Mediated cytoprotection is observed in cultured cells, evidence for a pharmacologically distinct receptor. PGE2, DDM-PGE2, and TPA all induce cytoprotection, supporting a role for this pathway in the cytoprotection, activation of protein kinase C, also induces cytoprotection. These data suggest that cytoprotection by PGE2-mediated cytoprotection in renal epithelial cells: evidence for a pharmacologically distinct receptor (12). Prostaglandin E2 (PGE2) is a major metabolite of arachidonic acid in mammalian kidney (34), where it modulates renal hemodynamics, metabolism, water and ion transport, and sympathetic nerve activity (18, 27). PGs also protect a variety of tissues, including the kidney (30), from a diverse array of toxicants, and this property has been termed “cytoprotection” (16, 32). Because PGE2-mediated cytoprotection is observed in cultured cells, a cellular mechanism of action has been proposed (30, 33), although the cellular and molecular mechanisms associated with this response remain unknown.

The pharmacological effects of PGE2 are primarily receptor mediated, and currently four PGE2 receptor [E-prostanoid (EP)] subtypes have been cloned, namely the EP1, EP2, EP3, and EP4 receptors (for a review, see Refs. 11, 12, 17, 31 and references therein). EP1 receptors are coupled to inositol phospholipid (IP)-related signal transduction, whereas cyclic nucleotide metabolism is regulated positively by EP1, EP2 receptors and negatively by EP3 receptors (31). EP3 alternative splice variants have been identified that differentially couple to cyclic nucleotide- or IP-related signal transduction (17). Modulation of adenosine 3',5'-cyclic monophosphate (cAMP) metabolism and IP turnover, in turn, regulates the activity of a cAMP-dependent protein kinase A (PKA) and PKC, respectively (22).

PKC represents a multigene family of protein kinases similar in size, structure, and function that transduce signals from a wide variety of stimuli, including growth factors, hormones, and neurotransmitters (for a review, see Ref. 28). The role of PKC in IP-related signaling has been firmly established. In this pathway, IPs are hydrolyzed by phospholipase C to the ultimate second messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (28). IP3 releases calcium from intracellular stores, whereas DAG is the endogenous activator of PKC (28). Endogenous DAG and exogenously added cell-permeant DAG analogs are rapidly metabolized, limiting their application in studying PKC-related signaling (28). For this reason, phorbol esters, metabolically stable tumor promoters that are potent activators of PKC, have been used as surrogates of DAG (4).

PKC plays an important role in the transduction of multiple signals into the nucleus (29), including activation of the activator protein-1 (AP-1) transcriptional complex, which is considered a “nuclear third messenger” in this pathway (23). The AP-1 transcription factor is a heterodimeric complex composed of c-fos (c-fos, c-jun, j un B, j un D) and c-fos (c-fos, F os B, Fra-1) protooncogene family members, as either a J un/j un homodimer or J un/F os heterodimer (23, 29). On formation, this complex specifically binds to a target DNA sequence (TGAC/GTCA) referred to as the 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) (23). TPA-induced binding of AP-1 to the TRE is independent of protein synthesis, suggesting this response is regulated by posttranslational modification of existing proteins (2, 3).

Conjugation of hydroquinone with glutathione (GSH) in the liver results in the formation of several isomeric...
nephrotoxic GSH conjugates (20). Histochemical analysis of kidney sections following administration of the most potent metabolite, 2,3,5-(trisglutathionyl-S-yl)-hydroquinone [2,3,5-(trisglutathionyl-S-yl)-HQ], shows cytotoxicity initially localized to proximal tubule epithelial cells in the S3 segment (24). The present studies were conducted to determine whether PGE2 affords cytoprotection against 2,3,5-(trisglutathionyl-S-yl)-HQ-mediated cytotoxicity in a renal proximal tubule epithelial cell line (LLC-PK1) and to determine the cellular and molecular nature of the cytoprotective response. We report that PGE2 offers cytoprotection against chemical-induced cytotoxicity in LLC-PK1 cells and that this event is mediated by a PKC-coupled receptor, which is pharmacologically distinct from currently classified EP receptor subtypes.

**MATERIALS AND METHODS**

Chemicals. 2,3,5-(Trisglutathionyl-S-yl)-HQ was synthesized as previously described (24) and was >99% pure as determined by high-performance liquid chromatography. PGE2, PGE2 methyl ester, 17-phenyltrinor PGE2 (PT-PGE2), 11-deoxy-16,16-dimethyl PGE2 (DDM-PGE2), sulprostone, PGE1, 11-deoxy PGE1, and PGA2 were obtained from Cayman Chemical (Ann Arbor, MI). N-[2-[3-(4-bromophenyl)-2-propanyl]-amino-ethyl]-5-isouquinolinesulfonamide (H-89) and calphostin C were purchased from Calbiochem (La Jolla, CA). Formaldehyde, glyacial acid, glycerol, and ethanol were purchased from Fisher Scientific (Houston, TX). TRE and AP2 consensus sequences were purchased from Promega (Madison, WI). [γ-32P]ATP (3,000 Ci/mmol) was obtained from NEN (Beverly, MA). Poly[dI-dC] was purchased from Boehringer-Mannheim (Indianapolis, IN). 4-bromo-A-23187 (4-Br-A-23187) was a product of Molecular Probes (Eugene, OR). All other chemicals were from Sigma Chemical (St. Louis, MO).

Cell culture. LLC-PK1 cells were obtained from the American Type Culture Collection (CL101) at passage 181. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (JRH Biosciences, Lenexa, KS) supplemented with 4 g/l d-glucose (Sigma) and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) in 5% CO2 (95% air) at 37°C. Cells were subcultured by trypsinization, and all experiments were conducted with 5-day postconfluent cultures at passage levels 187–200.

Pretreatment of LLC-PK1 cells with PGs. LLC-PK1 cells were seeded in 24-well plates and maintained in 10% FBS-DMEM until 5 days postconfluency, and media were replaced every 2 days. Postconfluent cultures were rinsed with phosphate-buffered saline (PBS) and exposed to PGs in 10% FBS-DMEM for specified periods of time. Prior to chemical challenge, media were aspirated, and cell monolayers were rinsed three times with PBS to remove residual PGs.

Neutral red assay. The neutral red assay employs lysosomal membrane integrity as an index of cell viability. This assay affords the most sensitive detection of chemical challenge in LLC-PK1 cells compared with other cytotoxicity assays, such as those that measure plasma membrane integrity (lactate dehydrogenase leakage) or mitochondrial function [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay] (26). Vehicle or PG-pretreated cells were rinsed three times with PBS and exposed to 2,3,5-(trisglutathionyl-S-yl)HQ (300 µM) in 0.1% FBS-DMEM supplemented with 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.4) for 2 h in a final volume of 0.5 ml. HEPES was required to maintain physiological pH following addition of 2,3,5-(trisglutathionyl-S-yl)-HQ due to the presence of small amounts of acetic acid in the purified product. After chemical challenge, cells were washed three times with PBS and exposed to 50 µg/ml neutral red in 0.1% FBS-DMEM supplemented with 25 mM HEPES (pH 7.4) for 1 h. To quantify neutral red uptake, monolayers were washed once with 1 ml of a 1% formaldehyde-1% calcium chloride solution followed by aspiration. Neutral red was extracted from the cells with 1 ml of a 1% glacial acetic acid-50% ethanol solution for 15 min at room temperature while protected from light. The extracted dye was quantified at 540 nm using a Shimadzu UV-160 spectrophotometer, and results are expressed as percent of control. For time-course studies, values are expressed as percent of protection rather than percent of control, using the following equation to calculate the value: [neutral red absorbanceC] – [neutral red absorbanceB] × 100/[neutral red absorbanceC]. Neutral red absorbanceC is treated with 2,3,5-(trisglutathionyl-S-yl)-HQ alone; and neutral red absorbanceB is absorbance for control groups.

Electrophoretic mobility shift assay (EMSA). EMSAs were carried out as described previously (6). LLC-PK1 cells were collected and lysed in buffer A [25 mM HEPES, pH 7.6, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.1 mg/ml phenylmethylsulfonyl fluoride] using 20 strokes with a Dounce homogenizer. The homogenate was centrifuged at 12,000 revolutions/min (rpm) in an Eppendorf microcentrifuge at 4°C for 5 min, and the supernatant was discarded. The remaining pellet was centrifuged for 10 s, and the residual supernatant was aspirated. The pellet was extracted with 40 µl of buffer A supplemented with 0.5 M KCl for 1 h on ice. Extracted pellets were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was designated the nuclear extract. Protein concentrations were determined by the method of Bradford (7) using bovine serum albumin as
METHODS. Values represent means ± SE (n = 3). *Significantly different from respective control, †significantly different from respective 2,3,5-(trisglutathion-S-yl)-HQ-treated group, P < 0.05. Similar results were observed in 2 separate experiments.

Fig. 2. Effect of pH on PGE2-induced cytoprotection. LLC-PK1 cells were pretreated with 10 µM PGE2 in 10% FBS-DMEM + HEPES buffered to pH 7.4 (open bars) or pH 7.8 (solid bars) for 24 h and subsequently challenged with 300 µM 2,3,5-(trisglutathion-S-yl)-HQ in 0.1% FBS-DMEM for 2 h. Cell viability measurements were obtained using a neutral red assay as described in MATERIALS AND METHODS. Values represent means ± SE (n = 3). *Significantly different from respective control, †significantly different from respective 2,3,5-(trisglutathion-S-yl)-HQ-treated group, P < 0.05. Similar results were observed in 2 separate experiments.

standard. For EMSAs, 10 µg of nuclear extract were incubated in a reaction mixture consisting of 18.8 mM HEPES, 40 mM KCl, 1.1 mM EDTA, 7.5% glycerol, 0.75 mM DTT, and 62.5 ng/µl poly[d(I-C)] for 15 min at 20°C to reduce interference by nonspecific DNA binding proteins. Addition of 3.5 nM TRE probe labeled with [γ-32P]ATP was added for 15 min to determine AP-1 binding activity. Bound TRE was separated on a 5% polyacrylamide nondenaturing gel for 2 h at 120 V, dried, and, exposed to Hyperfilm-MP (Amersham) for autoradiography.

PKC assay kit. Measurements of PKC activity were obtained using a PKC assay kit (GIBCO-BRL, Gaithersburg, MD), as described previously (37).

Statistics. Individual comparisons were made using the Student’s t-test or analysis of variance with a post hoc Student-Newman-Keuls test, as appropriate. P < 0.05 was accepted as significant.

RESULTS

The neutral red assay affords the most sensitive detection of quinone-thioether-mediated cytotoxicity in LLC-PK1 cells (26) and was therefore used in the present studies. Pretreatment of LLC-PK1 cells with 0.01–40 µM PGE2 for 24 h protected against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity in a concentration-dependent manner (Fig. 1, control values are 100%). In contrast, cotreatment of LLC-PK1 cells with PGE2 (0.1–40 µM) did not protect against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity (data not shown). PG-treated groups alone were not different from respective controls (data not shown).

In our initial studies with PGE2, the cytoprotective dose-response curve was variable, and we suspected this trend was secondary to chemical stability. It is well known that PGE2 undergoes β-elimination in basic solutions (pH > 7.4) to form the corresponding A- and B-series PGs (15, 19), and minor changes toward alkaline pH occur during routine handling of cells in culture. Thus LLC-PK1 cells were treated with PGE2 in media buffered to pH 7.4 or 7.8 to assess the pH and structural dependence of the cytoprotective response. Pretreatment of cells with 10 µM PGE2 for 24 h at pH 7.8 eliminates its cytoprotective properties, whereas pretreatment at pH 7.4 retains the cytoprotective properties (Fig. 2). Increasing the pH from 7.4 to 7.8 did not affect cell viability or 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity. These data suggest a structural requirement for cytoprotection and demonstrate that minor changes in pH can dramatically effect the cytoprotective response to PGE2.

In subsequent studies, cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity was evaluated in cells pretreated with EP receptor agonists (PT-PGE2, 11-deoxy PGE1, sulprostone), a synthetic stable PGE2 analog (DDM-PGE2), PGE1, or PGA2. Because synthetic analogs exhibit EP receptor subtype specificity and/or enhanced stability in basic solutions, they offer advantages over PGE2 for evaluating biological activity in vitro. PG pretreatment alone did not affect cell viability, relative to controls (DDM-PGE2, 109 ± 1.7%; PT-PGE2, 102 ± 0.6%; 11-deoxy PGE1, 100 ± 2.8%; sulprostone, 104 ± 1.7%; PGE1, 100 ± 3.1%; PGA2, 91 ± 5.2%). Cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ (300 µM; 2 h)-mediated cytotoxicity was only observed in cells pretreated with

Fig. 3. Prostaglandin-mediated cytoprotection is structurally limited. LLC-PK1 cells were pretreated with 17-phenyltrinor PGE2 (PT-PGE2, open bar), 11-deoxy PGE1 (solid bar), sulprostone (shaded bar), 11-deoxy-16,16-dimethyl PGE2 (DDM-PGE2, latticed bar), PGE1 (left hatched bar), or PGA2 (right hatched bar) in 10% FBS-DMEM for 24 h (all concentrations were 20 µM, except PGE1, which was 40 µM) and subsequently challenged with 300 µM 2,3,5-(trisglutathion-S-yl)-HQ in 0.1% FBS-DMEM for 2 h. Cell viability measurements were obtained using a neutral red assay as described in MATERIALS AND METHODS. Values represent means ± SE (n = 3). †Significantly different from the 2,3,5-(trisglutathion-S-yl)-HQ-treated group, P < 0.05. Similar results were observed in at least 2 separate experiments.
DDM-PGE₂ but not in cells pretreated with agonists for the EP₁, EP₄ receptor subtypes (PT-PGE₂, 11-deoxy PGE₁, sulprostone; all concentrations were 20 µM). In addition, neither 20 µM PGA₂ nor 40 µM PGE₁ induced cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity (Fig. 3).

Thus only DDM-PGE₂ reproduced PGE₂-mediated cytoprotection in LLC-PK₁ cells. This stable analog was therefore used to further investigate the mechanism of PGE₂-mediated cytoprotection. The induction of cytoprotection by DDM-PGE₂ exhibited time dependence (Fig. 4).

Cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity was first detected after an 8 h exposure to 1 µM DDM-PGE₂, with the maximal cytoprotective response occurring between 20 and 24 h.

Pretreatment of LLC-PK₁ cells with 0.001-40 µM DDM-PGE₂ for 24 h resulted in a concentration-related induction of cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity (Fig. 5). Cytoprotection was maximal between 0.1-1.0 µM DDM-PGE₂ in LLC-PK₁ cells and, therefore, all subsequent pretreatment protocols employed 1 µM DDM-PGE₂ for 24 h.

PGE₂-mediated responses are associated with cyclic nucleotide- or IP₃-related signal-transduction pathways in mammalian cells (17). To determine whether these pathways are associated with the cytoprotection by DDM-PGE₂ exhibited time dependence (Fig. 4). Cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity was first detected after an 8 h exposure to 1 µM DDM-PGE₂, with the maximal cytoprotective response occurring between 20 and 24 h.

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tive response, LLC-PK1 cells were pretreated with agents modulating cAMP- [forskolin, dibutyryl cAMP (DBcAMP)], calcium (4-Br-A-23187)-, or PKC (TPA)-related signaling. Pretreatment of cells with forskolin (0.1–30 µM), DBcAMP (0.0001–1.0 mM), or 4-Br-A-23187 (0.001–1.0 µM) for 24 h did not induce cytoprotection against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity (data not shown), consistent with the negative effects of the EP receptor agonists (see Fig. 3). The ability of forskolin to increase cAMP levels in LLC-PK1 cells was verified using a cAMP radioimmunoassay. Calcium-related signaling induced by 4-Br-A-23187 was verified by measuring the induction of calcium-responsive genes, including gadd 153 (data not shown). These data indicate that neither cAMP- or calcium-related signal-transduction pathways are involved in the cytoprotective response of LLC-PK1 cells to PGE2.

In contrast, pretreatment of cells with TPA (10 ng/ml, 24 h), a potent PKC activator, protected against 2,3,5-(trisglutathion-S-yl)-HQ-mediated cytotoxicity (data not shown), consistent with the negative effects of the EP receptor agonists (see Fig. 3). The ability of forskolin to increase cAMP levels in LLC-PK1 cells was verified using a cAMP radioimmunoassay. Calcium-related signaling induced by 4-Br-A-23187 was verified by measuring the induction of calcium-responsive genes, including gadd 153 (data not shown). These data indicate that neither cAMP- or calcium-related signal-transduction pathways are involved in the cytoprotective response of LLC-PK1 cells to PGE2.

To further differentiate between PKC and PKA signaling in the cytoprotective response to DDM-PGE2, LLC-PK1 cells were pretreated with the protein kinase inhibitor H-89, which inhibits PKA in the nanomolar range and PKC in the micromolar range (8). Inhibition of PKA by H-89 was observed in the concentration range of 0.05–10 µM DDM-PGE2 or TPA. Nuclear extracts from LLC-PK1 cells treated with 0.05–10 µM DDM-PGE2 or 0.1–100 ng/ml TPA were incubated with a 32P-labeled TRE in an EMSA as described in MATERIALS AND METHODS. Protein-DNA complexes were separated on a 5% native polyacrylamide gel and visualized by autoradiography. Similar results were observed in 2 separate experiments. AP-1, activator protein-1.
DDM-PGE<sub>2</sub> mediated cytoprotection exhibited a latency suggestive of the involvement of transcriptional activity or activities (Fig. 4). PKC regulates the activity of the AP-1 transcriptional complex (23), and, therefore, we examined the induction of TRE binding activity as preliminary evidence for a transcriptional component in the cytoprotective response and as a molecular marker of PKC activation (29). Peak TRE binding activity occurred 2 h after treatment of LLC-PK<sub>1</sub> cells with 1 µM DDM-PGE<sub>2</sub> (Fig. 7A) or 1 h after treatment with 10 ng/ml TPA (Fig. 7B). The induction of TRE binding activity in nuclear extracts from LLC-PK<sub>1</sub> cells treated with TPA. Protein-DNA complexes were separated on a 5% native polyacrylamide gel and visualized by autoradiography. Similar results were observed in 2 separate experiments. Top arrowhead, AP-1:TRE; bottom arrowhead, free TRE.

LLC-PK<sub>1</sub> cells, causing cell lysis within the first 4 h of treatment (data not shown).

DMN-PGE<sub>2</sub>-mediated cytoprotection exhibited a latency suggestive of the involvement of transcriptional activity or activities (Fig. 4). PKC regulates the activity of the AP-1 transcriptional complex (23), and, therefore, we examined the induction of TRE binding activity as preliminary evidence for a transcriptional component in the cytoprotective response and as a molecular marker of PKC activation (29). Peak TRE binding activity occurred 2 h after treatment of LLC-PK<sub>1</sub> cells with 1 µM DDM-PGE<sub>2</sub> (Fig. 7A) or 1 h after treatment with 10 ng/ml TPA (Fig. 7B). The induction of TRE binding activity in nuclear extracts from LLC-PK<sub>1</sub> cells treated with TPA. Protein-DNA complexes were separated on a 5% native polyacrylamide gel and visualized by autoradiography. Similar results were observed in 2 separate experiments. Top arrowhead, AP-1:TRE; bottom arrowhead, free TRE.

**Fig. 9.** Inhibition of DDM-PGE<sub>2</sub> (A)- or TPA (B)-inducible TRE binding activity by H-89. LLC-PK<sub>1</sub> cells were pretreated with H-89 prior to challenge with DDM-PGE<sub>2</sub> or TPA, and nuclear extracts were prepared and incubated with a <sup>32</sup>P-labeled TRE in an EMSA as described in MATERIALS AND METHODS. A: effects of 0.2–60 µM H-89 on inducible TRE binding activity in nuclear extracts from LLC-PK<sub>1</sub> cells treated with 1 µM DDM-PGE<sub>2</sub>. B: effect of 20 µM H-89 on inducible TRE binding activity in nuclear extracts from LLC-PK<sub>1</sub> cells treated with TPA. Protein-DNA complexes were separated on a 5% native polyacrylamide gel and visualized by autoradiography. Similar results were observed in 2 separate experiments. Top arrowhead, AP-1:TRE; bottom arrowhead, free TRE.

**Fig. 10.** TRE binding activity in nuclear extracts from LLC-PK<sub>1</sub> cells treated with PT-PGE<sub>2</sub>, DDM-PGE<sub>2</sub>, sulprostone, PGE<sub>1</sub>, or PGE<sub>2</sub>. Nuclear extracts from prostaglandin (20 µM)-treated LLC-PK<sub>1</sub> cells were incubated with a <sup>32</sup>P-labeled TRE in an EMSA as described in MATERIALS AND METHODS. Protein-DNA complexes were separated on a 5% continuous polyacrylamide gel and visualized by autoradiography. Specificity for the binding reaction was confirmed by addition of excess unlabeled TRE or nontarget DNA (AP2 consensus sequence). Similar results were observed in 2 separate experiments. DMSO, dimethyl sulfoxide. Top arrowhead, AP-1:TRE; bottom arrowhead, free TRE.
tion of nuclear extracts with unlabeled TRE or nontarget DNA (AP2 responsive element).

To rule out the possibility that DDM-PGE2-mediated TRE binding activity occurs via the direct activation of PKC by this analog, in a manner similar to phorbol ester, PKC activity was measured in isolated cell homogenates treated with TPA or DDM-PGE2. In contrast to TPA, DDM-PGE2 did not increase PKC activity in isolated LLC-PK1 cell homogenates (data not shown).

**DISCUSSION**

We have shown that PGE2 induces cytoprotection against quinone-thioether-mediated cytotoxicity in LLC-PK1, cells (Fig. 1). Moreover, structure-activity relationships do not support a role for any of the presently classified EP receptor subtypes in the cytoprotective response. In contrast, DDM-PGE2, a stable analog of PGE2, acts as a potent agonist for the putative receptor-mediated effects of PGE2 (Fig. 3). Evidence supporting the presence of a DDM-PGE2 receptor include 1) the induction of cytoprotection by PGE2 is not observed under pH-restrictive conditions (pH 7.8), suggesting a structural requirement for cytoprotection, thus implicating a receptor; 2) PGE2 and DDM-PGE2 induce the binding of nuclear proteins to a γ-32P-labeled TRE, suggesting this response is mediated by a common receptor; 3) DDM-PGE2 does not directly activate PKC in isolated LLC-PK1 cell homogenates (data not shown), in accordance with a mechanism of receptor-mediated TRE binding activity; and 4) cytoprotection by DDM-PGE2 occurs at concentrations comparable to those eliciting functional EP receptor-mediated responses, including cytoprotection in cortical neurons (1), sodium and water transport in renal cortical collecting ducts (18), and muscle relaxation in piglet saphenous veins (10).

PGE2 undergoes β-elimination to form A and B series prostaglandins at pH > 7.4 (15, 19). PGA2, but not PGB2, PGD2, PGE2, or PGF2α, increases the biosynthesis of γ-glutamylcysteine synthetase, which subsequently results in increased glutathione levels in L1210 and NIH/3T3 cells (21). Upregulation of antioxidant defense mechanisms would significantly impact quinone-thioether-mediated cytotoxicity, which exhibits a prominent oxidative stress-related component (26, 31). However, under conditions favoring PGE2 degradation (pH 7.8), cytoprotection is not observed (Fig. 2), consistent with the observation that exogenous PGA2 does not induce cytoprotection (Fig. 3). Although B-series prostaglandins were not tested in these studies, the fact that PGE2 degradation is associated with B-series formation, but not cytoprotection, argues against a role for B-series PGs in the cytoprotective response. In addition, the induction of cytoprotection by DDM-PGE2, an analog which is stable at pH ≤ 9.0, is pH insensitive (data not shown), suggesting that changes in pH per se cannot account for the loss of cytoprotection in PGE2-pretreated cells under pH-restrictive (pH 7.8) conditions. Collectively, these data implicate PGE2 in the cytoprotective response, and this prostanoid is known to elicit its effects through its interactions with EP receptors (12).

In contrast to PGE2, EP receptor subtype-specific agonists failed to induce cytoprotection against 2,3,5-(trisglutathion-S-y1)-HQ-mediated cytotoxicity (Fig. 3). However, PGE1 was also inactive in this system (Fig. 3), questioning a role for EP receptors in the cytoprotective response to PGE2. PGE2 and PGE1 share a cyclopentane ring structure with keto and hydroxyl groups in positions 9 and 11, respectively. PGE2 and PGE1 are equipotent at displacing [3H]PGE2 from EP2 (5) and EP3 (35) receptors in ligand binding studies, with PGE1 slightly less potent than using EP1 receptor preparations (14). The biological activities of PGE1 and PGE2 are nearly identical, and both induce cytoprotection against N-methyl-o-aspartate receptor-mediated glutamate cytotoxicity via EP2 receptors in cultured cortical neurons (1). Thus PGE1 would be expected to induce cytoprotection if the response is mediated by an EP receptor. Additional evidence supporting the differential effects of PGE2 and PGE1 include the observation that TRE binding activity is induced by PGE2 but not PGE1 (Fig. 10).

EP receptors have only recently been cloned, and alternative splice variants have been identified, raising the possibility that novel subtypes exist and may mediate the present cytoprotective response (12). EP1/EP3 receptors are coupled to IP-related signal transduction, whereas cyclic nucleotide metabolism is regulated positively by EP2/EP4 receptors and negatively by EP3 receptors (17). To facilitate identification of the PGE2/DDM-PGE2 receptor, we activated second messenger systems to define pathways capable of inducing cytoprotection. Although many actions of PGs involve cyclic nucleotide-related signaling, cytoprotection is not induced by agents that activate this pathway (DBcAMP, forskolin; data not shown). PG treatment is also associated with elevations in intracellular calcium (17), but pretreatment with a calcium ionophore (4-Br-A-23187) alone was ineffective. In contrast, IP turnover is associated with activation of PKC, and pretreatment of cells with TPA, a potent activator of PKC (28), induces cytoprotection against 2,3,5-(trisglutathion-S-y1)-HQ-mediated cytotoxicity (Fig. 6).

To further dissociate these pathways and provide additional support for protein kinase activity in the cytoprotective response to DDM-PGE2, the protein kinase inhibitor H-89 was employed. This inhibitor has the advantage of differentially inhibiting PKA and PKC activities as a function of concentration (K_{iPKA}, 48 nM; K_{iPKC}, 31.7 µM) (8). The cytoprotective responses to DDM-PGE2 and TPA are sensitive to H-89 at concentrations approaching the K_i for PKC but not at concentrations 40-fold higher than the K_i for PKA (Fig. 6), consistent with a PKC-dependent mechanism. H-89 alone did not modulate cell viability, suggesting that inhibition of cytoprotection and TRE binding activity is not secondary to overt cytotoxicity, as observed with the selective PKC inhibitor calphostin C (data not shown). The highest concentration of calphostin C, which was not cytotoxic (10 nM), marginally inhibited the cytopro-
tective response to DDM-PGE₂ (10–15%, data not shown), as would be predicted (half-maximal inhibitory concentration, 50 nM). The differential cytotoxicity of H-89 and calphin C in LLC-PK₁ cells suggests the presence of a critical PKC isoform(s) that regulates cell viability and suggest that H-89 may be a useful probe to identify this isoform(s).

PKC regulates the activity of the AP-1 transcriptional complex, which is considered a nuclear third messenger in this cascade. On activation, AP-1 specifically binds to a target DNA sequence referred to as the TRE (23). DDM-PGE₂ induces binding of nuclear proteins to a 32P-labeled TRE (Figs. 7–10), providing molecular evidence for the involvement of PKC in this response. Furthermore, the induction of TRE binding activity is observed in cells treated with cytoprotective agents (DDM-PGE₂ and PGE₂) but not by prostanoids known to interact with PKC-coupled EP receptors (PT-PGE₂, sulprostone, PGE₁) (Fig. 10). Consistent with a PKC-dependent mechanism, H-89 inhibits inducible TRE binding activity as the concentration approaches the Kᵢ for PKC (Fig. 9).

The mechanism of PG-mediated cytoprotection in vivo may involve modulation of renal hemodynamics (13), direct cellular actions (1), or perhaps a combination of both. The latency of the cytoprotective response to DDM-PGE₂ in LLC-PK₁ cells (Fig. 4) suggests cytoprotection occurs at the cellular level, consistent with reports elsewhere (1, 30, 33). Interestingly, inhibition of DDM-PGE₂-mediated cytoprotection (Fig. 6) and inducible TRE binding activity (Fig. 9) by H-89 were observed, and this correlation raises the possibility that cytoprotection is transcriptionally regulated. This suggestion is consistent with the delayed onset of this effect (Fig. 4) and with the observation that PGE₂ as a cotreatment does not induce cytoprotection (data not shown). Alternatively, the cytoprotective response was first detected 8 h after treatment of cells with DDM-PGE₂ (Fig. 4), a time point where inducible TRE binding activity was returning to control (Fig. 7). This correlation suggests the induction of cytoprotection is secondary to PKC downregulation. Studies are ongoing to delineate the mechanism of PG-mediated cytoprotection and the role of PKC in this response.

In conclusion, evidence is presented suggesting that cytoprotection by PGE₂ and DDM-PGE₂ in LLC-PK₁ cells is receptor mediated. The increased potency of DDM-PGE₂, relative to PGE₂, in the cytoprotective response may be related to the enhanced stability of this analog. Alternatively, modification of PGE₂ to the DDM-PGE₂ analog may increase its affinity for the putative receptor that mediates the cytoprotective response. The latter suggestion is consistent with the general decreased potency of PGE₂ with respect to the induction of cytoprotection and TRE binding activity. The observation that known EP receptor agonists and PGE₂ are inactive in this system suggest the involvement of a receptor unrelated to presently known EP receptor subtypes. The PGE₁/DDM-PGE₂ receptor is coupled to PKC as evidenced by 1) the induction of TRE binding activity by PGE₂ and DDM-PGE₂, which does not result from the direct activation of PKC; 2) the inhibition of DDM-PGE₂-mediated cytoprotection and TRE binding activity by H-89; and 3) the induction of cytoprotection by phorbol ester.

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