Polycyclic Aromatic Hydrocarbon *o*-Quinones Inhibit the Activity of the Catalytic Fragment of Protein Kinase C†

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ABSTRACT: Polycyclic aromatic hydrocarbons (PAHs) require metabolic activation to exert their carcinogenic effects. PAH *trans*-dihydrodiol proximate carcinogens are oxidized by aldo−keto reductases (AKRs) to their corresponding reactive and redox-active *o*-quinones which may have the properties of initiators and promoters. To determine whether these *o*-quinones target protein kinase C (PKC), their effects on human recombinant PKCα and PKC*δ* and the catalytic fragment of rat brain PKC were determined. Naphthalene-1,2-dione (NP-1,2-dione), benzo[*a*]pyrene-7,8-dione (BP-7,8-dione), and 7,12-dimethylbenz[*a*]anthracene-3,4-dione (DMBA-3,4-dione) potently inhibited (IC_{50} values 3−5 *μ*M) the basal and stimulated activity of the holoenzymes PKCα and PKC*δ* in a dose-dependent manner. Inhibition of PKC by BP-7,8-dione was observed irrespective of whether PKCα activity was stimulated with phorbol 12-myristate 13-acetate (PMA), phosphatidylserine (PS), or Ca^{2+} or whether PKC*δ* was stimulated with phorbol 12-myristate 13-acetate (PMA) or phosphatidylserine (PS), suggesting that the inhibition was not cofactor-specific. All three quinones inhibited the catalytic fragment of PKC in vitro, yielding identical IC_{50} values (3−5 *μ*M), indicating that they interact with the catalytic domain of PKC rather than the cofactor/activator sites. In contrast, no effect on either the holoenzyme or the catalytic fragment was observed with the corresponding PAH *trans*-dihydrodiols, indicating that inhibition was *o*-quinone-specific. Irreversible inhibition of the catalytic fragment of PKC was observed since activity could not be restored by dialysis, suggesting that arylation of the fragment had occurred. NP-1,2-dione and BP-7,8-dione also suppressed PKC activity in human breast cancer MCF-7 cell lysates which express PKCα, -*β*, -*δ*, -*ϵ*, -*ι*, and -*λ* isozymes. These data suggest that PAH *o*-quinones, generated by AKRs, may affect cellular signaling through suppression of the activity of PKC isoforms.

Polycyclic aromatic hydrocarbons (PAHs)[1] are ubiquitous environmental pollutants and may be the causative agents in human lung cancer. PAHs have several important properties. First, they are procarcinogens and require metabolic activation to exert their deleterious effects (*1*−*3*). Second, they can act as complete carcinogens in experimental animals and may act as initiators and promoters. While much is known about the metabolic activation of PAHs to electrophiles that alkylate DNA leading to initiation, relatively little is known about how PAHs act as tumor promotors.

Three pathways of metabolic activation of PAHs have been proposed. In the first pathway, the *trans*-dihydrodiols formed by the combined action of CYP1A1 and epoxide hydrolase are converted to diol epoxides by CYP1A1 (*4*−*7*). In the case of BP-7,8-diol, the end product is *anti*-BPDE. *anti*-BPDE is highly mutagenic and tumorigenic. PAH diol epoxides react readily with mutational hot spots in DNA to form stable adducts both in vitro and in vivo (*8*−*11*), which may result in misreplication and mutagenesis (*12*, *13*). *anti*-BPDE has been shown to mutate the proto-oncogene c-H-*ras* via G to T transversions (*14*) and target hot spots in the *p53* tumor suppressor gene for mutation (*15*).

In the second pathway, PAH radical cations are formed at the most electrophilic carbon by CYP peroxidase and react with DNA to form unstable depurinating adducts (*16*). The formation of apurinic sites if unrepaired can lead to G to T transversions in target genes. Radical cations have also been shown to mutate the proto-oncogene c-H-*ras* via G to T transversions (*17*).

In the third pathway, aldo−keto reductases (AKRs) oxidize PAH *trans*-dihydrodiol proximate carcinogens to their corresponding reactive and redox-active *o*-quinones (*18*). The

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[1] Abbreviations: PAH, polycyclic aromatic hydrocarbon; DD, dihydrodiol dehydrogenase; *anti*-BPDE, (±)-*anti*-7,8-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; AhR, aryl hydrocarbon receptor; AKR, aldo−keto reductase; CYP, cytochrome P450; ROS, reactive oxygen species; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; PC, phosphatidylcholine; NP-1,2-diol, (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene; NP-1,2-dione, naphthalene-1,2-dione; BP-7,8-diol, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP-7,8-dione, benzo[*a*]pyrene-7,8-dione; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBA-3,4-diol, (±)-*trans*-3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene; DMBA-3,4-dione, 7,12-dimethylbenz[*a*]anthracene-3,4-dione.

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AKRs are monomeric cytosolic NADP(H)-dependent oxidoreductases (~34 kDa) which are broadly expressed. The *o*-quinones generated by AKRs can form stable and depurinating covalent DNA adducts (*19*, *20*). Autoxidation of the intermediate catechols and/or subsequent redox cycling of the *o*-quinones generates reactive oxygen species (ROS) (*21*, *22*) which can lead to mutation of *p53* (*23*). Only the AKR pathway can generate a prooxidant state. Prooxidant states have been linked to tumor promotion (*24*), and ROS can have both inhibitory and stimulatory effects on PKC domains.

Protein kinase C (PKC) is a family of at least 11 related serine−threonine kinases that play a critical role in the control of cell proliferation and is a key regulator of tumor promotion (*25*). PKC is the major cellular target for the phorbol ester tumor promoters and the second messenger diacylglycerol. PKC isozymes possess two functional domains: an N-terminal regulatory and a C-terminal catalytic domain. Motifs for activators such as phorbol 12-myristate 13-acetate (PMA), phosphatidylserine (PS), and Ca^{2+} reside in the regulatory domain. The regulatory domain and catalytic domain can be obtained by limited digestion of the holoenzyme with trypsin. The catalytic fragment is highly conserved among PKC isoforms and many other kinases and is active without any activators (*25*−*27*).

Since the initiation and promotion of mouse skin papillomas require an initiating dose of PAH followed by subsequent repetitive treatment with PMA (*28*), we sought to examine the effects of PAH *o*-quinones on PKC activity. Previous structure−activity relationships indicate that non-K region *trans*-dihydrodiols in which the bay region is methylated are among the most potent proximate carcinogens known (*3*). We selected three pairs of *trans*-dihydrodiols and their corresponding *o*-quinones as potential modulators of PKC. Each pair represents a substrate and product for AKRs. They are (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene (NP-1,2-diol) and naphthalene-1,2-dione (NP-1,2-dione), (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) and benzo[*a*]pyrene-7,8-dione (BP-7,8-dione), and (±)-*trans*-3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene (DMBA-3,4-diol) and 7,12-dimethylbenz[*a*]anthracene-3,4-dione (DMBA-3,4-dione) (Figure 1). These pairs of PAH metabolites also permit the influence of a bay region and a methylated bay region to be examined. For example, the naphthalene series lacks a bay region, the benzo[*a*]pyrene series contains a bay region, and the DMBA series contains a methylated bay region. We now report the irreversible inhibition of the catalytic fragment of PKC by all three quinones in vitro and in MCF-7 cell lysates and discuss this finding in the context of PKC signal transduction, cell growth, and tumor promotion.

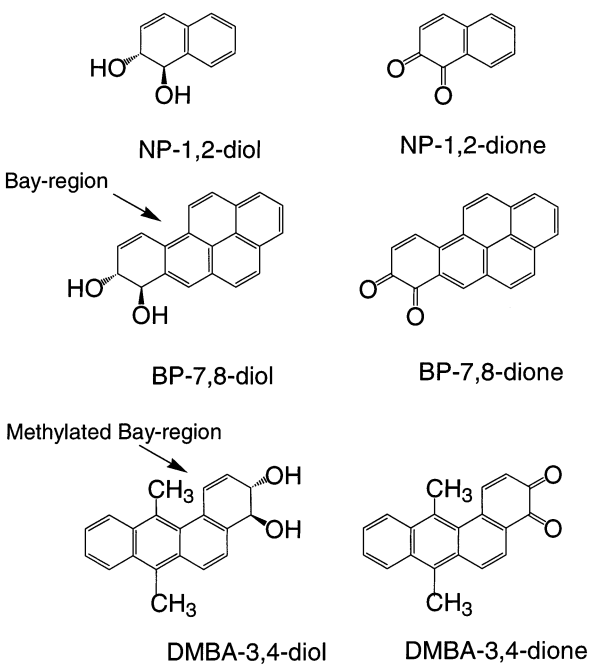

FIGURE 1: Structures of PAH metabolites: NP-1,2-diol, (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene; NP-1,2-dione, naphthalene-1,2-dione (no bay region); BP-7,8-diol, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP-7,8-dione, benzo[*a*]pyrene-7,8-dione (bay region); DMBA-3,4-diol, (±)-*trans*-3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene; DMBA-3,4-dione, 7,12-dimethylbenz[*a*]anthracene-3,4-dione (methylated bay region).

MATERIALS AND METHODS

Chemicals and Reagents. Adenosine 5′-triphosphate (ATP), PMA, PS, and phosphatidylcholine (PC) were purchased from Sigma (St. Louis, MO). The PKCα pseudosubstrate peptide (H_2N-Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val-CO_2H) and cell culture media were from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). Bis-(indolyl)maleimide I (inhibitor GF109203X) was obtained from Calbiochem (LaJolla, CA). [γ-^{32}P]ATP and enhanced chemiluminescence (ECL) Western blotting reagent were obtained from Amersham (Arlington Heights, IL). Recombinant PKCα and PKCδ were expressed in baculovirus-infected Sf9 cells according to Kazanietz et al. (*29*). PKCα had a final specific activity of 1.5 μmol of pseudosubstrate peptide phosphorylated min^{-1} mg^{-1} in the presence of 100 μg/mL PS and 1 μM PMA while PKCδ had a final specific activity of 0.75 μmol of pseudosubstrate peptide phosphorylated min^{-1} mg^{-1} in the presence of 100 μg/mL PS and 1 μM PMA. The catalytic fragment of PKC (55 kDa) was prepared from rat brain by trypsin digestion (Biomol, Plymouth Meeting, PA) as described (*30*, *31*) and was judged >95% pure by SDS−PAGE. The antibodies against PKC isoforms were purchased from Transduction Laboratories (Lexington, KY). The chromatography paper p81 was from Whatman International (Maidstone, England). NP-1,2-diol, NP-1,2-dione, BP-7,8-dione, DMBA-3,4-diol, and DMBA-3,4-dione were synthesized according to the published routes (*32*, *33*). BP-7,8-diol was obtained from the National Cancer Institute, Chemical Carcinogen Standard Reference Repository. Other reagents were of the highest grade available.

Caution: All PAHs are potentially hazardous and should be handled in accordance with NIH Guidelines for the Laboratory Use of Chemical Carcinogens.

Cell Culture. The human breast cancer cell line MCF-7 (ATCC no. HTB-22) was grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. For preparation of cell lysates, cells were harvested into lysis buffer containing 50 mM Tris-HCl, pH 7.4, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 4 μg/mL pepstatin A, and 5 μg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride and then lysed by sonication.

PKC Activity Assay. PKC activity was determined as described previously (*29*). Recombinant PKCα, recombinant PKCδ, the catalytic fragment of PKC from rat brain (1−10 ng), or cell lysates were incubated with PAH *o*-quinones or PAH *trans*-dihydrodiols at 30 °C for 5 min in the presence of 10 μM pseudosubstrate peptide, 0.25 mg/mL BSA, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 10 mM magnesium acetate. Unless otherwise indicated, the activators used were 1 μM PMA and/or 100 μg/mL phospholipids (20% phosphatidylserine plus 80% phosphatidylcholine) and/or 50 μM $CaCl_2$. PKCδ was assayed in an identical manner in the absence of calcium. The reaction was initiated by adding 25 μM ATP containing 0.4 μCi of [γ-^{32}P]ATP. The total reaction mixture was 50 μL. After 6 min incubation at 30 °C, 25 μL of the reaction mixture was applied to p81 cation chromatography paper. The paper was washed with 0.4% phosphoric acid three times. After being rinsed in acetone, the paper was air-dried and counted in a Tricarb scintillation counter. The counts per minute (cpm) was corrected for the $t_{1/2}$ life of ^{32}P. The PKC activity was expressed as micromoles of phosphate incorporated min^{-1} mg^{-1}.

Immunoblot Analysis. Lysates of 5×10^5 MCF-7 cells were subjected to SDS−PAGE. The proteins in the gels were then electrotransferred to PVDF membranes (Millipore, Bedford, MD) and probed with a panel of monoclonal antibodies against PKC isoforms. The bands were visualized with a chemiluminiscence detection kit (ECL) according to the manufacturers' instructions.

RESULTS

PAH o-Quinones Inhibit PKC Activity in the Holoenzyme. Recombinant PKCα was incubated with NP-1,2-dione (Figure 2, panel A), BP-7,8-dione (Figure 2, panel B), and DMBA-3,4-dione (Figure 2, panel C), and the activity was assayed in the absence of any activators or in the presence of either phospholipid or PMA plus phospholipid. All three quinones inhibited in a dose-dependent manner basal activity, the activity stimulated by 20% PS, and the activity stimulated by 1.0 μM PMA plus 20% PS. To determine whether this effect was PKC isoform-specific, parallel studies were performed on recombinant PKCδ, and identical results were observed (Figure 2, panels D−F).

Because PKC activators and cofactors (PMA, PS, and Ca^{2+}) have distinct motifs in the regulatory domain, we next determined whether inhibition was activator-specific. PKCα was stimulated with various concentrations of PMA (Figure 3, panel A), varied PS plus 50 μM Ca^{2+} (Figure 3, panel B), and varied Ca^{2+} plus 20% PS (Figure 3, panel C), and the inhibitory effect of BP-7,8-dione on kinase activity was determined. In each instance the kinase activity was inhibited by BP-7,8-dione in a dose-dependent fashion. Because recombinant PKC isoforms contain DTT in their storage buffer, there was concern that this thiol might obscure the real inhibitory potency of the reactive *o*-quinones due to the formation of thioether *o*-quinone conjugates. The final concentration of DTT in our enzyme assays was 30 μM. Experiments were replicated with PAH *o*-quinones following dialysis of recombinant PKCα, and identical IC_{50} values were observed (data not shown). Thus under the conditions of the PKC assay the potential scavenging of the *o*-quinone by micromolar concentrations of free thiol does not influence the inhibition profiles.

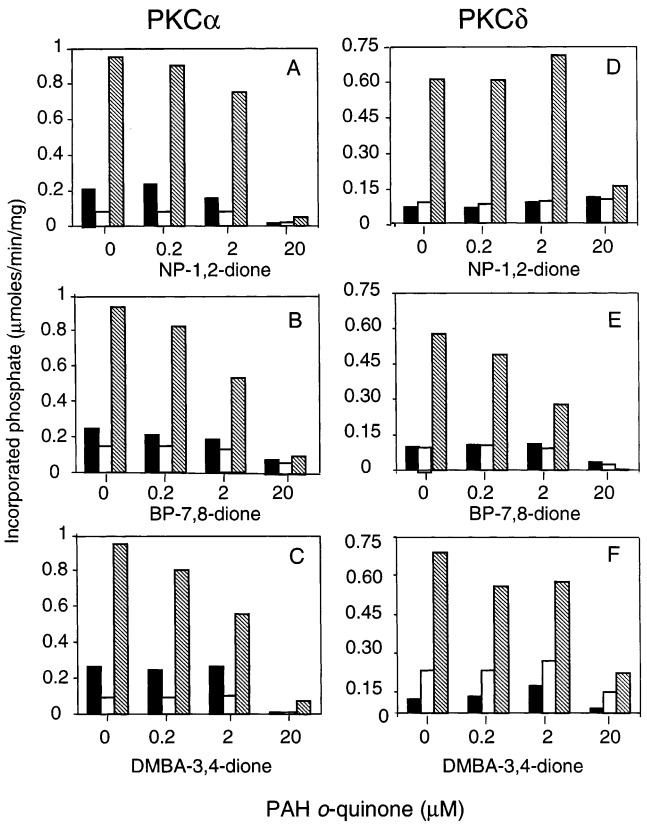

FIGURE 2: PAH *o*-quinones inhibit the activity of PKCα and PKCδ. Recombinant protein kinase Cα was incubated with 0, 0.2, 2, or 20 μM NP-1,2-dione (A), BP-7,8-dione (B), and DMBA-3,4-dione (C) at 30 °C for 5 min in the absence of cofactor/activator (filled bars), 20% PS (20 μg/mL PS plus 80 μg/mL PC, open bars), or 1 μM PMA + 20% PS (hatched bars). Recombinant protein kinase Cδ was incubated with 0, 0.2, 2, or 20 μM NP-1,2-dione (D), BP-7,8-dione (E), and DMBA-3,4-dione (F) at 30 °C for 5 min in the absence of cofactor/activator (filled bars), 20% PS (20 μg/mL PS plus 80 μg/mL PC, open bars), or 1 μM PMA + 20% PS (hatched bars). In each instance the reaction was started by adding ATP containing 0.4 μCi of [γ-^{32}P]ATP. After 6 min of incubation at 30 °C, aliquots of the mixture were removed and applied to p81 cation chromatography paper, washed, and counted. PKC activity was determined as described in Materials and Methods and expressed as μmol of phosphate incorporated min^{-1} mg^{-1}. All measurements were conducted in triplicate, and the experiments were repeated three times. Individual variation in the replicates was less than 10% of the SE within a given experiment.

Our data suggested that these *o*-quinones may interact with a common site in PKC, the candidate site being the catalytic domain. In contrast, the corresponding PAH *trans*-dihydrodiols (Figure 4) were unable to inhibit PKCα activity in the presence of PMA and PS, indicating that the inhibitory effect was *o*-quinone-specific.

PAH o-Quinones Irreversibly Inhibit the Catalytic Fragment of PKC. To determine whether the PAH *o*-quinones inhibit the catalytic fragment of PKC, these *o*-quinones were incubated with a proteolytic fragment of PKC purified from rat brain. NP-1,2-dione, BP-7,8-dione, and DMBA-3,4-dione all inhibited the catalytic fragment of PKC in a dose-dependent manner (Figure 5). In each case the PAH *o*-quinones gave identical IC_{50} values of 3−5 μM. No inhibition of the PKC catalytic fragment was observed with the corresponding *trans*-dihydrodiols, indicating that inhibition was again quinone-specific.

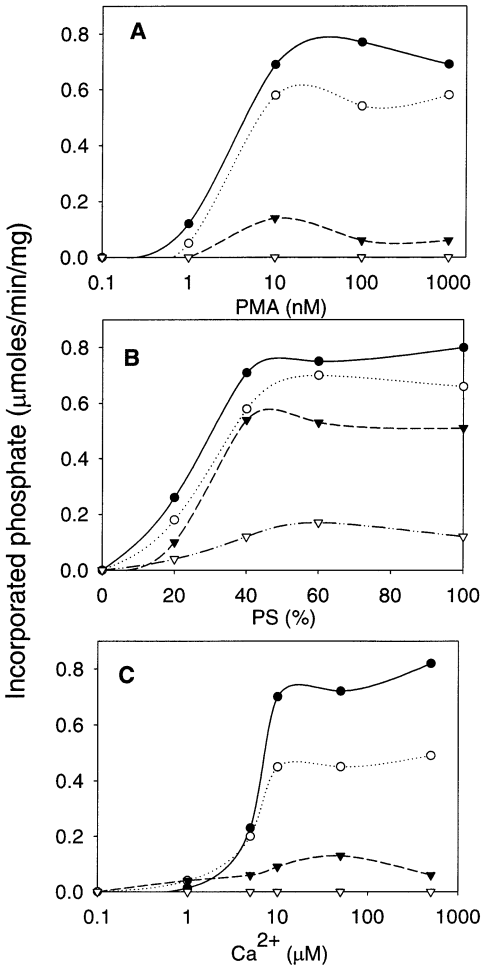

FIGURE 3: BP-7,8-dione inhibits PKCα activity stimulated with PMA, phosphatidylserine, or Ca^{2+}. Recombinant PKCα activity was determined as described in Figure 2 in the presence of 0 (●), 0.2 (○), 2 (▼), or 20 (▽) μM BP-7,8-dione using different activators: varied PMA plus 20% PS (A), varied PS plus 50 μM Ca^{2+} (B), and varied Ca^{2+} plus 20% PS (C). All measurements were conducted in triplicate, and the experiments were repeated three times. Individual variation in the replicates was less than 10% of the SE within a given experiment.

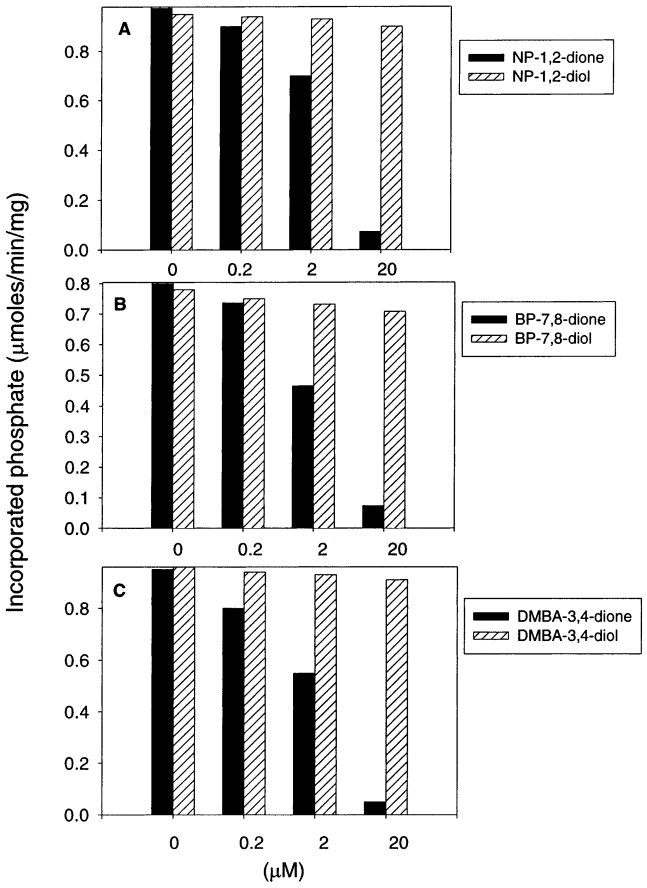

FIGURE 4: Inhibition of PKCα activity is PAH *o*-quinone-specific. Recombinant PKCα activity was determined as described in Figure 3 in the presence of 0, 0.2, 2, or 20 μM NP-1,2-dione or NP-1,2-diol (A), BP-7,8-dione or BP-7,8-diol (B), and DMBA-3,4-dione or DMBA-3,4-diol (C) using 1 μM PMA plus 20% PS as activators. All measurements were conducted in triplicate, and the experiments were repeated three times. Individual variation in the replicates was less than 10% of the SE within a given experiment.

We next examined whether the PAH *o*-quinones were reversible inhibitors of the catalytic fragment of PKC and noticed mixed-type inhibition (data not shown). The ability of PAH *o*-quinones to reduce V_{max} prompted us to examine whether they could act as irreversible enzyme inhibitors. PAH *o*-quinones are Michael acceptors and have the capability of forming covalent adducts with proteins, raising the possibility that they could arylate PKC. To address this issue, we conducted time-dependent inactivation studies but found that upon addition of the quinone the activity of the catalytic fragment was lost instantaneously. Next the catalytic fragment of PKC was preincubated with 20 μM NP-1,2-dione, BP-7,8-dione, or DMBA-3,4-dione (IC_{90}) for 5 min and then dialyzed extensively against 3 mM DTT to scavenge excess *o*-quinone (100 μL versus three changes of 200 mL of buffer over 2 h). The inclusion of high concentrations of DTT was an important aspect of the experiment since PAH *o*-quinones have high bimolecular rate constants for the addition of thiols, suggesting that any free quinone would be eliminated with an estimated $t_{1/2} = 3$ min (*34*). Aliquots of the dialyzed catalytic fragment were then assayed to determine the amount of kinase activity remaining. While the DMSO solvent control showed complete retention of enzyme activity, the kinase activity did not return to the *o*-quinone-treated catalytic fragments, showing that the inhibition was irreversible (Figure 6). As an additional control we preincubated the catalytic fragment with solvent, BP-7,8-dione, or bis-(indolyl)maleimide I (a competitive inhibitor for the ATP-binding site) and then conducted dialysis. While the inhibitory effect of bis(indolyl)maleimide I was reversed by dialysis, the inhibition by BP-7,8-dione was not reversed by this procedure (data not shown). These data imply that the catalytic fragment of PKC was covalently modified via arylation by the *o*-quinones.

PAH o-Quinones Inhibit PKC Activity in MCF-7 Cell Lysates. The data so far showed that PAH *o*-quinones are inhibitors of PKC in vitro using recombinant enzyme and that the effect was at the level of the catalytic fragment. To assess whether the effect is seen in cells, the ability of NP-1,2-dione and BP-7,8-dione to inhibit PKC activity in MCF-7 cell lysates was examined. IC_{50} values of 15−20 μM were observed (Figure 7A). Since MCF-7 cells are likely to express multiple PKC isoforms, we used immunoblot analysis to determine the PKC expression profile in these cells. MCF-7 cells were found to express PKCα, -β, -δ, -ϵ, -ι, and -λ isoforms (Figure 7B). The ability of PAH

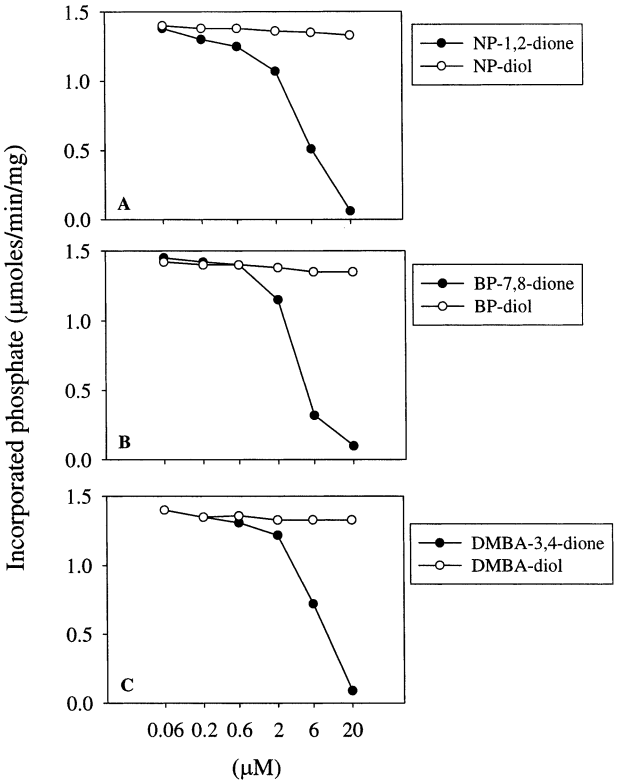

FIGURE 5: PAH *o*-quinones inhibit the activity of the catalytic fragment of PKC. The activity of the catalytic fragment of PKC was determined in the presence of 0.06, 0.2, 0.6, 2, 6, or 20 μM NP-1,2-dione or NP-1,2-diol (A), BP-7,8-dione or BP-7,8-diol (B), and DMBA-3,4-dione or DMBA-3,4-diol (C), as described in Materials and Methods. All measurements were conducted in triplicate, and the experiments were repeated three times. Individual variation in the replicates was less than 10% of the SE within a given experiment.

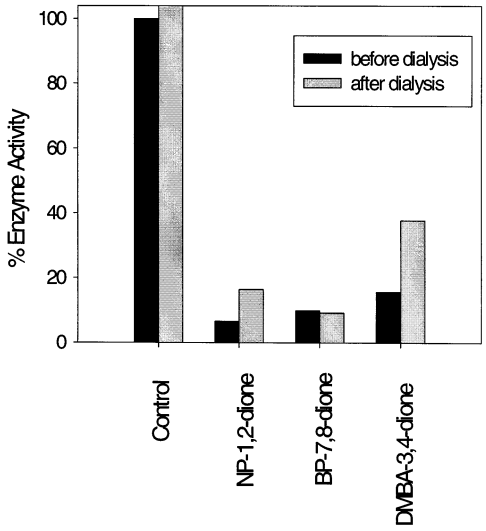

FIGURE 6: PAH *o*-quinones irreversibly inactivate the catalytic fragment (kinase domain) of PKC. The catalytic fragment of PKC (70 ng) was incubated with 8% DMSO (control), 20 μM NP-1,2-dione, BP-7,8-dione, or DMBA-3,4-dione in 20 mM Tris-HCl, pH 7.5 at 30 °C, for 5 min, and an aliquot (10 μL) was assayed for kinase activity (i.e., before dialysis). The remaining mixture (90 μL) was dialyzed against 200 mL of 20 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 3 mM DTT, and 10% glycerol for 2 h at 4 °C. After dialysis a second aliquot was assayed for kinase activity.

o-quinones to inhibit PKC activity in lysates containing multiple isoforms suggests that the inhibitory effect of the quinones may be seen across isozyme.

DISCUSSION

PAH *o*-quinones are unique among the activated metabolites of PAH since they are both reactive electrophiles and redox active. Their ability to enter into 1 and 2 e^- futile redox cycles provides a route to the amplification of ROS and generation of a prooxidant state. Prooxidant states have been linked to the activation of PKC and tumor promotion (*23*). As part of our ongoing studies to determine whether PAH *o*-quinones and the ROS they generate activate or inhibit PKC, we now describe *o*-quinone-specific inhibition of PKC isoforms via irreversible inhibition of the catalytic fragment. Moreover, the effect is seen in cell lysates which express multiple PKC isoforms, suggesting that the phenomenon may occur across PKC isoforms.

This is the third report that PAH *o*-quinones generated by AKRs affect receptors and therefore have epigenetic effects. Low micromolar concentrations of PAH *o*-quinones cause the translocation and *trans*-activation of the aryl hydrocarbon (AhR) receptor, suggesting that they are ligands for this transcription factor (*35*). A consequence of the nuclear translocation of the AhR is that this may provide a route by which the genotoxic PAH *o*-quinones can be targeted to the nucleus with specificity. Consequences of AhR *trans*-activation include increased induction of CYP1A1. This will enhance the amount of PAH *trans*-dihydrodiol substrate that is available for either the CYP1A1-catalyzed formation of diol epoxides or the AKR-catalyzed formation of *o*-quinones.

Subsequently, it has been shown that low micromolar concentrations of BP-7,8-dione activated the SERCA-1 transporter (the ryanodine receptor), while other known metabolites of benzo[*a*]pyrene, including BP-7,8-diol, *anti*-BPDE, BP-1,6-dione, BP-3,6-dione, BP-6,12-dione, and BP-7,8-dione, were without effect (*35*). The activation of the ryanodine receptor was biphasic, leading to a rapid increase in the frequency of channel opening and the length of channel opening, resulting in a Ca^{2+} efflux, while prolonged exposure to BP-7,8-dione led to channel inactivation. The ryanodine receptor is known to contain redox-sensitive cysteines, and channel activation may result from the redox cycling of BP-7,8-dione and the production of ROS, while long-term inactivation of the receptor may be due to arylation by Michael addition of the quinone. The ryanodine receptor is known to be broadly expressed (*35*), providing a mechanism by which PAH *o*-quinones could produce a Ca^{2+} transient to activate classical PKC isozymes (α, βI, βII, and γ). It thus becomes apparent that PAH *o*-quinones may not only have direct effects on PKC isozymes but also produce two intracellular signals that could effect PKC activity, e.g., ROS and intracellular Ca^{2+}.

PKC isozymes have two domains, the catalytic domain and the regulatory domain. We show that PAH *o*-quinones potently and irreversibly inhibit the catalytic domain of PKC. The rapid inactivation observed allows us to speculate that PAH *o*-quinones arylate the reactive and redox-sensitive cysteines in the catalytic domain. PAH *o*-quinones generated in situ can also enter into redox cycles and generate ROS. The ROS formed could also target redox-sensitive cysteines in the catalytic domain leading to PKC inhibition (*36*, *37*).

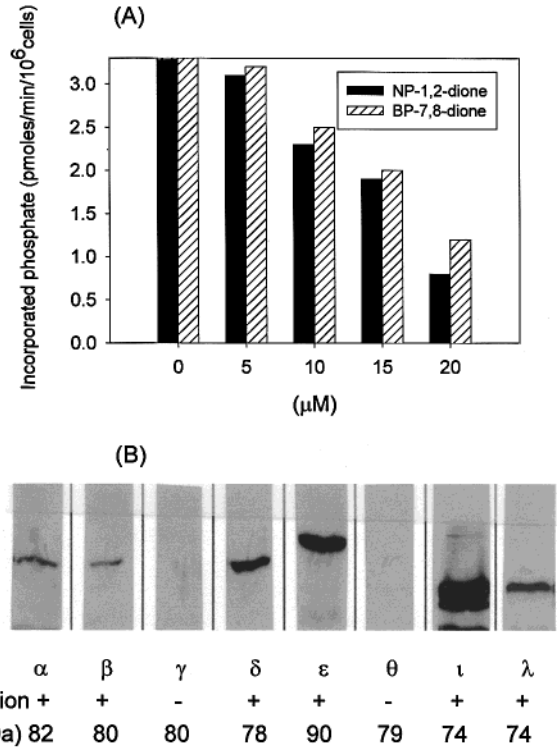

FIGURE 7: PAH *o*-quinones inhibit PKC activity in MCF-7 cell lysates. (A) Lysates of human breast cancer MCF-7 cells were incubated with 0, 5, 10, 15, or 20 μM NP-1,2-dione or BP-7,8-dione in the presence of 1 μM PMA, 10 μM substrate peptide, and 100 μg/mL PS at 30 °C for 20 min. The reaction was started by adding ATP containing 0.4 μCi of [γ-^{32}P]ATP. The result was expressed as μmol of phosphate incorporated min^{-1} (10^6 cells)$^{-1}$. (B) Immunoblot analysis of PKC isoforms in MCF-7 cells. MCF-7 cell lysates (50 μg of protein from 5×10^5 cells) were subjected to SDS−PAGE and blotted with specific antibodies for the PKC isozymes indicated. Antibodies were obtained from Transduction Laboratories.

Cellular consequences of PAH *o*-quinone formation may be further impacted by the finding that PKC isozymes also contain redox-sensitive cysteines in the regulatory domain (C1). When these cysteines are modified, a conformational change results in the activation of the kinase. In the experiments described PAH *o*-quinones were used under non-redox cycling conditions, but, clearly, the production of ROS by PAH *o*-quinones generated in a cellular context and their subsequent impact on PKC needs to be examined.

Because there are 11 PKC isozymes that can be differentially expressed with discrete roles in each cell type, the consequences of PKC activation or inhibition on cell growth may be difficult to predict. Work in diverse cell types has demonstrated that PKC can participate in either positive or negative regulation of cell proliferation and apoptosis (*38*). Emerging evidence increasingly points to roles for PKCα and PKCδ in negative growth regulation. PKCδ not only reduces cell growth but also promotes apoptosis in many cell types (*39*). We show that PAH *o*-quinones potently inhibit both PKCα and PKCδ. In such systems PKC inhibition by PAH *o*-quinones is anticipated to either reduce the inhibition of cell growth or reduce apoptosis, leading to growth stimulation. Paradigms involving ectopic expression of discrete PKC isoforms will have to be used to dissect the role of PAH *o*-quinone formation on individual PKC activity within a cellular context.

MCF-7 cells express multiple PKC isoforms as demonstrated by immunoblot analysis. When MCF-7 cell lysates were incubated with PAH *o*-quinones, the total cellular PKC activity was suppressed, and at concentrations of 20 μM quinone almost all activity was lost. This suggests that quinones have the ability to inhibit multiple PKC isoforms in vitro. Whether each PKC isozyme possesses different sensitivity to PAH *o*-quinones is a subject of current investigation. This raises the issue of what the effect of PAH *o*-quinone inhibition on PKC isoforms might be in vivo. Experimentally, administration of PMA following a tumor-initiating dose of PAH has been linked to a tumor promoting effect. However, it is less clear whether this effect is due to activation of PKC or its downregulation. Evidence exists that the tumor promoting effects of PMA occur due to a depletion of growth inhibitory isozymes, such as PKCδ. First, the tumor promoting effects of PMA in rat fibroblasts expressing the c-Src proto-oncogene correlate with a depletion of expressed PKC isoforms. Second, bryostatin 1 which inhibits PMA-induced downregulation of PKCδ, blocks the tumor promoting effect of PMA. Third, expression of a dominant-negative form of PKCδ in cells expressing the Src proto-oncogene leads to cell transformation (*40*). These observations suggest that a potential consequence of PAH *o*-quinone-mediated PKC inhibition is an increase in tumor promotion.

An unresolved question in PAH carcinogenesis is how PAHs can act as complete carcinogens (initiators and promoters). While much is known about the activation of PAH to electrophiles that form DNA lesions, less is known about their tumor promoting properties. High dose or repetitive PAH dosing will cause tumor formation in animal models. Our data suggest that either PAH *o*-quinones or quinones in general may have tumor promoting properties. Few studies have been performed in animals to test this hypothesis. Single topical application of 1,4-naphthoquinone and its 5-hydroxy analogue (juglone) was found to produce a dose-dependent increase in ornithine decarboxylase activity and promote papilloma formation in SENCAR female mice following an initiating dose of 7,12-DMBA (*42*).

In our study non-bay region quinones (naphthalene-1,2-dione), bay region quinones (benzo[*a*]pyrene-7,8-dione), and methylated bay region quinones (DMBA-3,4-dione) were equally potent as irreversible inhibitors of the catalytic fragment of PKC, suggesting that all three quinones may act equally well as tumor promoters. However, these *o*-quinones also vary dramatically in their ability to redox cycle (*43*), suggesting that cellular and animal models need to be used to test the tumor promoting properties of these compounds further. Elucidation of the molecular effects of PAH metabolites on signal transduction pathways will greatly contribute to our understanding of tumor promoter mechanisms.

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