Evidence of Protein Kinase C Involvement in Phorbol Diester-stimulated Arachidonic Acid Release and Prostaglandin Synthesis*

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Judy Parker†, Larry W. Daniel, and Moseley Waite
From the Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

Many stimulators of prostaglandin production are thought to activate the Ca**+-- and phospholipid-dependent protein kinase C first described by Nishizuka and his colleagues (Takai, Y., Kishimoto, A., Iwasa, Y., Kawanara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695. In this paper we report evidence that the activation of protein kinase C caused by 12-O-tetradecanoylphorbol-13-acetate (TPA) is involved in the increased prostaglandin production induced by 12-O-tetradecanoylphorbol-13-acetate in Madin-Darby canine kidney (MDCK) cells. We have shown that TPA activates protein kinase C in MDCK cells with similar dose response curve as observed for TPA induction of arachidonic acid release in MDCK cells. Activation of protein kinase C was associated with increased phosphorylation of proteins of 40,000 and 48,000 daltons. We used two compounds (1-Octadecyl-2-O-methyl-1-rac-glycero-3-phosphocholine (ET-18-OMe) and 1-(5-isoquinolinesulfonfyl)piperazine known to inhibit protein kinase C by different mechanisms to further examine if activation of protein kinase C was involved in the increased synthesis of prostaglandins in TPA-treated MDCK cells. We found that both compounds inhibited protein kinase C partially purified from MDCK cells and that ET-18-OMe inhibited the phosphorylation of proteins by protein kinase C in the intact cells. Addition of either compound during or after TPA treatment decreased both release of arachidonic acid from phospholipids and prostaglandin synthesis. Release of [3H]arachidonic acid from phosphatidylethanolamine in TPA-treated cells was blocked by ET-18-OMe or 1-(5-isoquinolinesulfonfyl)piperazine addition. However, arachidonic acid release stimulated by A23187 is not blocked by Et-18-OMe. When assayed in vitro, treatment of cells with Et-18-OMe did not prevent the enhanced conversion of arachidonic acid into prostaglandins induced by pretreatment of cells with TPA. Our results suggest that the stimulation of phospholipase A2 activity by TPA occurs via activation of protein kinase C by TPA.

The prostaglandins, hydroxyeicosatetraenoic acids, thromboxanes, and leukotrienes (the eicosanoids) are important local modifiers of biological function and are synthesized from arachidonic acid by cellular enzymes. However, before PGH synthase (cyclooxygenase) or lipooxygenase converts arachidonic acid into the various eicosanoids, it is thought that arachidonic acid must be released from cellular lipids where it is esterified predominantly into phospholipids (1). Activation of arachidonic acid release is currently considered the principal mechanism for stimulation of eicosanoid synthesis. Epidermal growth factor (2), hormones (3, 4), and tumor promoters (5-7) are thought to increase the synthesis of eicosanoids by activation of arachidonic acid release. However, production of eicosanoids is also controlled by the activity of PGH synthase and lipooxygenase which catalyze the first step in the synthesis of prostaglandins/thromboxanes and HETEs leukotrienes, respectively. The activity of PGH synthase is increased by epidermal growth factor (8), TPA (9, 10), and bradykinin (11). Previous studies in our laboratory have shown that prolonged stimulation of PGH synthase activity by TPA in MDCK cells was blocked by inhibitors of protein synthesis (9), whereas the stimulation of arachidonic acid release by TPA was independent of protein synthesis (12). Thus, TPA appears to have two mechanisms of increasing synthesis of prostaglandins: 1) increased release of arachidonic acid and 2) enhanced conversion of arachidonic acid to prostaglandins. Only the latter TPA effect is sensitive to cycloheximide, which indicates that TPA either stimulates the synthesis of PGH synthase or of an activator of this enzyme (9).

Many stimuli that cause production of eicosanoids also activate protein kinase C including tumor promoters, growth factors, hormones, and neurotransmitters (5-7, 13). Protein kinase C is a ubiquitous enzyme involved in the transduction of biological signals (reviewed in Ref. 13). It is possible that activation of protein kinase C might mediate the increase in prostaglandin synthesis caused by this diverse group of stimuli. Indeed, protein kinase C has been suggested to phosphorylate and thus modulate the activity of a family of proteins (lipocortins) that inhibit phospholipase A2 (14-17). Here we examine the role of protein kinase C in the stimulation of release of arachidonic acid and of prostaglandin synthesis by TPA.

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† Postdoctoral fellow under National Research Service Award T32 CA 09422 from the National Cancer Institute. To whom correspondence should be addressed: Dept. of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, 300 South Hawthorne Rd., Winston-Salem, NC 27103.

§ The abbreviations used are: PGH2, prostaglandin H2; TPA, 12-O-tetradecanoylphorbol-13-acetate; ET-18-OMe, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; protein kinase C, Ca**+/phospholipid-dependent protein kinase; EGTA, ethylene glycol bism(2-aminoethyl)ether)-N,N',N"-tetraacetic acid; SDS, sodium dodecyl sulfate; MDCK, Madin-Darby canine kidney; TES, 2-[1-hydroxyethyl]glycine N-trimethonium salt; 1,1-bis(hydroxymethyl)ethylaminoethanesulfonic acid.
MATERIALS AND METHODS

Chemicals—Arachidonic acid was from NuChek Prep. Inc., Elyria, OH. 125I-labeled heparin was from Behring Diagnostics, and indomethacin and prostaglandins from Sigma. All phospholipids were from Serdary Research Laboratories Inc. (London, Ontario, Canada), except ET-18-Ome which was a gift from Dr. Wolfgang E. Berdel, Munich, Federal Republic of Germany. The 1-(5-isoquinolinesulfonyl)piperazine from Dr. Craig Gerard, Department of Medicine, Bowman Gray School of Medicine was prepared as described (18). DEAE-Sepharose was from Pharmacia L-P Biochemicals. Histone type II, bovine serum albumin, β-mercaptoethanol and phenylmethylsulfonyl fluoride were from Sigma. Dye was from Serdary Research Laboratories Inc. Carrier-free 32P04 (approximately 285 Ci/mmol) was from New England Nuclear Research Products. [3H]Arachidonic acid (135 Ci/mmol) was from Amersham Corp. Silica Gel 60 plates were prepared by E. Merck, Darmstadt, Federal Republic of Germany (5721-7) and En3Hancce was from NEN Research Products. Fetal bovine serum was from Gibco Laboratories, whereas medium and antibiotics for tissue culture were from Flow Laboratories, Rockville, MD. All other chemicals were reagent grade or better.

Growth of Madin-Darby Canine Kidney Cells—MDCK cells were obtained from Flow Laboratories. The cells were grown in plastic flasks in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (heat inactivated), penicillin 100 units/ml, and streptomycin sulfate 100 µg/ml.

To study the effect of the toxicity of ET-18-Ome on confluent MDCK cells were removed from a flask by treatment with 0.05% trypsin, 0.02% EDTA, and then soaked in trypsin solution for 15 min at 37°C. The cells were counted and 5 x 10⁴ cells were added to all wells of a 96-well microtiter plate. After incubation at 37°C overnight, the medium was removed and replaced with dilutions of ET-18-Ome in the normal culture medium (final ethanol concentration was 0.1% or less and did not inhibit cell growth). After 4-6 h of incubation at 37°C, the medium was removed from wells, the wells rinsed with trypsin solution, and then sonked in trypsin solution for 15 min at 37°C to detach the cells from the plastic surface. The detached cells were mixed vigorously and an aliquot diluted with trypsin blue (0.1% in saline) and then counted with a hemocytometer. All samples were assayed in triplicate. For long term assays of growth, 3 x 10⁵ cells were seeded in each well and cells were exposed to ET-18-Ome, trypsinized, and counted as above at daily intervals.

Preparation of Protein Kinase C—MDCK cells were grown in 100-mm plastic dishes for 4 days. The monolayer of cells was then washed with ice-cold 0.9% NaCl and the cells scraped off with a rubber policeman. After centrifugation at 600 x g for 5 min at 4°C the cell pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride. The cells were then sonicated for 20 s with a stepped microprobe. Unbroken cells were removed by centrifugation at 120,000 x g for 90 min at 4°C. The supernatant from this step (cytosol) was then fractionated on a 1 x 8-cm DEAE-Sepharose column after addition of sucrose to give 10% final concentration. The DEAE column was equilibrated in 20 mM Tris, pH 7.5, 0.2 mM EDTA, 50 mM β-mercaptoethanol, 10% sucrose. After the sample was loaded onto the column, unbound material was washed through with 40 ml of the equilibration buffer. Then protein kinase C was eluted by a gradient from 0–0.5 M NaCl in equilibration buffer. Fractions of 1 ml were collected at 25 ml/h and then 0.05-ml aliquots from fractions assayed for protein kinase C as below. The fractions with the most activity were pooled and used in further experiments. The particulate fraction pelleted by centrifugation at 120,000 x g was extracted by sonication in the same buffer with 1% Triton X-100 added. The Triton X-100 extract was then centrifuged at 120,000 x g for 90 min at 4°C. The supernatant from this step (extracted particulate) was purified by DEAE column chromatography as above except that 0.1% Triton X-100 was added to the buffer.

Assay of Protein Kinase C—The assays were done in a total volume of 0.25 ml and all tubes contained 25 mM Tris, pH 7.5, 10 mM MgCl2, 40 µg of histone, 10 µM ATP (including 1 µCi of [γ-32P]ATP), 0.6 mM NaN3, 20 µM from phosphodiesterase, and 2 µg/ml diolamine plus 0.05 ml of protein kinase C. Protein kinase C activity was determined as the incorporation of 32P from [γ-32P]ATP into histone in the presence of Ca++, phosphatidylsersine, and diolamine minus the incorporation in the absence of these activators. Reactions were initiated by the addition of protein kinase C and halted after 20 min at 30°C by the addition of 0.05 ml of bovine serum albumin (10 mg/ml) and 1 ml of 25% trichloroacetic acid (ice-cold). The tubes were kept on ice and then filtered in a Millipore vacuum box using Millipore HA filters and washed with 25% trichloroacetic acid. The radioactivity bound to the filters was determined in 5 ml of Budge Solution by scintillation. The amount of enzyme used was shown to result in linear activity for at least 20 min, and the assay was linearly dependent on the amount of enzyme used.

ET-18-Ome or 1-(5-isoquinolinesulfonyl)piperazine was added directly to the reaction mixture before the addition of protein kinase C. As a control for the small amount of ethanol from the ET-18-Ome stock solution, 0.1% ethanol was included in the control experiment but no ET-18-Ome. In addition to the standard assay components 0.01% Triton X-100 was included in these experiments.

Analysis of the Phosphorylation of Proteins by TPA—MDCK cells were seeded into 35-mm dishes and grown for 3 or 4 days. The medium was changed to 1 ml of minimum Eagle's medium without phosphate and replaced with 1 ml of fresh medium containing 0.5 µCi of 32P04, 30 min later, and each dish was incubated at 37°C for 30 min. The labeling medium was then removed and 1 ml of 20 mM TES, 5 mM KC1, 1 mM MgCl2, 0.15 M NaCl, 10 mM glucose, pH 7.4, added to all dishes; TPA was then added to dishes to give 50 nM or other final concentration. After 30 min of incubation the dishes were washed three times with the above TES buffer including 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride at 4°C. Cells were scraped off the dishes in this buffer and collected by centrifugation at 600 x g for 5 min at 4°C. The supernatant was discarded, the cell pellet dissolved in 0.1% SDS, 50 mM Tris, pH 6.25 mM Tris, pH 6.8, and then boiled for 5 min in a water bath. ET-18-Ome, when present, was given 10 min before the TPA. Proteins were analyzed by polyacrylamide gel electrophoresis in 12.5% resolving gel with 5% stacking gel according to Laemmli (19). The gels were fixed in 30% methanol, 10% acetic acid, dried onto filter paper, and an autoradiograph prepared by exposing Kodak X-Omat AR or SB-5 film to the gels. Autoradiographs were scanned with a Zeinhe soft laser scanning densitometer (Model SL TRFF).

Detection of Changes Induced in Arachidonic Acid Metabolism by TPA or A23187—MDCK cells were grown for 2–3 days under standard conditions in 35-mm plastic dishes. The medium in each dish was replaced with 1 ml of fresh medium containing 0.5 µCi of [3H] arachidonic acid and the cells grown for another 18–20 h. The labeling medium was then removed and the dishes washed with medium. The cells were then exposed to TPA or A23187 in 1 ml of fresh medium. After further incubation at 37°C, the cells and medium from each dish were separated, the lipids extracted, and the radioactivity in various lipids determined by thin layer chromatography as described (20). The only modifications made were that silica Gel 60 plates were used for all chromatography and a fluorogram prepared by spraying the thin layer chromatography plate with En3Hancce and then exposing Kodak SB-5 film to the plate at –70°C to aid in localizing the lipid spots for subsequent analysis. Prostaglandins synthesized by the MDCK cells include PGF2α, PGF2α, PGI2, PGF2β, and PGD2, as previously confirmed by us using high pressure liquid chromatography (20). ET-18-Ome, 1-(5-isoquinolinesulfonyl)piperazine, or indomethacin were dissolved in ethanol and then diluted in medium. Addition of these compounds was directly into the medium at the same time as the stimulus (TPA or A23187) unless otherwise indicated. Final concentration of ethanol was 0.1% or less and had no effect on arachidonic acid metabolism.

Assay of Prostaglandin Synthesis in Broken Cells—Prostaglandin synthesis in sonicated MDCK cells was determined as previously described (9). Samples were assayed with and without 10 µg/ml indomethacin and the supernatant radioactivity converted into prostaglandins from added [3H]arachidonic acid in the absence of indomethacin minus that in the presence of indomethacin determined. The identity of the radioactive products was determined routinely by thin layer chromatography or by high pressure liquid chromatography (1). Arachidonic acid was used as a saturating concentration, and included 0.5 µCi of [3H]arachidonic acid in each 1 ml of reaction mixture. Under the conditions employed, the reaction was linear with time and concentration of protein (9). Where indicated the cells were pretreated for 6 h with TPA or TPA and ET-18-Ome.

Effect of TPA on Distribution of Protein Kinase C in MDCK Cells—Protein kinase C activity was not detected in the cytosol or in a Triton X-100 extract from the particulate fraction of MDCK cells. However, after the crude samples were partially purified by DEAE ion exchange column chromatography, protein kinase C activity could be detected (Fig. 1). In control cells most of the protein kinase C activity was found in the cytosol, whereas in TPA-treated cells protein kinase C activity was found in the Triton X-100 extract of the particulate fraction (Fig. 1). The protein kinase C activity from MDCK cells eluted from DEAE columns with the concentration of NaCl (80–100 mM) previously reported to elute the enzyme from bovine brain (21) and was dependent on Ca²⁺ and phosphatidylserine. Thus as previously reported for other cells (22–24) addition of TPA to MDCK cells altered the distribution of protein kinase C between cytosolic and particulate fractions.

Protein Phosphorylation Is Stimulated by TPA in MDCK Cells—Addition of TPA stimulated the phosphorylation of two proteins of 40,000 and 48,000 daltons in the MDCK cells (Fig. 2). In order to quantitate the extent of TPA-stimulated phosphorylation of these two proteins, autoradiograms were scanned with a densitometer (see example in Fig. 5). The density of the film image from the 40,000-dalton protein was then compared to that of a protein whose phosphorylation was not stimulated by TPA (Ref.). TPA stimulation of the phosphorylation of the 40-kDa protein was dependent on the dose of TPA (Fig. 2) and on time (Fig. 3). The effect of TPA on the 48-kDa protein was similar (not shown). Occasionally phosphorylation of a protein of 29,000 daltons was also stimulated by TPA but this was not always seen perhaps due to overlap with a nearby heavily phosphorylated protein.

Protein Kinase C from MDCK Cells Is Inhibited by ET-18-OMe and 1-(5-Isoquinolinesulfonyl)piperazine—The partially purified protein kinase C from MDCK cells was inhibited by addition of either ET-18-OMe or 1-(5-isooquinolinesulfonyl)piperazine (Fig. 4). Half-maximal inhibition was observed at about 10 μM of each compound. In other in vitro assays ET-18-OMe inhibited protein kinase C 50% at 12 μM (28), whereas 1-(5-isooquinolinesulfonyl)piperazine required 20 μM for 50% inhibition (18). Addition of high concentrations of ET-18-OMe failed to completely inhibit the enzyme, perhaps because ET-18-OMe is amphipathic and a nonhomogenous mixture forms at high concentrations.

Addition of ET-18-OMe during the TPA exposure inhibited the phosphorylation of all three proteins (48-, 40-, and 29-kDa) stimulated by TPA (Fig. 5). Furthermore, the dose dependence of ET-18-OMe for inhibition of TPA-stimulated protein phosphorylation was similar to that required for the inhibition of protein kinase C (Fig. 5). Thus, ET-18-OMe inhibited protein phosphorylation in the intact cells which resulted from TPA addition.

Protein Kinase C Inhibitors Reduce Arachidonic Acid Release and Prostaglandin Synthesis in TPA-treated MDCK Cells—MDCK cells release [3H]arachidonic acid and prostaglandins from prelabeled cells into the medium upon stimulation by TPA (6). Addition of ET-13-OMe decreased both the TPA-stimulated release of arachidonic acid and synthesis of prostaglandins (Fig. 6). Addition of 1-(5-isoquinolinesulfonyl)piperazine had a similar effect (not shown). The inhibition of arachidonic acid release and prostaglandin synthesis by either protein kinase C inhibitor continued for 24 h, the longest period tested. Addition of increasing concentrations of either ET-18-OMe or 1-(5-isooquinolinesulfonyl)piperazine caused a dose-dependent reduction in the changes in arachidonic acid metabolism induced by TPA (Fig. 7). A maximum of 50–60% of the release of arachidonic acid or the production of prostaglandins induced by TPA could be inhibited by either inhibitor, no additional inhibition occurred with higher concentrations. In most experiments we used 100 nM TPA to get maximum release of arachidonic acid (6), although ET-18-OMe also inhibited arachidonic acid release and prostaglandin production with 1 or 10 nM TPA. Furthermore, ET-18-OMe could be added after TPA and still inhibited arachidonic acid release (not shown) and prostaglandin synthesis (Fig. 8), which was maintained for hours. Regardless of the time of ET-18-OMe addition there was always some arachidonic acid release and prostaglandin synthesis stimulated by TPA which was not inhibited by ET-18-OMe.

ET-18-OMe Inhibition of TPA-induced Prostaglandin Synthesis Is Not the Result of Reduction of Prostaglandin H Synthase Activity—Since both arachidonic acid release and prostaglandin synthesis were reduced by ET-18-OMe or 1-(5-isooquinolinesulfonyl)piperazine we examined the effect of ET-18-OMe on the synthesis of prostaglandins in cell homogenates. TPA-treated homogenates had elevated capacity for prostaglandin synthesis (Table I), reflecting the increase in de novo synthesis of PGH synthase or an activator of this enzyme (9, 10). There was some variation in the prostaglandin synthesis of different preparations from TPA-treated and control cells, probably because PGH synthase activity is affected by unknown variables such as growth factors in the serum (2) and the age of the cell culture. However, TPA consistently increased prostaglandin synthesis 3–5-fold in all
experiments. The addition of ET-18-OMe during TPA treatment of the cells failed to block the increase in prostaglandin synthesis (Table I). No diminution of the TPA enhancement of conversion of arachidonic acid to prostaglandins occurred even with 26 μM ET-18-OMe. Thus, ET-18-OMe fails to block the second effect of TPA, that is, the stimulation of the conversion of arachidonic acid into prostaglandins. As previously published (9), however, we do not known if this stimulation of prostaglandin synthesis by TPA is the result of enhanced synthesis of PGH synthase or of a co-factor required for activation of the PGH synthase.

We confirmed that ET-18-OMe blocked only the release of arachidonic acid but not its metabolism to prostaglandins since addition of ET-18-OMe directly into the in vitro assay did not inhibit prostaglandin synthesis by preparations from either control or TPA-treated cells (not shown). Furthermore, in the presence of indomethacin, free arachidonic acid accumulated in the TPA-treated MDCK cells since the indomethacin blocked the conversion of arachidonic acid to prostaglandins (Fig. 9A). However, in cells exposed to TPA and ET-18-OMe, arachidonic acid did not accumulate in the presence of indomethacin (Fig. 9B). This indicates that conversion of arachidonic acid to prostaglandins is not the limiting step in arachidonic acid metabolism when ET-18-OMe is present and the reduction of prostaglandin synthesis caused by ET-18-OMe occurs before the action of PGH synthase.

Effect of ET-18-OMe and 1-(5-isoquinolinesulfonyl)-piper-
Fig. 5. ET-18-OMe inhibition of TPA-induced phosphorylation of MDCK cell proteins. Left, autoradiogram of the SDS-polyacrylamide gel electrophoresis separation of MDCK proteins labeled with \(^{32}P\) and then stimulated for 60 min with TPA or TPA plus ET-18-OMe as described under “Materials and Methods.” Left arrows indicate migration of standard proteins of molecular weights shown. Right arrows indicate the 48-, 40-, and 29-kDa bands which are stimulated by TPA. Densitometer scans are from a portion of the autoradiogram indicated by the bracket. Letter designations correspond to those of the inset. Arrows mark 48-, 40, and 29-kDa bands which are stimulated by TPA exposure. REF arrow marks a band that does not vary with TPA exposure. Lane a, 0-time; lane b, 0.005% dimethyl sulfoxide; lane c, 50 nM TPA plus 40 \(\mu\)M ET-18-OMe; lane d, 50 nM TPA. Right, MDCK cells were labeled with \(^{32}P\) as above and then stimulated with 50 nM TPA for 30 min in the presence of different concentrations of ET-18-OMe. Autoradiograms of SDS-polyacrylamide gels were scanned and the density in the 40,000- or 48,000-dalton protein plotted as a ratio to the REF band. •, TPA; ■, control; ▲, ET-18-OMe 50 \(\mu\)M without TPA; \(K\), kDa.

azine on Deacylation of MDCK Phospholipids—We next determined the effect of ET-18-OMe and 1-(5-isoquinolinesulfonyl)piperazine on the deacylation of arachidonic acid from the cellular lipids of MDCK cells. The source of the arachidonic acid released by TPA was studied using cells prelabeled with \([\text{H}]\)arachidonic acid. At the end of the labeling period, the \([\text{H}]\)arachidonic acid was distributed among the cellular lipids: phosphatidylcholine, 18%; phosphatidylethanolamine, 57%; phosphatidylinositol, and phosphatidylserine, 17%; arachidonic acid, 0.6%; triglycerides, 1-3%; other 3-6%. As previously observed (6), the bulk of the \([\text{H}]\)arachidonic acid released with TPA was from phosphatidylethanolamine with lesser amounts derived from phosphatidylinositol and phosphatidylcholine (Fig. 10). In TPA-treated cells, ET-18-OMe inhibited the loss of \([\text{H}]\)arachidonic acid from phosphatidylethanolamine without effecting the loss from the other phospholipids. The same result was obtained with 1-(5-isoquinolinesulfonyl)piperazine (not shown). Thus, when release of arachidonic acid was reduced by addition of the protein kinase C inhibitors there was a retention of \([\text{H}]\)arachidonic acid in phosphatidylethanolamine.

Effect of ET-18-OMe on A23187-induced Arachidonic Acid Release—We also examined the effect of ET-18-OMe on the deacylation of arachidonic acid from phospholipids caused by a second stimulus. The calcium ionophore A23187-stimulated arachidonic acid release and prostaglandin synthesis in MDCK cells; however, ET-18-OMe did not alter the release of arachidonic acid stimulated by A23187 (Fig. 11). In fact, addition of ET-18-OMe increased the release of arachidonic acid induced by A23187, although ET-18-OMe itself did not stimulate arachidonic acid release. This may be because ET-18-OMe also inhibits reacylation of arachidonic acid into phospholipids. The treatment of MDCK cells with A23187 also caused the release of the majority of the \([\text{H}]\)arachidonic acid from phosphatidylethanolamine (not shown). These results demonstrate that ET-18-OMe is not a direct inhibitor of phospholipase activity and that the mechanisms of TPA and A23187 stimulation of arachidonic acid release are distinct.

Toxicity of ET-18-OMe to MDCK Cells—In order to ensure that the effect of ET-18-OMe was unrelated to a change in cell number, we exposed confluent cultures of MDCK cells to ET-18-OMe, thus duplicating the conditions used in the other experiments. The number of intact cells in cultures treated with ET-18-OMe for up to 6 h was no different than control

\(^3\)J. Parker, L. W. Daniel, and M. Waite, unpublished results.
Arachidonic Acid Release and Protein Kinase C

Fig. 6. ET-18-OMe inhibits TPA-induced release of arachidonic acid and prostaglandin synthesis. MDCK cells prelabeled with [3H]arachidonic acid were exposed to TPA with or without ET-18-OMe. Radioactive lipids accumulating in the medium were analyzed as described under "Materials and Methods." A, arachidonic acid; B, prostaglandins (sum of PGE₂, PGG₂, PGF₂, and PGD₂). A typical experiment is shown with the results represented as percent of the total radioactivity in the cells at the start of the experiment (1 × 10⁶ dpm). •, 100 nM TPA; ■, 100 nM TPA plus 20 μM ET-18-OMe.

Fig. 7. Protein kinase C inhibitors ET-18-OMe and 1-(5-isooquinolinesulfonyl)piperazine inhibit arachidonic acid and prostaglandin release in TPA-treated MDCK. MDCK cells were prepared as in Fig. 6, except the concentration of each inhibitor was varied and all samples extracted at 2 h. Radioactivity is expressed as the percent of the sum of arachidonic acid and prostaglandins with TPA alone (control). Prostaglandins (O–O), arachidonic acid (■–■), and sum of arachidonic acid + prostaglandins (■–■).

Fig. 8. ET-18-OMe added after TPA inhibits prostaglandin synthesis. MDCK cells were labeled with [3H]arachidonic acid as in Fig. 6 and stimulated by addition of 1 nM TPA at zero time. ET-18-OMe (40 μM) was added at zero time (O–O), 2 h (△–△), 4 h (●–●), 8 h (▲–▲), or 12 h (■–■). Cells treated only with TPA are also shown (O–O). Radioactivity in prostaglandins was quantitated as for Fig. 6 and is given as percent of total radioactivity at the start of the experiment. Arrows indicate the time of addition of ET-18-OMe.

TABLE I
ET-18-OMe fails to prevent the TPA-induced stimulation of the conversion of arachidonic acid to prostaglandins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostaglandin synthesis pmol min⁻¹ mg protein⁻¹</th>
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<tbody>
<tr>
<td>DMEM, 10% fetal calf serum</td>
<td>186 ± 153 (8)</td>
</tr>
<tr>
<td>10 nM TPA</td>
<td>700 ± 410* (7)</td>
</tr>
<tr>
<td>10 nM TPA + 17 μM ET-18-OMe</td>
<td>805 ± 446' (3)</td>
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* Values are the mean ± standard deviation.
' Number of separate preparations assayed.
' Different from DMEM, 10% fetal calf serum by Student’s t test, p < 0.006.

DISCUSSION
We have shown that TPA has the same effect on protein kinase C in MDCK cells as it has in many other cell types. In cultures. No change in the number of intact cells was observed at 6 h even with 40 μM ET-18-OMe. In other experiments ET-18-OMe was added to subconfluent cells and the growth of the cells observed for several days. These experiments showed that ET-18-OMe decreased the ability of the cells to proliferate; however, this effect occurs much later than the effects we observed on protein phosphorylation and arachidonic acid release. Since the inhibition of arachidonic acid release caused by ET-18-OMe in TPA-treated cells is observed even before 1 h, the effect of ET-18-OMe on arachidonic acid release is not due to cell death and must represent a change in cellular metabolism.

The MDCK cells TPA caused the redistribution of protein kinase C to the particulate fraction as previously observed for other cells (21–23). Furthermore, TPA stimulated the phosphorylation of 40- and 48-kDa proteins in the MDCK cells. In platelets a 40-kDa protein has been shown to be a substrate for protein kinase C (26, 27), and TPA-stimulated phosphorylation of 48-kDa protein has been reported in neutrophils (28). However, the MDCK cells respond to TPA more slowly than platelets and neutrophils in the phosphorylation of the 40- and 48-kDa proteins. Since the dose response for the stimulation of protein phosphorylation by TPA is the same for MDCK cells as for other (26, 29–31), we feel the slower response of MDCK cells is probably due to higher levels of protein kinase C inhibitors in the MDCK cells which activation by TPA can only slowly overcome. This would be consistent with our inability to detect protein kinase C in crude cell fractions. Furthermore, protein kinase C activity is low
in the MDCK cells which agrees with previous reports that kidney has less protein kinase C relative to platelets (21). The dose response for stimulation of protein phosphorylation by TPA in MDCK cells is similar to that for the release of arachidonic acid stimulated by TPA in these cells (6). Furthermore, both 1-oleyl-2-acetyl-rac-glycerol and 1,2-dioctanoyl-rac-glycerol, two other activators of protein kinase C, stimulated arachidonic acid release and prostaglandin synthesis equivalent to that induced by TPA.\(^3\) Since all three activators of protein kinase C stimulate prostaglandin synthesis, it appears that protein kinase C is involved in one or more steps of prostaglandin synthesis.

We have used two compounds known to be protein kinase C inhibitors to further test the involvement of protein kinase C in the release of arachidonic acid and synthesis of prostaglandins stimulated by TPA. We found that addition of ET-18-OMe or 1-(5-isoquinolinesulfonyl)piperazine decreased both the release of arachidonic acid and the synthesis of prostaglandins in TPA-treated MDCK cells. The concentrations of these compounds which inhibited arachidonic acid release and prostaglandin synthesis were similar to those required to inhibit partially purified protein kinase C from MDCK cells and to inhibit phosphorylation of proteins stimulated by TPA in intact cells. Since the protein kinase C inhibitors we used have distinct structures and unrelated modes of inhibition of protein kinase C (25, 32), it is unlikely that they share other effects on MDCK cells. Furthermore, although the isoquinolinesulfonamides inhibit other protein kinases (32), ET-18-OMe is unable to inhibit cAMP- and cGMP-dependent kinases (25). ET-18-OMe did not inhibit conversion of arachidonic acid into prostaglandins in the intact cells and was unable to prevent the TPA-induced stimulation of the conversion of arachidonic acid into prostaglandins. The latter result suggests that TPA increases the conversion of arachidonic acid to prostaglandins by a mechanism which is independent of the ability of TPA to activate protein kinase C. However, since we found that protein kinase C was not completely inhibited by inhibitors, it is possible that residual low level of protein kinase C activity suffices for the TPA enhancement of the conversion of arachidonic acid into pros-

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**Fig. 9.** Indomethacin causes arachidonic acid to accumulate in TPA-treated but not in TPA- and ET-18-OMe-treated MDCK cells. MDCK cells were labeled with \([\text{H}]\text{arachidonic acid} as in Fig. 3. The cells were stimulated for 3 h at 37 °C with various combinations of TPA, ET-18-OMe, and indomethacin, and then the lipids in the medium were analyzed as described under "Materials and Methods." The percent of total radioactivity in prostaglandins or arachidonic acid was determined and then all other values were normalized to that of the sum of prostaglandins and arachidonic acid from TPA-treated samples set to 100% (8.2% of total radioactivity). A, TPA 100 nM; B, TPA + ET-18-OMe 40 \(\mu\)M cells. O--O, prostaglandins; □--□, arachidonic acid; and Δ--Δ, arachidonic acid and prostaglandins.

**Fig. 10.** Effect of ET-18-OMe on deacylation of \([\text{H}]\text{arachidonic acid} from phospholipids in TPA-treated MDCK cells. The extracted lipids of the cells from Fig. 6 were separated by thin layer chromatography and radioactivity in various phospholipids quantitated by scraping appropriate areas of the plate and counting the silica in scintillation fluid. Results are presented as percent of total radioactivity in the cells at the start of the experiment. O--O and □--□, phosphatidylethanolamine; O--□ and □--□, phosphatidylycholine; and O--○ and □--○, phosphatidlyinositol + phosphatidylyserine. 100 nM TPA (circles); 100 nM TPA + 20 \(\mu\)M ET-18-OMe (squares).

**Fig. 11.** ET-18-OMe fails to inhibit A23187-induced arachidonic acid and prostaglandin release. MDCK cells labeled with \([\text{H}]\text{arachidonic acid} as for Fig. 6 were transferred into fresh DMEM, 10% fetal calf serum (O--O), 10 \(\mu\)M A23187 (□--□), ET-18-OMe 40 \(\mu\)M (○--○), or A23187 + ET-18-OMe (Δ--Δ). At times shown, medium was removed from the cells and the lipids of the medium extracted into chloroform:methanol. Results are presented as percent of total radioactivity in cells at start of the experiment. A, arachidonic acid; B, prostaglandins.
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taglandins. We think the best interpretation of our results is that inhibition of protein kinase C in MDCK reduces the release of arachidonic acid caused by TPA without decreasing the ability of the cells to synthesize prostaglandins from arachidonic acid.

Previous work from this laboratory suggested that a phospholipase \( A_2 \), which primarily cleaves phosphatidylethanolamine is activated by TPA in MDCK cells (33). The results reported here suggest that the activity of a phospholipase \( A_2 \), which is responsible for the release of arachidonic acid from phosphatidylethanolamine in MDCK cells, is regulated by protein kinase C. There are some reports of regulation of a phospholipase \( A_2 \) by a mechanism which involves phosphorylation in other cells. Addition of cAMP, ATP, and Mg\(^{++}\) stimulated phospholipase \( A_2 \) activity in a preparation from bovine brain synaptic vesicles (34). Wightman et al. (35) observed a similar activation of phospholipase \( A_2 \), by ATP and Mg\(^{++}\) or Ca\(^{++}\) in sonicates of murine macrophages and showed direct activation of phospholipase \( A_2 \) by cAMP-dependent kinase from bovine heart. Furthermore, phospholipase \( A_2 \) has previously been suggested to be regulated by phosphorylation-dephosphorylation of a regulatory protein (14, 36, 37). Alkaline phosphatase of human platelets known to be a substrate for protein kinase C. This is supported by the fact that in combination TPA and the Ca\(^{++}\) ionophore A23187 overcomes the inhibition of the protein kinase C by ET-18-OMe. A23187 causes an influx of Ca\(^{++}\) into the cell and leads to an increase in the activity of phospholipase \( A_2 \) (38), probably because phospholipase \( A_2 \) activity is dependent on calcium (39). The activation of phospholipase \( A_2 \) by an increase in intracellular Ca\(^{++}\) appears to be distinct from the activation induced by TPA through protein kinase C. This is supported by the fact that in combination TPA and A23187 induce more release of arachidonic acid from MDCK cells than either stimuli alone (12). Our results are consistent with the presence of two mechanisms to activate release of arachidonic acid in MDCK cells. It is difficult to predict whether a single phospholipase \( A_2 \) is activated via multiple pathways or if separate phospholipases are involved in each pathway. Indeed, it is possible that the action of the protein kinase C on phospholipase \( A_2 \) activity is mediated through a change in the concentration of Ca\(^{++}\) required to activate the phospholipase. If this were the case it would explain why the Ca\(^{++}\) ionophore A23187 overcomes the inhibition of the protein kinase C by ET-18-OMe. The Ca\(^{++}\)-independent arachidonic acid release stimulated by vasopressin (40), angiotensin II (40), and thrombin (41) might also be mediated by activation of phospholipase \( A_2 \) by protein kinase C since these stimuli are thought to activate protein kinase C (13, 27, 42). Thus, there appear to be two mechanisms (which may or may not be part of the same pathway) for the activation of phospholipase \( A_2 \) in kidney cells: one probably controlled by protein kinase C and the other a result of an increase in the concentration of intracellular Ca\(^{++}\).

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