiation, however, is also accompanied by an increase in c-jun expression and enhanced AP-1 binding activity (22). Furthermore, ectopic expression of c-jun leads to differentiation of P19 embryonal carcinoma cells in the absence of RA (23), suggesting a critical role for c-Jun in RA-induced differentiation.

We examined the possibility that, similar to F9 teratocarcinoma cells, RA increases the expression of one of the members of the AP-1 transcription complex. RA did not increase the expression of any fos or jun family member, but instead decreased the expression of c-fos mRNA and c-Fos, Fos B, and fra-1 proteins. Busam et al. (30) previously reported that RA decreased mitogen-induction of c-fos mRNA. They also found that the induction of c-jun mRNA was suppressed, but required higher concentrations of RA and a longer period of incubation. We found that the mitogen, PDB, transiently increased c-Fos family member protein expression in B16 cells. Interestingly, Fos B and fra-1 increased more slowly than c-Fos. Delayed expression of fra-1 and fra-2 with serum stimulation has previously been reported (31). A possible explanation may lie in the observation that c-Fos can transactivate the fra-1 and fra-2 genes (32). These data suggest that the RA-induced increase in AP-1 transcriptional activity may not involve a c-Jun/c-Fos heterodimer.

Since an increase in members of the AP-1 transcription complex could not explain the RA-induced increase in AP-1 transcriptional activity, we examined the possibility that RA altered AP-1 DNA binding activity. Using a consensus oligonucleotide from the AP-1 site in the collagenase gene and nuclear proteins from untreated B16 cells, we observed five protein-DNA complexes. Instead of enhancing binding, RA in-
hibited the appearance of complex 5 and with longer times of incubation (48 h) also inhibited the appearance of complex 1. Also, the intensity of complexes 2 and 4 was reduced by a 48-h treatment of the B16 cells with RA. Short term (2 h) treatment of cells with PDB increased the intensity of complex 2, but with longer times of treatment this change was reversed, and the intensity of complexes 4 and 5 dramatically increased. Since a Fos antiserum diminished the intensity of complexes 1 and 2 and caused a “supershifted” complex, we conclude that these complexes contain members of the Fos family. A variety of Jun antisera failed to diminish the appearance or to “supershift” any complex. Some of these same antisera successfully recognized c-Jun on Western blots. Since the electrophoretic mobility shift assay is conducted under nondenaturing conditions, one explanation for these results is that the epitope recognized by the antibodies is unavailable under the electrophoretic mobility shift assay conditions. Alternatively, c-Jun may not be involved in AP-1 complexes using our assay conditions (B16 melanoma nuclear extracts, specific AP-1 oligonucleotide). The acute PDB-induced increase in complex 2 and its decrease in RA-treated cells probably reflects the opposite effect of these agents on the expression of c-Fos. Since we have shown that chronic treatment with PDB depletes B16 cells of PKCα, the major isotype expressed by these cells (33), the decrease in complex 2 and the increase in complexes 4 and 5 may reflect a change in the expression of AP-1-associated transcription factors regulated through the PKC pathway. Overall, these results suggest that the RA-induced increase in AP-1 transcriptional activity cannot be explained by an increase in binding activity.

Finally, we examined the role of PKC in the RA-induced increase in AP-1 transcriptional activity. We found that down-regulation of PKC, through chronic PDB treatment, inhibited both acute PDB and RA-induced increase in AP-1 transcrip-

![Figure 7](http://www.jbc.org/)

**Fig. 7.** The effect of inhibition of PKC enzyme activity on RA- and PDB-induced AP-1 transcriptional activity. A, cells were harvested, and PKC-enriched fractions were obtained by ion-exchange chromatography. Equal amounts of protein (20 μg) were assayed as described under “Experimental Procedures” plus or minus different concentrations (0.1, 0.5, and 1 μM) of bisindolylmaleimide. PKC-specific activity is expressed as picomoles of 32P transferred to peptide substrate/min/μg of protein. B, B16 cells were transfected with the 4× AP-1 reporter plasmid and the β-galactosidase plasmid as described previously. Following transfection, cells were treated with 1 μM PDB (2 h), 10 μM RA (24 h), 2.5 μM bisindolylmaleimide (24 h), bisindolylmaleimide + RA (24 h), or bisindolylmaleimide + PDB (24 h and 2 h, respectively). Cells were harvested 48 h after transfection, and luciferase activity was measured and normalized to the β-galactosidase activity. This experiment was performed in triplicate, and the entire experiment was repeated four times. The data presented are the mean ± S.E. of the mean (error bars) of triplicate dishes for each treatment group from a representative experiment. C, cells were treated with 10 μM RA, 2.5 μM bisindolylmaleimide, or a combination of these two agents for 24 h. Total cell extracts (75 μg) were then analyzed for the amount of PKCα by Western blotting using an anti-PKCα monoclonal antibody from Upstate Biotechnology, Inc.
tional activity. However, the PKC-specific enzyme inhibitor, bisindolylmaleimide, enhanced RA-induced AP-1 transcriptional activity, while inhibiting acute PDB stimulation of AP-1 transcriptional activity by 60%. These data lead to a tentative conclusion that PKC protein, but not PKC enzyme activity, is required for the RA induction of AP-1 activity. It has been reported that PKC stimulates phospholipase D activity through a nonenzymatic mechanism (34). Also PKC binds to proteins other than substrates (35, 36). Inhibition of PKC enzyme activity also increased the amount of PKCα protein (Fig. 7C). A likely explanation for this result is the enhanced proteolysis of activated PKC (37), which would be diminished in bisindolylmaleimide-treated cells. The finding that inhibition of PKC enzyme activity enhanced RA-stimulated AP-1 transcriptional activity correlates with other data presented in this study. It reinforces the conclusion that activation of PKC enzyme activity (via PDB) increases AP-1 activity by a different pathway than that induced by RA. Our data also suggest that the two pathways are antagonistic to each other. This might provide a molecular explanation for the antagonistic action of phorbol esters and RA on B16 melanoma growth and differentiation (10, 33). It is important to note that, while both agents provide a molecular explanation for the antagonistic action of the two pathways are antagonistic to each other. This might

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