Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation and reactive oxygen formation by bile acids.

P A Craven, … , J Pfanstiel, F R DeRubertis

J Clin Invest. 1987;79(2):532-541. https://doi.org/10.1172/JCI112844.

Research Article

Deoxycholate (DOC), chenodeoxycholate, 12-O-tetradecanoyl phorbol-13-acetate (TPA), or 1-oleoyl-2-acetyl-glycerol (OAG) activated colonic epithelial protein kinase C as reflected by translocation from the soluble to the particulate cell fraction. Activation of protein kinase C was correlated with stimulation of enhanced proliferative activity of colonic mucosa and reactive oxygen production. TPA and OAG, but not DOC, directly activated soluble protein kinase C in vitro. However, DOC rapidly increased labeled inositol phosphate and diacylglycerol accumulation in colonic epithelial cells. Retinoic acid inhibited protein kinase C activity and suppressed DOC-, TPA-, and OAG-induced increases in reactive oxygen production. The results support a role for protein kinase C in the stimulation of colonic epithelial proliferative activity and reactive oxygen production induced by bile acids, TPA and OAG. In contrast to TPA and OAG, which activate protein kinase C directly, bile acids appear to activate protein kinase C indirectly by increasing the diacylglycerol content of colonic epithelium.

Role of Activation of Protein Kinase C in the Stimulation of Colonic Epithelial Proliferation and Reactive Oxygen Formation by Bile Acids

Patricia A. Craven, James Pfanstiel, and Frederick R. DeRubertis
Department of Medicine, Veterans Administration Medical Center and the University of Pittsburgh, Pittsburgh, Pennsylvania 15240

Abstract

Deoxycholate (DOC), chenodeoxycholate, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), or 1-oleoyl-2-acetyl-glycerol (OAG) activated colonic epithelial protein kinase C as reflected by translocation from the soluble to the particulate cell fraction. Activation of protein kinase C was correlated with stimulation of enhanced proliferative activity of colonic mucosa and reactive oxygen production. TPA and OAG, but not DOC, directly activated soluble protein kinase C in vitro. However, DOC rapidly increased labeled inositol phosphate and diacylglycerol accumulation in colonic epithelial cells. Retinoic acid inhibited protein kinase C activity and suppressed DOC-, TPA-, and OAG-induced increases in reactive oxygen production. The results support a role for protein kinase C in the stimulation of colonic epithelial proliferative activity and reactive oxygen production induced by bile acids, TPA and OAG. In contrast to TPA and OAG, which activate protein kinase C directly, bile acids appear to activate protein kinase C indirectly by increasing the diacylglycerol content of colonic epithelium.

Introduction

Bile acids stimulate the proliferative activity of colonic epithelium (1–5), an action likely related to the ability of these agents to act as tumor promoters in experimental (6, 7) and, possibly, in human (7–10) colon carcinogenesis. The mechanisms by which bile acids enhance colonic epithelial proliferation are not known. However, one of the striking cellular actions of certain bile acids, such as deoxycholate (DOC)[1] and chenodeoxycholate which are potent stimuli of proliferative activity, is enhancement of membrane phospholipid turnover (4, 11).

Numerous studies in recent years have linked enhanced turnover of membrane phosphoinositides to activation of Ca^{2+} and phospholipid-dependent protein kinase C (12). Hydrolysis of inositol phospholipids by phospholipase C results in an increased cellular content of diacylglycerol which, in turn, is thought to serve as the endogenous activator of protein kinase C by increasing the affinity of the enzyme for Ca^{2+} (12). Phorbol esters and other structurally unrelated tumor promoters directly activate protein kinase C in a manner analogous to diacylglycerol (13–15). Activation of protein kinase C by these agents has now been linked to increased proliferative activity in several cell systems (16–21). Activation of protein kinase C has also been correlated with mitosis, viral transformation, and periods of rapid growth in cultured cells (16). More recently, activation of protein kinase C has been implicated in the mediation of increases in superoxide production induced in neutrophils by phorbol esters (22). In view of these considerations, we examined the possibility that bile acids may activate protein kinase C in colonic epithelium, and that activation of this enzyme may play a role in both the stimulation of epithelial proliferative activity and the enhancement of reactive oxygen production which we have previously described in colonic epithelium in response to bile acids (5).

Methods

Determination of ornithine decarboxylase activity and tritiated thymidine incorporation into colonic mucosal DNA. Female Sprague-Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) were fasted for 8 h before surgery and throughout the study period. Rats were anesthetized with pentobarbital (50 mg/kg i.p.), and the colon was exposed through a midline abdominal incision. Test agents were administered intracolonically as previously described in detail (4, 5). For determination of ornithine decarboxylase activity (ODC), rats were sacrificed at 4 h after bile acid or saline. For determination of tritiated thymidine ([^{3}H]dThd) incorporation into DNA, [^{3}H]dThd (100 μCi/kg) was injected intraperitoneally 22 h after the bile salt or saline instillation had been made. Rats were then killed 2 h after the injection of [^{3}H]dThd.

Isolation of colonic crypts. Colonic crypts were isolated from rat colon by a method previously reported for crypt isolation in the mouse colon (23), and employed earlier by our laboratory in colon (5).

Measurement of chemiluminescence. Chemiluminescence (CL) was measured as described previously (4, 5) in a Packard beta scintillation counter (model 3380, Packard Instrument Co., Downers Grove, IL) maintained at room temperature and operated in the out of coincidence mode (gain 100%, window A to infinity; discriminators 0–1,000). All values shown represent peak values obtained 10 s after addition of the crypt suspension or test agents and have been corrected for the background CL obtained with the phosphate-buffered saline plus luminol.

Measurement of O_2^- production by cytochrome c reduction. O_2^- production by colonic crypts was measured by the reduction of acetylated cytochrome *c* as previously described in detail (5). Assays routinely contained 75 μM acetylated cytochrome *c*, 50 μg/ml catalase, 1 mM phosphate buffer, pH 7.4, and when indicated 20 μg/ml superoxide dismutase. The production of O_2^- from colonic crypts was linear with time (0–2.5 min) and crypt protein concentration (2–6 mg) under the conditions employed.

Determination of ODC. Rats were killed 4 h after intraluminal instillation of bile acids. Enzyme activity was determined in the soluble fraction of mucosal scrapings from the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine as previously described (4, 5). ODC was linear with time for 60 min with 20–200 μg of protein under all conditions of study.

Address reprint requests to Dr. DeRubertis, VA Medical Center, University of Pittsburgh, University Drive C, Pittsburgh, PA 15240.

Received for publication 20 June 1986 and in revised form 24 September 1986.

1. *Abbreviations used in this paper:* CL, chemiluminescence; dThd, deoxythymidine; DOC, sodium deoxycholate; IP, inositol-1-phosphate; IP_2, inositol-1,4,-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; KRBG, Krebs-Ringer bicarbonate buffer; OAG, 1-oleoyl-2-acetyl glycerol; ODC, ornithine decarboxylase activity; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

The Journal of Clinical Investigation, Inc.
Volume 79, February 1987, 532–541

Preparation and partial purification of soluble protein kinase C activity. The procedure used for homogenization of tissue, solubilization of particulate activity, and DEAE cellulose column chromatography is the same as that reported previously (17, 18).

Assay of protein kinase C. Protein kinase C activity was determined as previously described (18). Unless otherwise indicated, reaction mixtures contained 20 mM Tris, 10 mM $MgCl_2$, 400 μg/ml histone (type III-S), 50 μM [^{32}P]ATP (1 μCi), 1 mM $CaCl_2$, and, where indicated, 80 μg/ml phosphatidylserine and 2 μM 1-oleoyl-2-acetyl-glycerol (OAG) in a final volume of 75 μl. The contribution of EGTA and EDTA to the assay mixture from the column elution buffer was 0.17 and 0.66 mM, respectively. Ca^{2+} was routinely present in the assay mixture at 1 mM in excess of EDTA plus EGTA. When OAG and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were added directly to the in vitro enzyme assay, Ca^{2+} chelators were removed from the enzyme solution by passage through a Sephadex G-25 column and 10 μM Ca^{2+} was added to the enzyme assay. The use of 10 μM rather than 1 mM Ca^{2+} was required to demonstrate the stimulatory effect of OAG and TPA on enzyme activity (13). Aliquots of phosphatidylserine (10 mg/ml ethanol) and OAG (50 mg/ml ethanol) were evaporated under N_2 and sonicated in 20 mM Tris, pH 7.6, before addition to the enzyme assay mixtures. Reactions were begun by the addition of 25 μl of enzyme preparation to 50 μl of assay mixture. Incubations were for 5 min at 30°C. Reactions were stopped by pipetting 50 μl of the assay mixtures onto a square (1 × 1 cm) of filter paper (Whatman 31 Whatman Inc., Clifton, NJ) which had been dipped in 10% trichloroacetic acid (TCA), 2 mM NaH_2PO_4. Filter papers were then washed with agitation in 250 ml of ice-cold 10% TCA for 15 min followed by four changes of 10% TCA at room temperature. The papers were soaked in 95% ethanol for 5 min followed by ether for an additional 5 min and allowed to air dry before counting. Enzyme activity was linear for 2–5 min under all conditions of assay employed.

Determination of labeled diacylglycerol. Labeled diacylglycerol was determined in colonic crypts which had been prelabeled with [^{3}H]arachidonate. Colonic crypts (9 mg protein/5 ml) were preincubated with [^{3}H]arachidonate (100 μCi/5 ml) for 60 min at 37°C. The suspension was washed by centrifugation in Krebs-Ringer bicarbonate buffer (KRBG), which contained 50 mg/ml fatty acid free albumin, resuspended in KRBG and incubated (5 ml per flask) at 37°C with or without test agents for the times indicated in the text. At the end of the incubation the crypt suspensions were extracted. The neutral lipid fraction was isolated by silicic acid chromatography as previously described (24). Aliquots of each extract were applied to silica gel G thin-layer plates and developed with hexane/ethyl ether/acetic acid 80:20:2 as previously described (25). Lipids were located with I_2 vapor and the spot corresponding to diacylglycerol standard was transferred to a scintillation vial and counted.

Determination of labeled inositol phosphates and phosphoinositides. Colonic crypts were incubated for 2 h with 3 μCi/2 ml of [^{3}H]myoinositol (sp act 80 Ci/mmol). Excess label was removed by washing the crypt suspension three times with KRBG. The crypts were then resuspended in KRBG plus 10 mM LiCl and incubated at 37°C for 3 min with or without 5 mM DOC. At the end of 3 min the incubation was stopped by addition of 0.67 ml of 10% $HClO_4$ and the mixture was homogenized in a grinding vessel on ice. After centrifugation, inositol-1-phosphate (IP), inositol-1,4-bisphosphate (IP_2), and inositol-1,4,5-triphosphate (IP_3) were separated by Dowex formate chromatography as previously described in detail (26). [^{3}H]IP, [^{3}H]IP_2, and [^{3}H]IP_3 were employed as standards. Phosphoinositides were isolated from particulate fractions as previously described (27). Unlabeled phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP_2) were added as carriers. The samples were then chromatographed on oxalate impregnated silica gel G thin-layer plates developed in $CHCl_3/CH_3OH$/4 N NH_4O_4 45:35:10. The lipids were visualized by exposure to I_2 vapor, scraped, and counted.

Statistics. Statistical significance of differences between mean values was determined by the *t* test for unpaired data. For ODC and [^{3}H]TdR incorporation into DNA, studies were conducted on six rats in each experimental group. Between 8 and 12 rats could be studied on any given day. Experiments were thus repeated until the indicated number of rats in each group had been studied. For the purposes of statistical analysis, the average of replicate determinations for any parameter from a single rat colon was entered as a single value ($n = 6$, degrees of freedom = 10 comparing any two conditions by independent *t* test). Unless otherwise indicated, in vitro incubations for determination of diacylglycerol, CL, and protein kinase C activity were performed in duplicate. Experiments were performed three times. The average value of duplicate determinations of diaclyglycerol, CL, and protein kinase C activity from each experiment was entered as a single value for the purposes of statistical analysis ($n = 3$, degrees of freedom = 4 comparing any two experimental conditions by independent *t* test).

Materials. TPA, 4a phorbol 12,13 didecanoate (4aPDD), and phorbol were obtained from Sigma Chemical Co. (St. Louis, MO). [1,2-^{3}H(*N*)]Myo-inositol, PIP [inositol-2,3-^{3}H(*N*)], PIP_2 [inositol-2,3-^{3}H(*N*)], and PI [arachidonyl-1-^{14}C] were obtained from New England Nuclear, (Boston, MA). Myo-[2-^{3}H]IP, myo-[2-^{3}H]IP_2, and myo-[2-^{3}H]IP_3 were obtained from Amersham Corporation (Arlington Heights, IL). The sources of all other reagents have been previously reported (4, 5, 25).

Results

Ca^{2+} and phospholipid-dependent protein kinase C activity was not detectable in the crude 100,000-*g* soluble fraction of colonic crypt homogenates. However, as illustrated in Fig. 1, partial purification of the crude soluble fraction on a DEAE cellulose column resulted in a sharp peak of activity which eluted at between 0.035 and 0.05 M NaCl. Protein kinase C activity is defined here as the difference between activity measured in the presence of Ca^{2+} plus phosphatidylserine and that measured with Ca^{2+} alone. As illustrated in Fig. 1, in the presence of Ca^{2+}, activity was stimulated approximately eightfold by the addition of phosphatidylserine to assay mixtures.

Table I illustrates the effects of incubation of isolated colonic crypts for 10 min at 37°C with DOC, chenodeoxycholate, TPA, or OAG on the soluble-particulate distribution of protein kinase C in homogenates. As shown, exposure of colonic crypts to 1 and 5 mM DOC, 5 mM chenodeoxycholate, 1 μM TPA, or 5 mM OAG resulted in a 48%–90% fall in soluble protein kinase C activity. In each case, the reduction in soluble enzyme activity was accounted for by a rise in enzyme activity in the particulate fraction. Such translocation of soluble protein kinase C to the

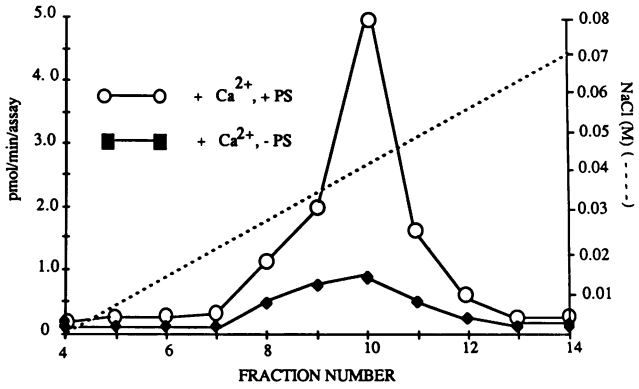

Figure 1. DEAE-cellulose purification of soluble protein kinase C activity from colonic crypts. The 100,000-*g* soluble fraction of crypt homogenates was prepared and applied to a DEAE-cellulose column. Fractions were eluted with a 0–0.07 M NaCl gradient (*dotted line*) and assayed for protein kinase activity in the presence of Ca^{2+} plus phosphatidylserine (80 μg/ml) (*open circles*) or with Ca^{2+} alone (*filled squares*). Results shown are from a single representative experiment repeated twice.

Table I. Effects of Addition of Test Agents to Isolated Colonic Crypts on the Subcellular Distribution of DEAE-Cellulose-purified Protein Kinase C Activity

Addition to incubated colonic crypts	Fraction	
	Soluble	Particulate
	pmol ^{32}P/min per mg crypt protein	
None	2.7±0.4	1.0±0.2
DOC, 5 mM	0.65±0.08*	3.3±0.5*
DOC, 1 mM	1.4±0.2*	2.1±0.3*
Chenodeoxycholate, 5 mM	1.1±0.2*	2.2±0.5*
Chenodeoxycholate, 1 mM	2.9±0.5	0.8±0.1
TPA, 1 μM	0.28±0.05*	3.6±0.5*
OAG, 5 mM	0.95±0.2*	2.4±0.3*
4aPDD, 10 μM	2.5±0.3	1.2±0.2
Phorbol, 10 μM	2.4±0.3	1.0±0.2

Colonic crypts were incubated for 10 min at 37°C with the additions shown. The 100,000-*g* soluble and particulate fractions of crypt homogenates were partially purified by DEAE-cellulose chromatography. The results shown represent the difference between enzyme activity assayed in the presence of Ca^{2+} and phosphatidylserine and activity measured with Ca^{2+} alone. Results shown are means±SE of determinations from three separate experiments ($df = 4$ comparing any two values by independent *t* test).
* $P < 0.05$ compared with corresponding value in the absence of an addition.

particulate fraction has been employed as an index of enzyme activation in intact cell systems (16). Consistent with previous results that demonstrated that chenodeoxycholate was less effective than DOC as a stimulus for ornithine decarboxylase and [^{3}H]dThd incorporation into DNA (4), chenodeoxycholate was less effective than DOC at mediating the translocation of soluble protein kinase C to the particulate fraction when both bile acids were tested at 5 mM. Moreover, chenodeoxycholate did not induce protein kinase C translocation when tested at 1 mM. In contrast to the effects of TPA, incubation of colonic crypts with 10 μM of 4aPDD or phorbol, which are not tumor promoters (12), had no effect on the subcellular distribution of protein kinase C activity in colonic crypts (Table I).

Table II illustrates the effects of addition of phosphatidylserine or phosphatidylserine plus OAG, TPA, or DOC to the enzyme assay on DEAE-cellulose-purified soluble protein kinase C activity from colonic crypts. As shown, phosphatidylserine increased enzyme activity sevenfold compared with that observed in the presence of Ca^{2+} (10 μM) alone. Addition of OAG (2 μM) or TPA (0.2 μM) to enzyme assay mixtures that contained phosphatidylserine plus 10 μM Ca^{2+} further increased activity twofold above that seen with phosphatidylserine and Ca^{2+} alone. By contrast, 4aPDD or phorbol had no effect on protein kinase C activity. As is also shown in Table II, raising the Ca^{2+} concentration from 10 μM to 1 mM increased protein kinase C activity twofold. Addition of phosphatidylserine further increased enzyme activity fivefold compared with that seen with 1 mM Ca^{2+} alone. DOC had a slight inhibitory effect on enzyme activity when tested in either the presence or absence of phosphatidylserine, possibly due to its ability to bind Ca^{2+} (28).

Table III illustrates the effects of DOC, chenodeoxycholate, TPA, or OAG on labeled diacylglycerol formation by incubated colonic crypts. Colonic crypts were prelabeled with [^{3}H]arachidonic acid. Where indicated, 1 and 5 mM DOC, 5 mM chenodeoxycholate, 10 μM TPA, or 5 mM OAG was added to the crypt suspension for the final 1 or 10 min of incubation. As shown in Table III, 5 mM DOC increased labeled diacylglycerol content approximately twofold when examined after either 1 or 10 min of exposure to DOC. 1 mM DOC was less effective than 5 mM DOC (50% increase in diacylglycerol). As is also shown in Table III, 5 mM chenodeoxycholate increased

Table II. Effects of Test Agents on Partially Purified Soluble Ca^{2+} and Phospholipid-sensitive Protein Kinase C Activity from Colonic Crypts

Additions to assay	Activity
	pmol ^{32}P/min per mg protein
Calcium, 10 μM	0.52±0.06
+ Phosphatidylserine, 80 μg/ml	3.6±0.2
+ OAG, 2 μM	7.3±0.4*
+ TPA, 0.2 μM	7.7±0.3*
+ 4aPDD, 0.2 μM	3.4±0.1
+ Phorbol, 0.2 μM	3.4±0.2
Calcium, 1 mM	1.2±0.1
+ DOC, 1 mM	0.8±0.1‡
+ Phosphatidylserine, 80 μg/ml	6.5±0.4
+ DOC, 1 mM	5.1±0.3*‡

Soluble protein kinase C activity (100,000 *g*) was purified from colonic crypt homogenates by DEAE-cellulose chromatography before assay. Results are means±SE of assays of three different enzyme preparations and are expressed as a function of milligrams of crude 100,000-*g* soluble protein ($df = 4$).
* $P < 0.05$ compared with corresponding value with phosphatidylserine alone.
‡ $P < 0.05$ compared with corresponding value without DOC.

Table III. Effects of Test Agents on Labeled Diacylglycerol Formation by Incubated Colonic Crypts

Additions to crypts	Time of exposure to test agent (min)	
	1	10
	dpm/mg protein	*dpm/mg protein*
None	25±4	—
DOC, 5 mM	63±12*	57±9*
DOC, 1 mM	43±6*	40±5*
Chenodeoxycholate, 5 mM	39±5*	37±6*
TPA, 10 μM	21±4	24±4
OAG, 5 mM	30±5	28±5

Colonic crypts were prelabeled with [^{3}H]arachidonate (1 μCi/5 ml) for 60 min at 37°C. The suspension was washed by centrifugation, resuspended in KRBG, and incubated (5 ml per flask) at 37°C for 20 min. Additions shown were made to the flasks for the final 1 or 10 min of incubation. At the end of the incubation, labeled diacylglycerol was extracted, isolated by thin layer chromatography as described in the methods, and counted. Results shown are means±SE of determinations on three colonic crypt incubates ($df = 4$).
* $P < 0.05$ compared with corresponding value in the absence of an addition to the crypt incubation.

Table IV. Effects of DOC on Phosphoinositide Hydrolysis in Isolated Colonic Crypts

Additions to colonic crypts	Inositol 1-phosphate	Inositol 1,4-bisphosphate	Inositol 1,4,5-trisphosphate	Phosphatidyl inositol	Phosphatidyl inositol-4-phosphate	Phosphatidyl inositol-4,5-bisphosphate
	% of total cpm	*% of total cpm*	*% of total cpm*	*% of total cpm*	*% of total cpm*	*% of total cpm*
None	5.8±0.8	2.8±0.5	0.37±0.06	83±2	5.9±1.1	4.8±0.6
5 mM DOC	9.2±3.0	6.9±0.8*	0.74±0.12*	76±5	5.6±0.7	1.1±0.1*

Colonic crypts were prelabeled with [^{3}H]inositol (3 μCi/2 ml) for 2 h at 37°C. The suspension was washed by centrifugation, resuspended in KRBG plus 10 mM LiCl, and incubated (2 ml per flush) at 37°C for 3 min with or without 5 mM DOC. Labeled phosphoinositides and inositol phosphates were extracted, separated, and counted as described in Methods. Results shown are means±SE of duplicate determination from three experiments ($df = 4$). * $P < 0.05$ compared with corresponding value in the absence of DOC.

diacylglycerol content of colonic crypts. However, chenodeoxycholate was less effective than DOC when tested at the same concentration. In contrast to the bile acids, neither TPA or OAG had any effect on labeled diacylglycerol content when tested under the same conditions of incubation as the bile salts and at concentrations greater than those which directly stimulated protein kinase C (Table II).

Table IV illustrates the effects of DOC on the accumulation of labeled inositol phosphates in incubates of colonic crypts that had been prelabeled with [^{3}H]inositol. As shown, 5 mM DOC significantly increased the accumulation of IP_2 and IP_3 approximately twofold. The accumulation of IP was also increased by DOC. However, the latter increase was not statistically significant. As is also illustrated in Table IV, a reduction in labeled PIP_2 was observed in colonic crypt incubates that contained 5 mM DOC compared with incubations conducted in the absence of DOC. The reduction in PIP_2 was sufficient to account for the rise in IP_2 and IP_3 observed. In contrast to PIP_2, no significant change in PI or PIP was observed with DOC.

Table V shows the effects of intracolonic instillation of DOC, chenodeoxycholate, TPA, or OAG on soluble and particulate protein kinase C activity in colonic mucosa. In these studies test agents were surgically instilled into the colons of rats. After 10 min the colonic mucosa was scraped and homogenized and the 100,000-*g* soluble fraction was prepared. Protein kinase C activity was purified by DEAE cellulose chromatography prior to assay. As illustrated, exposure of colons to 5 mM DOC or to 5 or 25 mM chenodeoxycholate resulted in translocation of soluble protein kinase C to the particulate fraction. Chenodeoxycholate was less effective than deoxycholate. As is also shown in Table V, intracolonic instillation of 10 or 1 μM TPA for 10 min also resulted in a 93% and 48% loss of soluble protein kinase C, respectively, whereas 0.1 μM TPA was not effective. Intracolonic instillation of 5 mM OAG similarly reduced soluble protein kinase C by 45% (Table V). By contrast, 4aPDD or phorbol had no effect on soluble protein kinase C activity when tested under the same conditions as TPA. Under all conditions shown in Table V, which led to a decrease in soluble protein kinase C, an analogous increase in particulate activity was observed. Qualitatively similar responses to each test agent were observed when colonic mucosa was harvested at 4 h rather than 10 min after intracolonic instillation of each test agent (data not shown).

Table VI illustrates the effects of intracolonic instillation of TPA, OAG, or DOC on ODC and [^{3}H]dThd incorporation into colonic mucosal DNA. Consistent with previous results, 5 mM DOC increased mucosal ODC ninefold and [^{3}H]dThd incorporation into mucosal DNA 2.5-fold. TPA had no effect on proliferative activity when tested at 0.1 μM but increased ornithine decarboxylase and [^{3}H]dThd incorporation into DNA 2- and 1.5-fold, respectively, when tested at 1 μM. Increasing the concentration of TPA to 10 μM resulted in a further increase in ornithine decarboxylase and [^{3}H]dThd incorporation into DNA to twofold above that observed in rats treated with saline alone. When tested at 1 mM, OAG increased ornithine decarboxylase and [^{3}H]dThd incorporation into DNA 70% and 40%, respectively (Table VI). Increasing the concentration of OAG to 5 mM further increased ornithine decarboxylase and [^{3}H]dThd

Table V. Effects of Intracolonic Instillation of Test Agents on the Subcellular Distribution of Protein Kinase C Activity in Colonic Mucosa

Test agents	Fraction	
	Soluble	Particulate
	pmol ^{32}P/min/mg protein	
Saline	2.4±0.5	0.71±0.14
DOC, 5 mM	0.64±0.11*	2.2±0.3*
Chenodeoxycholate, 5 mM	1.2±0.2*	1.8±0.3*
Chenodeoxycholate, 25 mM	0.62±0.14*	2.4±0.4*
TPA, 0.1 μM	2.6±0.4	0.53±0.08
TPA, 1 μM	1.1±0.2*	1.6±0.2*
TPA, 10 μM	0.19±0.03*	3.2±0.5*
OAG, 5 mM	1.2±0.2*	1.9±0.3*
4aPDD, 10 μM	2.7±0.5	0.51±0.10
Phorbol, 10 μM	3.1±0.5	0.62±0.13

Test agents were instilled into the colons of three rats in each group. DOC and sodium chenodeoxycholate were dissolved in saline. TPA, OAG, 4a PDD, and phorbol were dissolved in ethanol, an aliquot was added to saline, and the mixture was sonicated with a Branson probe sonicator for 30 s at the highest setting. The concentration of ethanol in saline employed (0.5%) did not influence the subcellular distribution of protein kinase C when tested alone. The pH of all solutions instilled was 8.0. After 10 min, colonic mucosa was removed by scraping and scrapings from three rats in each group were combined and homogenized. The 100,000-*g* soluble and particulate fractions were then prepared and purified by DEAE-cellulose chromatography as described in the legend to Fig. 1. Activity is expressed as a function of milligrams of protein in the mucosal homogenate. Results shown are means±SE of determinations from three separate experiments ($df = 4$).

* $P < 0.05$ compared with corresponding value in rats treated with saline.

Table VI. Effects of Intracolonic Instillation of Test Agents on ODC and [^{3}H]dThd Incorporation into Colonic Mucosal DNA

Intracolonic instillation	ODC	[^{3}H]dThd
	pmol CO_2/min per mg protein × dpm/μg DNA	
Saline	1.9±0.3	9±1
DOC, 5 mM	17±4*	22±3*
TPA, 0.1 μM	2.3±0.4	8±2
1 μM	4.1±0.6*	14±2*
10 μM	5.9±0.9*‡	20±3*‡
OAG, 1 mM	3.2±0.5*	13±2*
5 mM	4.3±0.5*‡	19±3*‡
4aPDD, 10 μM	1.8±0.4	10±2
Phorbol, 10 μM	1.9±0.3	10±2

Test agents, dissolved in 2 ml of saline, were instilled into rat colons as described in the footnote to Table IV. ODC was determined in the 100,000-*g* soluble fractions of homogenates of mucosal scrapings prepared 4 h after instillation of each agent. [^{3}H]dThd incorporation into DNA was determined 24 h after instillation of each test agent. Instillation of 0.5% ethanol in saline, which was the vehicle for TPA, OAG, 4aPDD, and phorbol, had no effect on ornithine decarboxylase or [^{3}H]dThd incorporation into DNA. Results shown for ODC represent the means±SE of duplicate determinations on mucosal supernatants from six rats in each group (n = 6). Results shown for [^{3}H]dThd incorporation into DNA represent determinations on three mucosal scrapings from each of six rats in each group (n = 6).

* $P < 0.05$ compared with the corresponding value with saline alone (df = 10).

‡ $P < 0.05$ comparing 10 μM TPA with 1 μM TPA or 5 mM OAG with 1 mM OAG.

incorporation into DNA to values that were twofold greater than those observed in rats that received saline alone. By contrast, 4aPDD and phorbol were without effect on ornithine decarboxylase or [^{3}H]dThd incorporation into DNA when tested at 10 μM.

Fig. 2 compares the concentration response relationship between DOC and ODC with that between DOC and the percent soluble protein kinase C activity in homogenates of colonic mucosal scrapings 4 h after intracolonic instillation of DOC. As shown in Fig. 2 (*upper panel*), exposure of colons to concentrations of DOC from 1 to 25 mM resulted in a progressive loss (47%–92%) of protein kinase C activity from the 100,000-*g* soluble fraction. The lowest concentration of DOC that caused a significant reduction in protein kinase C activity was 1 mM. As shown in Fig. 2 (*lower panel*), the concentration response relationship between DOC and ODC was similar to that between DOC and percent soluble protein kinase C activity.

Our previous studies had indicated a link between increased reactive oxygen production induced by bile salts and the subsequent increase in proliferative activity of colonic epithelium (5). Other studies have implicated activation of protein kinase C in the increases in superoxide production induced by phorbol esters in neutrophils (22). Accordingly, we examined the relationship between activation of protein kinase C and increases in reactive oxygen production in isolated colonic crypts. Fig. 3 (*upper panel*) illustrates the concentration response relationship between TPA and luminol-dependent CL in colonic crypts. A three- to fourfold increase in CL was observed in colonic crypts with 10 nM TPA. A 25-fold increase was observed with 1 μM TPA, and represented the maximal response to this agent. As is also shown in Fig. 3, 1 nM TPA was without effect on CL. Moreover, in contrast to TPA, 4aPDD or phorbol were without effect on CL in isolated colonic crypts. As is also shown in Fig. 3 (*lower panel*), concentrations of TPA from 10 to 1,000 nM caused a progressive shift in the subcellular distribution of protein kinase C from the soluble to the particulate fraction. Analogous to results obtained with CL, a significant decrease in percent soluble protein kinase C activity was observed with 10 nM TPA. Increasing the concentration of TPA to 1,000 nM resulted in a progressive decline in the percent soluble protein kinase C activity to 10%. 1 nM TPA was without effect on the subcellular distribution of PKC. As is also shown in Fig. 3 (*lower panel*), 4aPDD or phorbol failed to alter soluble protein kinase C activity at any of the concentrations tested.

Table VII illustrates the effects of DOC, TPA, and OAG on CL of colonic crypts. Consistent with previous observations (4), 5 mM DOC increased CL approximately fivefold. Increasing the concentration of DOC to 25 mM further increased CL in colonic crypts to values that were 25-fold higher than the value observed in colonic crypts in the absence of a stimulus. TPA at 1 μM also increased CL in colonic crypts ~ 25-fold to values that were not significantly different from those observed with an optimal concentration of DOC. As is also illustrated in Table VII, OAG (0.1–50 μM), increased CL in colonic crypts 5–25-fold. Optimal effects on CL were seen with 50 μM OAG. No further increases in CL were observed when the concentration

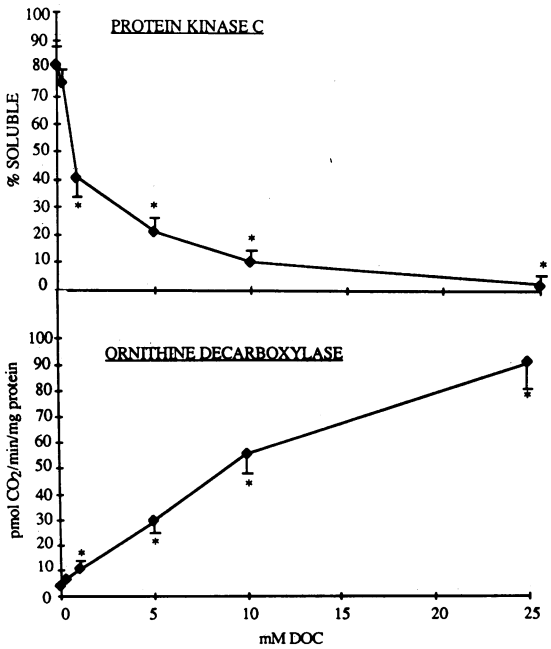

Figure 2. Concentration-response relationship between DOC and ODC or percent soluble protein kinase C activity in colonic mucosa. The percent soluble protein kinase C activity and ODC were determined in homogenates of colonic mucosal scrapings 4 h after intracolonic instillation of DOC as described in the footnotes to Tables IV and V, respectively. Results shown for ODC represent the mean±SE of duplicate determinations on mucosal supernatants from six rats (n = 6). Results for protein kinase C represent means±SE of duplicate determinations from three different enzyme preparations (n = 3). *$P < 0.05$ compared with value obtained in rats receiving saline alone.

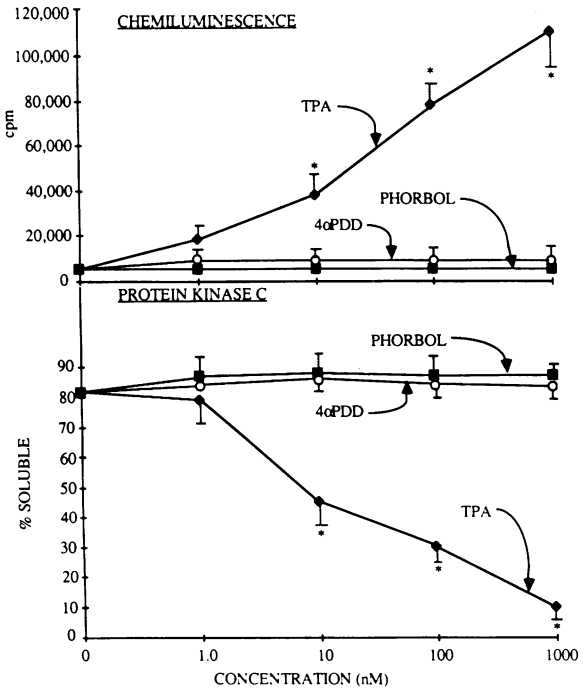

Figure 3. Concentration-response relationship between TPA, 4aPDD, or phorbol and CL (*upper panel*) or soluble protein kinase C (*lower panel*). Studies were conducted in vitro in isolated colonic crypt epithelium. Protein kinase C activity was determined as described in the footnote to Table I. Results shown for CL are means±SE of duplicate (with test agent) or eight (without test agent) counting rates from three separate experiments obtained 10 s after addition of colonic crypts or test agent to the vial (n = 3). Results shown for protein kinase C are means±SE of duplicate determinations on three different enzyme preparations (n = 3). *$P < 0.05$ compared to corresponding value in the absence of test agent.

of OAG was raised to 100 μM. CL is a nonspecific measure of reactive oxygen production. Accordingly, we examined the effects of DOC, TPA, and OAG on O_2^- production employing the more specific cytochrome *c* reduction assay. As previously reported (5), in the absence of a stimulus, O_2^- production is not detectable in colonic crypts. However, significant cytochrome *c* reduction by colonic crypts was observed in response to 5 mM DOC, (0.76±0.06); 1 μM TPA, (0.85±0.09); or 50 μM OAG, (0.96±0.08 nmol/min per mg protein).

Fig. 4 (*upper panel*) illustrates the concentration response relationship between DOC and CL in colonic crypts. Consistent with previous observations (5), a significant increase in CL was seen with 1 mM DOC. Increasing the concentration of DOC to 25 mM further increased CL to 25-fold above that seen with tissue alone in the absence of DOC. As is also illustrated in Fig. 4 (*lower panel*), analogous to results obtained with CL, a fall in percent soluble protein kinase C activity was evident after exposure of crypts to 1 mM DOC. Increasing the concentration of DOC to 25 mM resulted in a further reduction in soluble protein kinase C activity.

Retinoic acid has previously been shown to suppress CL or O_2^- production in neutrophils (22, 29, 30) and cultured epidermal cells (31). These actions of retinoic acid are thought to be due to inhibition of protein kinase C activity (22). We examined the influence of retinoic acid on basal CL and increases induced by DOC, TPA, and OAG in colonic crypts. In these studies, retinoic acid was preincubated with colonic crypts for 15 min at 0°C. Retinoic acid was then removed by washing the crypts by centrifugation. This was necessary due to intense CL caused by retinoic acid. As shown in Table VII, preincubation of colonic crypts with 100 μM retinoic acid had no influence on basal CL but abolished increases in CL induced by DOC, TPA, or OAG. By contrast, as is also shown, CL induced by addition of xanthine plus xanthine oxidase to the colonic crypts was not suppressed by retinoic acid, suggesting that any residual retinoic acid remaining in the crypt suspension was not acting as a scavenger of reactive oxygen.

Table VIII illustrates the effects of retinoic acid on TPA- and OAG-induced increases in soluble protein kinase C activity partially purified from colonic crypts. Consistent with results presented in Table II, phosphatidylserine increased protein kinase C activity sevenfold above that observed in the presence of Ca^{2+} (10 μM) alone. Addition of 0.2 μM TPA or 2 μM OAG to assays that contained Ca^{2+} and phosphatidylserine further increased protein kinase C activity twofold over that observed with Ca^{2+} plus phosphatidylserine alone. As is also shown in Table VIII, retinoic acid (25–100 μM) had no effect on enzyme activity assayed without phosphatidylserine but suppressed activity measured in the presence of phosphatidylserine by 55%–90%. Retinoic acid (25–100 μM) also markedly suppressed or abolished TPA- and OAG-induced increases in soluble protein kinase C activity from colonic crypts (Table VIII).

Table IX illustrates the effects of retinoic acid on DOC-induced alterations in the subcellular distribution of protein

Table VII. Effects of Retinoic Acid on Basal CL and Increases Induced by DOC, TPA, OAG, or Xanthine Oxidase in Colonic Crypts

Test agents	None	Retinoic acid (100 μM)
	cpm	*cpm*
None	4,873±589	4,436±483
DOC, 5 mM	56,592±7,034*	5,104±831‡
DOC, 25 mM	124,000±18,074*	6,059±905‡
TPA, 1 μM	120,394±21,743*	4,963±724‡
OAG, 0.1 μM	22,524±3,519*	4,271±731‡
OAG, 1 μM	51,369±7,246*	5,346±689‡
OAG, 10 μM	129,563±18,620*	4,891±702‡
OAG, 50 μM	127,404±16,593*	5,072±653‡
Xanthine + xanthine oxidase	61,952±9,043*	64,709±8,562

CL was determined as described in the legend to Fig. 2. Where indicated, retinoic acid was dissolved in ethanol and incubated with the colonic crypt suspension for 15 min at 0°C. Excess retinoic acid was then removed by centrifugation before final resuspension of the crypts. TPA and OAG were also dissolved in ethanol. Exposure of crypts to ethanol under the same conditions used to test retinoic acid, TPA and OAG had no effect on basal CL or increases induced by DOC. Where indicated, the concentration of xanthine was 1 mM and of xanthine oxidase was 1 μU/2 ml. Results shown are means±SE of duplicate (with test agent) or 16 (without test agent) counting rates from three separate experiments (n = 3) (df = 4).

* $P < 0.05$ compared with corresponding value in the absence of a test agent.

‡ $P < 0.05$ compared with corresponding value in the absence of retinoic acid.

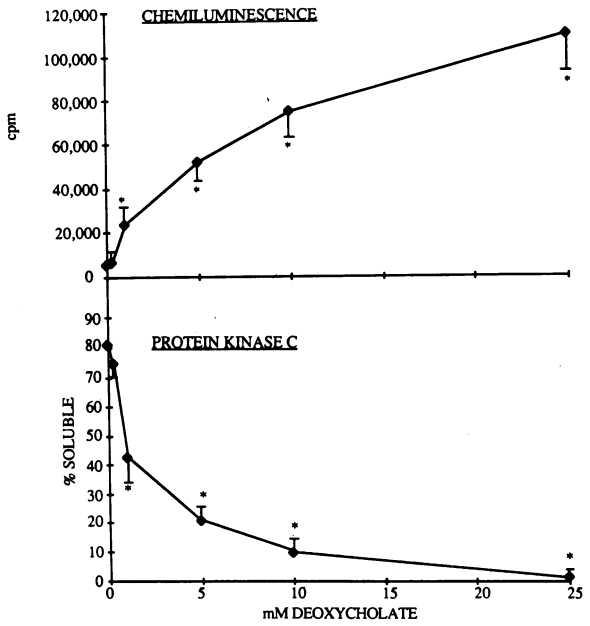

Figure 4. Concentration-response relationship between DOC and CL or percent soluble protein kinase C in isolated colonic crypts. CL and percent soluble protein kinase C were determined as described in the legend to Fig. 3 and the footnote to Table I, respectively. Results for CL are means±SE of duplicate (with DOC) or 10 (without DOC) counting rates from three separate experiments (n = 3). Results for protein kinase C are means±SE of duplicate determinations from three separate enzyme preparations (n = 3). *$P < 0.05$ compared with corresponding value in the absence of DOC.

kinase C activity in colonic crypts. As shown, DOC induced a marked shift in the distribution of enzyme activity from the soluble to the particulate fraction. When tested at 100 μM, retinoic acid completely prevented the DOC-induced shift in protein kinase C activity (Table IX).

Table VIII. Effects of Retinoic Acid on TPA and OAG-induced Increases in Soluble Protein Kinase C Activity from Colonic Crypts

		Retinoic acid (μM)	
Additions to assay	None	25	100
	pmol/min per mg protein		
None	0.51±0.06	0.48±0.05	0.54±0.06
Phosphatidylserine, 80 μg/ml	3.8±0.3	1.7±0.1‡	0.48±0.04‡
+TPA, 0.2 μM	7.9±0.7*	2.9±0.2*‡	0.52±0.06‡
+OAG, 2 μM	7.5±0.6*	2.4±0.2*‡	0.51±0.04‡

Protein kinase C activity was determined as described in the footnote to Table II. The 100,000-*g* soluble fraction of colonic crypt homogenates was partially purified by DEAE-cellulose before assay. Retinoic acid (dissolved in ethanol) was added to the assay mixture immediately before the enzyme preparation. Ethanol alone had no effect on enzyme activity under any of the conditions employed. Results shown are means±SE of determinations from three separate experiments and are expressed as a function of mg of crude 100,000-*g* soluble protein (df = 4).
* $P < 0.05$ compared with corresponding value in the presence of phosphatidylserine alone.
‡ $P < 0.05$ compared with corresponding value in the absence of retinoic acid.

Table IX. Effects of Retinoic Acid on DOC-induced Alterations in the Subcellular Distribution of Protein Kinase C Activity in Colonic Crypts

	Fraction	
Test agents	Soluble	Particulate
	pmol [^{32}P]/min per mg crypt protein	
None	3.2±0.5	0.81±0.09
DOC, 5 mM	0.92±0.02*	2.8±0.5*
DOC, 5 mM + retinoic acid, 100 μM	2.9±0.4‡	0.74±0.02‡

Studies were conducted as described in the footnote to Table I. Colonic crypts were preincubated at 37°C with 100 μM retinoic acid or no additions as indicated for 15 min before addition of DOC. Results shown are means±SE of determinations from three separate experiments (df = 4).
* $P < 0.05$ compared with corresponding value in the absence of test agent.
‡ $P < 0.05$ compared with corresponding value with DOC alone.

Discussion

Demonstration of protein kinase C in colonic epithelium. Protein kinase C activity has previously been demonstrated in small intestinal epithelium (32), and a role for this enzyme in intestinal secretion has been proposed (33–35). The results of the present study clearly demonstrate the presence of Ca^{2+} and phospholipid-dependent protein kinase C activity in colonic epithelium. Some of the properties of soluble colonic epithelial protein kinase C are analogous to those previously reported for this enzyme in other tissues (12, 13, 16–18). Consistent with previous studies in some other tissues, Ca^{2+} and phospholipid-dependent protein kinase C activity was not demonstrable in crude 100,000-*g* soluble fractions of colonic epithelial cell homogenates (16–18, 36). This may be due to the presence of inhibitory phosphatases (37) or to the presence of high levels of other protein kinase activities, such as Ca^{2+} or cyclic nucleotide-dependent protein kinases, which obscure Ca^{2+} and phospholipid-dependent activity.

Relationship between protein kinase C activation and translocation of enzyme activity to the particulate fraction of colonic epithelium. Activation of protein kinase C results in the formation of an enzyme phospholipid complex both in cell-free and in intact cell systems (38). Moreover, translocation of protein kinase C activity from the soluble to the particulate fraction has been repeatedly observed after exposure of intact cells to TPA, OAG, or thyrotropin-releasing hormone (16–18, 36, 39, 40). The ability of all of these agents to cause translocation of protein kinase C from the soluble to the particulate fraction is presumed to be due to their actions to activate protein kinase C. Thus, TPA and OAG activate protein kinase C activity directly (38, 41), whereas thyrotropin releasing hormone is known to induce inositol phospholipid breakdown resulting in an increase in the endogenous protein kinase C activator diacylglycerol (39).

In the present study, exposure of colonic epithelium to TPA, OAG, or bile salts induced a rapid and dramatic loss of soluble protein kinase C activity which was quantitatively recovered in the particulate fraction. The ability of TPA and OAG to induce

the translocation of soluble protein kinase C to the particulate fraction was likely a result of their ability to directly activate the enzyme. Thus, analogous to protein kinase C activity from other sources, soluble protein kinase C activity from partially purified colonic crypt epithelium was stimulated in vitro by TPA and OAG. Moreover, TPA induced the translocation of protein kinase C from the soluble to the particulate fraction in intact cells at concentrations that were effective at stimulating protein kinase C upon direct addition to the soluble fraction of colonic crypt homogenates. In contrast to TPA and OAG, DOC failed to activate soluble protein kinase C directly in vitro. However, DOC increased phosphoinositide turnover as measured by increased accumulation of IP_2 and IP_3 and a reciprocal fall in PIP_2. DOC also increased labeled diacylglycerol content of colonic epithelium. The concentration response relationship between DOC and increases in colonic epithelial diacylglycerol content was analogous to that between DOC and the translocation of soluble protein kinase C to the particulate fraction. These results suggest that DOC may activate protein kinase C indirectly by stimulating phosphoinositide breakdown. The mechanism by which DOC stimulates phosphoinositide breakdown and activates protein kinase C is not known, but could be related to its action as a Ca^{2+} ionophore (42). In this regard, the Ca^{2+} ionophore A23187 has been reported to increase phosphoinositide turnover (43) and high concentrations of Ca^{2+} may activate protein kinase C directly (12). Alternatively, DOC actions on membrane phosphoinositide breakdown could be related to its detergent properties, which may alter the interaction of phospholipase C with its membrane phopholipid substrates and thus increase their susceptibility to metabolism. DOC stimulates polyphosphoinositide hydrolysis by platelet cell membranes (11) and hepatic microsomes (44) and by homogeneous preparations of hepatic soluble phospholipase C (45). Of note, in the latter study, the ability of DOC to stimulate phosphoinositide hydrolysis by homogeneous preparations of phospholipase C was not mimicked by Triton X-100 (45), suggesting some specificity for DOC actions.

Relationship of protein kinase C activation to increased proliferative activity in colonic epithelium. Several lines of evidence suggest that activation of protein kinase C is associated with increased cell growth. Thus, a fall in soluble enzyme activity is associated with mitosis, viral transformation, and periods of rapid growth in cultured cells (16). Moreover, activation of protein kinase C by TPA and OAG is associated with an increase in [^{3}H]dThd incorporation into DNA and an increase in ODC in several cell systems (16, 19–21). The results of the present study provide support for an association between protein kinase C activation and increased proliferative activity in colonic epithelium. Increases in colonic mucosal protein kinase C induced by bile acids and other activators in vivo were evident within 10 min and clearly preceded enhancement of proliferative activity. Thus, changes in protein kinase C were not simply a concurrent of cell replication in colonic epithelium. In our in vivo studies, the concentration response relationship between DOC and loss of protein kinase C from the soluble fraction of colonic mucosa was analogous to that between DOC and stimulation of mucosal proliferation as reflected by increases in ODC and DNA synthesis demonstrated in the present and earlier studies (4, 5). Moreover, intracolonic instillation of TPA (1–10 μM) and OAG (1–5 mM) increased colonic mucosal ODC and [^{3}H]dThd incorporation into mucosal DNA at concentrations that were also effective at inducing a soluble to particulate translocation of the enzyme in vivo. By contrast, intracolonic instillation of 0.1 μM TPA was without effect on either proliferative activity or the percent soluble protein kinase C. The concentration of TPA (1–10 μM) employed in our in vivo studies was 40–400-fold higher than that required for optimal effects of these agents on soluble protein kinase C activity in vitro. This may be related to the fact that the effective concentration of these lipid soluble agents in the colonic lumen is much lower than that in the in vitro enzyme assay due to dilution of TPA in membrane lipids. In this regard, previous in vivo studies of TPA on intestinal secretion also required 10 μM TPA to demonstrate effects (33). Similarly, the concentration of OAG (1–5 mM) required for optimal effects on colonic proliferative activity in vivo was 20–1,000 fold higher than that required to obtain optimal stimulation of soluble protein kinase C activity in the in vitro assay system employing the partially purified soluble enzyme. This may be related to metabolism of OAG (36) in vivo. However, we cannot rule out the possibility that the ability of TPA and OAG to increase colonic proliferative activity in vivo is due to actions of high concentrations of these agents other than activation of protein kinase C.

Relationship of protein kinase C activation to reactive oxygen production. Our previous studies had indicated that reactive oxygen production plays a role in bile salt induced increases in colonic mucosal proliferative activity (5). Work from other laboratories has implicated reactive oxygen production in the expression of the actions of TPA to increase proliferative activity in mouse skin (31, 44) and cultured hepatocytes (46) and to promote tumor formation in mouse skin (47). TPA and OAG increase O_2^- production in neutrophils (22, 29, 30) and cultured epidermal cells (31). Recent evidence suggests that these actions of TPA and OAG require activation of protein kinase C (22, 48). As clearly demonstrated in the present study, when tested at very low concentrations, TPA and OAG also increased superoxide dismutase inhibitable CL and O_2^- production in colonic crypts. The increases in these parameters in response to optimal concentrations of TPA and OAG were analogous to those seen with optimal concentrations of DOC. The concentration response relationship between DOC and CL was analogous to that between DOC and translocation of soluble protein kinase C to the particulate fraction in isolated colonic crypts. Moreover, the concentration response relationship between TPA and CL was also similar to that observed between TPA and percent soluble protein kinase C activity in isolated crypts. The CL responses to DOC, OAG, and TPA were all abolished by 100 μM retinoic acid. The failure of the concentration of retinoic acid tested to block CL generated via the xanthine-xanthine oxidase system implied that retinoic acid was not simply acting as a scavenger of reactive oxygen. The ability of retinoic acid to inhibit TPA-induced O_2^- production but not that mediated by xanthine-xanthine oxidase has been previously observed (22, 49). Retinoic acid blocked DOC-, TPA-, and OAG-induced increases in CL in colonic crypts at concentrations that were effective at blocking the translocation of protein kinase C activity from the soluble to the particulate fraction of colonic crypt homogenates. The results are consistent with a role for protein kinase C in expression of the actions of DOC, TPA, and OAG to increase reactive oxygen formation in colonic crypts.

Thus, our findings implicate activation of protein kinase C in the process by which bile acids rapidly increase reactive oxygen formation and, subsequently, enhance the proliferative activity

of colonic epithelium. The mechanism(s) by which the activation of protein kinase C and the burst of reactive oxygen induced by bile acids or phorbol esters ultimately lead to the stimulation of the proliferation of the colonic epithelium observed in response to these agents remains to be determined.

Acknowledgments

The authors are indebted to Kathy Daum for excellent secretarial assistance.

This work was supported by grant CA31680 from the National Cancer Institute through the Large Bowel Cancer Project.

References

1. Takano, S., M. Matshushima, E. Erturk, and G. T. Bryan. 1981. Early induction of rat colonic epithelial ornithine and *S*-adenosyl-L-methionine decarboxylase activities by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and bile salts. *Cancer Res.* 41:624–628.

2. Bull, A. W., L. J. Marnett, E. J. Dawe, and N. D. Nigro. 1983. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis.* 4:207–210.

3. Wargovich, M. J., V. W. S. Eng, H. L. Newmark, and W. R. Bruce. 1983. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis.* 4:1205–1207.

4. DeRubertis, F. R., P. A. Craven, and R. Saito. 1984. Bile salt stimulation of colonic epithelial proliferation: evidence for involvement of lipoxygenase products. *J. Clin. Invest.* 74:1614–1624.

5. Craven, P. A., J. Pfanstiel, and F. R. DeRubertis. 1986. Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. *J. Clin. Invest.* 77:850–859.

6. Reddy, B. S., K. Watanabe, J. H. Weissburger, and E. L. Wynder. 1977. Promoting effect of bile acids in colon carcinogenesis in germ free and conventional F344 rats. *Cancer Res.* 37:3238–3242.

7. Bresalier, R. S., and Y. S. Kim. 1985. Diet and colon cancer. *N. Engl. J. Med.* 313:1413–1414.

8. Reddy, B. S., A. R. Hedges, K. Laakso, and E. L. Wynder. 1978. Metabolic epidemiology of large bowel cancer: fecal bulk and constituents of high risk North American and low-risk Finnish population. *Cancer.* 42:2832–2838.

9. Reddy, B. S. 1981. Dietary fat and its relationship to large bowel cancer. *Cancer Res.* 41:3700–3705.

10. Lipkin, M., and H. Newmark. 1985. Effect of added dietary calcium on colonic epithelial cell proliferation in subjects at high risk for familial colonic cancer. *N. Engl. J. Med.* 313:1381–1384.

11. Plantavid, M., L. Rossignol, H. Chap, and L. Douste-Blazy. 1986. Studies of endogenous polyphosphoinositide hydrolysis in human platelet membranes: evidence that polyhosphoinositides remain inaccessible to phosphodiesterase in the native membrane. *Biochim. Biophys. Acta.* 875: 147–156.

12. Nishizuka, Y., Y. Takai, A. Kishimoto, U. Kikkawa, and K. Kaibuchi. 1984. Phospholipid turnover in hormone actions. *Rec. Prog. Horm. Res.* 40:301–345.

13. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.

14. Miyake, R., Y. Tanaka, T. Tsuda, K. Kaibuchi, U. Kikkawa, and Y. Nishizuka. 1984. Activation of protein kinase C by non-phorbol tumor promoter, mezerein. *Biochem. Biophys. Res. Commun.* 121:649–656.

15. Fujiki, H., Y. Tanaka, R. Miyake, U. Kikkawa, Y. Nishizuka, and T. Sugimura. 1984. Activation of calcium-activated phospholipid-dependent protein kinase (protein kinase C) by new classes of tumor promoters: Teleocidin and debromoaplysiatoxin. *Biochem. Biophys. Res. Commun.* 120:339–343.

16. Anderson, W. B., A. Estival, H. Tapiovaara, and R. Gopalkrishma. 1985. Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumor promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 19:287–306.

17. Kraft, A. S., and W. B. Anderson. 1983. Increase the amount of Ca^{2+} phospholipid dependent protein kinase associated with plasma membrane. *Nature (Lond.).* 301:621–623.

18. Kraft, A. S., W. B. Anderson, H. C. Cooper, and J. J. Sando. 1982. Decrease in cytosolic calcium/phospholipid dependent protein kinase activity following phorbol ester treatment of EL 4 thymoma cells. *J. Biol. Chem.* 257:13193–13196.

19. Rozengurt, E., A. Rodrigues-Pena, M. Coombs, and J. Sinnett-Smith. 1984. Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: role of Ca^{2+} sensitive phospholipid dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 81:5748–5752.

20. Otani, S., I. Matsui, A. Kuramoto, and S. Mousawa. 1985. Induction of ornithine decarboxylase in guinea pig lymphocytes synergistic effect of diacylglycerol and calcium. *Eur. J. Biochem.* 147:27–31.

21. Jetten, A. M., B. R. Ganong, G. R. Vandenbark, J. E. Shirley, and R. M. Bell. 1985. Role of protein kinase C in diacylglycerol-mediated induction of ornithine decarboxylase and reduction of epidermal growth factor binding. *Proc. Natl. Acad. Sci. USA.* 82:1941–1945.

22. Fujita, I., K. Irita, K. Takeshige, and S. Minakami. 1984. Diacylglycerol, 1-oleoyl-2-acetyl-glycerol, stimulates superoxide generation from human neutrophils. *Biochem. Biophys. Res. Commun.* 120:318–324.

23. Bjerknes, M., and H. Cheung. 1981. Methods for the isolation of intact epithelium from the mouse intestine. *Anat. Rec.* 199:565–574.

24. Bell, R. L., D. A. Kennerly, N. Stanford, and P. W. Majerus. 1979. Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA.* 76:3238–3241.

25. Craven, P. A., and F. R. DeRubertis. 1983. Ca^{2+}-calmodulin-dependent release of arachidonic acid for renal medullary prostaglandin synthesis. *J. Biol. Chem.* 258:4814–4823.

26. Berridge, M. J. 1983. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* 212:849–858.

27. Walson, S. P., R. T. McConnell, and E. G. Lapetina. 1984. The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.* 259:13199–13203.

28. Newmark, H. L., M. J. Wargovich, and W. R. Bruce. 1984. Colon cancer and dietary fat, phosphate and calcium: a hypothesis. *J. Natl. Cancer Inst.* 72:1323–1325.

29. Kensler, T. W., and M. A. Trush. 1981. Inhibition of phorbol ester-stimulated chemiluminescence in human polymorphonuclear leukocytes by retinoic acid and 5,6 epoxyretinoic acid. *Cancer Res.* 41:216–222.

30. Witz, G., B. D. Goldstein, M. Amoruso, D. S. Stone, and W. Troll. 1980. Retinoid inhibition of superoxide anion radical production by human polymorphonuclear leukocytes stimulated with tumor promoters. *Biochem. Biophys. Res. Commun.* 97:883–888.

31. Fischer, S. M., and L. M. Adams. 1985. Suppression of tumor promoter induced chemiluminescence in mouse epidermal cells by several inhibitors of arachidonic acid metabolism. *Cancer Res.* 45:3130–3136.

32. DeJonge, H. R., and S. M. Lohmann. 1985. Mechanisms by which cyclic nucleotides and other intracellular mediators regulate secretion. *Ciba Found. Symp.* 112:116–138.

33. Weikel, C. S., J. J. Sando, and R. L. Guerrant. 1985. Stimulation of porcine jejunal ion secretion in vivo by protein kinase C activators. *J. Clin. Invest.* 76:2430–2435.

34. Chang, E. B., N. S. Wang, and M. C. Rao. 1985. Phorbol ester stimulation of active anion secretion in intestine. *Am. J. Physiol.* 249: C356–C361.

35. Fondacaro, J. D., and L. S. Henderson. 1985. Evidence for protein kinase C as a regulator of intestinal electrolyte transport. *Am. J. Physiol.* 249:G422–G426.

36. McCaffrey, P. G., B. A. Friedman, and M. R. Rosner. 1984. Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 259:12502–12507.

37. Sahyoun, N., H. LeVine III, R. McConnell, D. Bronson, and P. Cuatrecasas. 1983. A specific phosphoprotein phosphatase acts on histone H1 phosphorylated by protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80: 6760–6764.

38. Kikkawa, U., Y. Takai, Y. Tanaka, R. Miyake, and Y. Nishizuka. 1983. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.* 258:11442–11445.

39. Drust, D. S., and T. F. J. Martin. 1985. Protein kinase C translocates from cytosol to membrane upon hormone activation: effects of thyrotropin-releasing hormone in GH_3 cells. *Biochem. Biophys. Res. Commun.* 128:531–537.

40. Wooten, M. W., and R. W. Wrenn. 1985. Redistribution of phospholipid/calcium-dependent protein kinase and altered phosphorylation of its soluble and particulate substrate proteins in phorbol ester-treated rat pancreatic acini. *Cancer Res.* 45:3912–3917.

41. Kishimoto, A., Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka. 1980. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* 255:2273–2276.

42. Maenz, D. D., and G. W. Forsyth. 1982. Ricinoleate and deoxycholate are calcium ionophores in jejunal brush border vesicles. *J. Membr. Biol.* 70:125–133.

43. Rittenhouse, S. E. 1984. Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclooxygenase products and ADP. *Biochem. J.* 222:103–110.

44. Irvine, R. F., A. J. Lechter, and R. M. C. Dawson. 1984. Phosphatidylinositol-4,5-bisphosphate phosphodiesterase and phosphomonoesterase activities of rat brain. *Biochem. J.* 218:177–185.

45. Takenawa, T., and Y. Nagai. 1981. Purification of phosphatidylinositol-specific phospholipase C from rat liver. *J. Biol. Chem.* 256: 6769–6775.

46. Armato, U., P. G. Andreis, and F. Romano. 1984. Exogenous Cu, Zn-superoxide dismutase suppresses the stimulation of neonatal rat hepatocytes' growth by tumor promoters. *Carcinogenesis.* 5:1547–1555.

47. Kensler, T. W., D. M. Bush, and W. J. Kozumbo. 1983. Inhibition of tumor promotion by a biomimetic superoxide dismutase. *Nature (Lond.).* 221:75–77.

48. Gerard, C., L. C. McPhail, A. Marfat, N. P. Stimler-Gerard, D. A. Bass, and C. E. McCall. 1986. Role of protein kinases in stimulation of human polymorphonuclear leukocyte oxidative metabolism by various agonists. *J. Clin. Invest.* 77:61–65.

49. Taffet, S. M., A. R. L. Greenfield, and M. K. Haddox. 1983. Retinal inhibits TPA activated calcium dependent phospholipid dependent protein kinase. *Biochem. Biophys. Res. Commun.* 114:1194–1199.