1,25-Dihydroxyvitamin D₃ and 12-O-Tetradecanoyl Phorbol 13-Acetate Cause Differential Activation of Ca²⁺-dependent and Ca²⁺-independent Isoforms of Protein Kinase C in Rat Colonocytes

Marc Bissonnette, Ramesh K. Wali, Susanne C. Hartmann, Sharon M. Niedziela, Hemant K. Roy, Xiao-Ying Tien, Michael D. Sitrin, and Thomas A. Brasitus

Department of Medicine, University of Chicago, Chicago, Illinois 60637

Abstract

Considerable evidence that alterations in protein kinase C (PKC) are intimately involved in important physiologic and pathologic processes in many cells, including colonic epithelial cells, has accumulated. In this regard, phorbol esters, a class of potent PKC activators, have been found to induce a number of cellular events in normal or transformed colonocytes. In addition, our laboratory has demonstrated that the major active metabolite of vitamin D₃, 1,25(OH)₂D₃, also rapidly (seconds–minutes) activated PKC and increased intracellular calcium in isolated rat colonocytes. These acute responses, however, were lost in vitamin D deficiency and partially restored with the *in vivo* repletion of 1,25(OH)₂D₃. The Ca²⁺-independent or novel isoforms of PKC expressed in the rat colon and the isoform-specific responses of PKC to acute treatment with phorbol esters or 1,25(OH)₂D₃ have not been previously characterized. Moreover, the effects of vitamin D status on PKC isoform expression, distribution, and response to agonists are also unknown. In the present experiments, in addition to PKC- α , rat colonocytes were found to express the novel isoforms δ , ϵ , and ζ by Western blotting using isoform-specific PKC antibodies. The tumor-promoting phorbol ester, 12-O-tetradecanoyl phorbol 13-acetate, caused time- and concentration-dependent translocations of all these isoforms except PKC- ζ . In vitamin D deficiency, there were no alterations in colonic PKC isoform expression but significant changes in the subcellular distribution of PKC- α , δ , and ζ . Acute treatment of colonocytes from D-sufficient, but not D-deficient, rats with 1,25(OH)₂D₃ caused a rapid transient redistribution of only PKC- α from the soluble to the particulate fraction. The alterations in PKC isoform distribution and PKC- α responsiveness to 1,25(OH)₂D₃ in vitamin D deficiency were partially, but significantly, restored with 5–7 d *in vivo* repletion of this secosteroid. Both 12-O-tetradecanoyl phorbol 13-acetate and 1,25(OH)₂D₃ activated endogenous PKC, as assessed by inhibition of myristoylated alanine-rich C kinase substrate back-phosphorylation by exogenous

PKC. These studies indicate that PKC- α , δ , and/or ϵ likely mediate important phorbol ester-stimulated events described in the rat colon. In contrast, PKC- α is implicated in the rapid (s–min) PKC-dependent events initiated by 1,25(OH)₂D₃ in rat colonocytes. (*J. Clin. Invest.* 1995; 95:2215–2221.) Key words: PKC • calcitriol • phorbol ester • vitamin D deficiency • myristoylated alanine-rich C kinase substrate

Introduction

Protein kinase C (PKC)¹ consists of a family of lipid-dependent serine/threonine kinases that are involved in regulating cell growth and differentiation in many organs, including the colon (1, 2). Molecular cloning analysis has demonstrated that protein kinase C constitutes a family of at least 10 isozymes, including Ca²⁺-dependent (α , $\beta_{1,II}$, and γ) and Ca²⁺-independent or novel isoforms (δ , ϵ , η , λ , θ , and ζ) (3, 4). Although the PKC isozymes are homologous, sharing highly conserved domains, they differ in tissue expression, subcellular distribution, substrate specificity, and activating cofactor requirements, suggesting they have different functions (3, 5). In this regard, Craven and DeRubertis (6) have recently demonstrated that the normal rat colon possesses PKC- α but not β or γ .

In recent years the possibility of using dietary anticarcinogens as a strategy to prevent colon cancer has also been recognized (7). One such potential colonic anticarcinogen, vitamin D₃, and particularly its major biologically active metabolite, 1,25(OH)₂D₃, has received increased attention in this regard. In addition to its well-established role in regulating mineral homeostasis, 1,25(OH)₂D₃ has been shown to inhibit proliferation and induce differentiation in a number of normal and transformed cells (8), including cells of the large intestine (9). Other studies, using experimental models of colon cancer, have demonstrated that alterations in the vitamin D status of rats can influence the colonic malignant transformation process in this organ (10–12).

During the past several years, in an attempt to further elucidate the roles of vitamin D in the physiologic regulation and pathophysiologic derangements of important cellular processes in the colon, our laboratory has studied the effects of 1,25(OH)₂D₃ and other metabolites of vitamin D₃ in rat colonic epithelial cells and in Caco-2 cells, a human colon cancer-derived cell line. We have demonstrated that 1,25(OH)₂D₃ rapidly (seconds–minutes) stimulated membrane polyphosphoinositide hydrolysis, increased intracellular calcium, and translo-

Address correspondence to Thomas A. Brasitus, Department of Medicine, GI Section, University of Chicago, 5841 S. Maryland Avenue, MC 4076, Chicago, IL 60637. Phone: 312-702-9898; FAX: 312-702-2182.

Received for publication 19 September 1994 and in revised form 12 December 1994.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/95/05/2215/07 \$2.00

Volume 95, May 1995, 2215–2221

1. Abbreviations used in this paper: MARCKS, myristoylated alanine-rich C kinase substrate; M_r , relative molecular weight; PKC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol 13-acetate.

cated PKC from the soluble to the particulate fraction in both rat colonic epithelia (13) and Caco-2 cells (14, 15). Furthermore, we have described 1,25(OH)₂D₃-induced alterations in cellular proliferation and Na⁺–H⁺ exchange in these cells (13, 14, 16). Moreover, all these secosteroid-initiated events were lost in colonocytes prepared from vitamin D–deficient animals and partially restored by the in vivo repletion of 1,25(OH)₂D₃ (16). These derangements accompanying D deficiency are of particular interest since, as noted above, vitamin D status can influence the development of experimental colon cancer (11, 12).

While a number of studies have demonstrated phorbol ester– (17–19) or 1,25(OH)₂D₃-induced (13, 20) important events in normal or transformed colonocytes in association with PKC activation, the specific isoforms of PKC activated by these agents in these cells have not been established. In addition, an analysis of the Ca²⁺-independent isoforms of PKC in the normal rat colon has not been reported. In the present experiments, we have further characterized the PKC isoforms expressed in rat colonocytes and investigated the effects of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), as well as 1,25(OH)₂D₃, on these isoforms. We have also examined the effect of vitamin D status on the expression and distribution of these isoforms and their responsiveness to acute treatment with 1,25(OH)₂D₃ in rat colonocytes.

Methods

Materials. 1,25(OH)₂D₃ and 25(OH)D₃ were purchased from Steroids, Ltd. (Chicago, IL), and stock solutions were prepared in ethanol and maintained in the dark under N₂ at 4°C. 24,25(OH)₂D₃ was a gift from Dr. M. R. Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). TPA (4-β-TPA) was obtained from LC Services Corp. (Woburn, MA), and a stock solution was prepared in ethanol and stored at 4°C. Ammonium persulfate, *N,N,N,N'*-tetramethylethylenediamine, and 2-mercaptoethanol were obtained from Serva Biochemicals (Hauppauge, NY). Electrophoretic grade acrylamide, bisacrylamide, Tris, SDS, and prestained molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, CA). The *M_r* of these markers were 205,000 (myosin), 116,500 (β-galactosidase), 80,000 (BSA), and 49,500 (ovalbumin). Eastman Kodak Co. (Rochester, NY) supplied the film (X-OMAT™ AR). NP-40, 4-(2-aminoethyl)-benzenesulfonyl fluoride, and purified rat brain PKC (1,000 U/mg) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Purified rat brain PKC-α (1,500 U/mg) and isoform-specific monoclonal antibodies to PKC-α and PKC-β were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antipeptide antibodies to PKC-γ, -δ, -ε, and -ζ were obtained from Gibco BRL (Gaithersburg, MD). Polyvinylidene difluoride membranes (Immobilon-P) were obtained from Millipore Corp. (Bedford, MA). The enhanced chemiluminescence kit for Western blotting protein detection and the peroxidase-coupled sheep anti-mouse and donkey anti-rabbit antibodies were supplied by Amersham Corp. (Arlington Heights, IL). Protein A–Sepharose, carbamylated isoelectric point standards, and ampholines were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Rabbit polyclonal antibodies to bovine myristoylated alanine-rich C kinase substrate (MARCKS) were generously provided by Dr. Angus Narin (The Rockefeller University, New York). Unless otherwise noted, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA) and were of the highest purity available.

Isolation of rat colonocytes. Male albino Sprague-Dawley rats, initially weighing 40–60 g, were obtained as weanlings from partially vitamin D–deficient mothers and placed on vitamin D–sufficient or vitamin D–deficient diets, respectively. The latter group was housed in a facility devoid of fluorescent light. All diets were purchased from ICN

Nutritional Biochemicals (Cleveland, OH) and have been described in detail previously (13, 16). After 15–20 wk, a time interval required to achieve vitamin D depletion [serum 25(OH)D₃ levels < 5 ng/mL], one half of the D-deficient animals received 10 ng/100 g body wt of 1,25(OH)₂D₃ by mini osmotic pumps implanted subcutaneously (2001-2002; Alza, Palo Alto, CA) for 7 d, whereas the others received vehicle in these pumps as described (16). Serum 25(OH)D₃ and 1,25(OH)₂D₃ levels were assayed as previously described by our laboratory (13, 16). For the experiments described below, animals weighing 250–300 g in all three groups were fasted overnight except for water ad lib. On the day of the experiment, animals were killed, their colons were removed, and colonocytes were harvested using a technique that combined chelation of divalent cations with mild mechanical dissociation as previously described (13). For each experiment, isolated colonocytes were pooled from three rat colons, collected by centrifugation at 500 g, and resuspended in KRB (pH 7.4) containing 1.2 mM CaCl₂ and 180 mg% glucose (KRBG). Trypan blue exclusion in ≥ 90% of cells was observed for at least 75 min after isolation.

Detection of PKC isoforms by Western blotting in rat colonocytes. Isoform-specific antibodies were used to identify PKC isoforms present in isolated rat colonocytes. The monoclonal antibodies, PKC-α and PKC-β, were prepared using as immunogens PKC-α and -β purified from rabbit brain cytosol. The antibodies reacted with rat PKC-α and -β, respectively, as identified by Western blotting of rat brain. The polyclonal antipeptide antibodies to PKC-γ, -δ, -ε, and -ζ were prepared from peptides with sequences deduced from isoform-specific rat brain cDNAs. Each of these antipeptide antibodies reacted in an isoform-specific manner with the respective rat PKC isoforms as identified by Western blotting of rat brain and elimination of immunoreactivity by preincubating the antibodies with the isoform-specific peptide used as immunogen.

To identify the PKC isoforms present in rat colonocytes and examine changes in their subcellular distributions in response to phorbol esters or 1,25(OH)₂D₃, isolated colonocytes (10⁷ cells/ml) were incubated at 37°C in 2 ml of KRBG with the indicated agent or vehicle (ethanol, final concentration < 0.05%). In the case of 1,25(OH)₂D₃, a concentration of 10 nM was chosen, as we have previously demonstrated significant activation of PKC in rat colonocytes at this concentration (13). At the indicated times the cells were rapidly chilled to 4°C by the addition of 10 vol of ice-cold KRBG and collected by centrifugation. Cells were disrupted by sonication on ice in 0.8 ml homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.5 mM benzamide, 5 mM 2-mercaptoethanol, and 10 mg/l leupeptin. After a low speed centrifugation, the supernatant was separated into soluble and particulate fractions by centrifugation at 100,000 g for 60 min. Aliquots of the soluble and particulate fractions were boiled in Laemmli SDS-containing buffer (21), and proteins were measured (22). Isoform-specific PKC expression was quantified as previously described (15). Rat brain homogenates (10 μg protein) were included as positive controls where appropriate. PKC isoforms in the particulate or soluble fractions were expressed as percentage of total (soluble + particulate) PKC isoform present. PKC isoform expression in colonic homogenates (50 μg protein) prepared from vitamin D–deficient or D-repleted animals was expressed as percentage of isoform-specific expression in homogenates from vitamin D–sufficient animals.

MARCKS immunoprecipitation and back-phosphorylation with exogenous PKC. Colonocytes were prepared as described above and aliquots (1 ml, 10⁷ cells/ml) incubated at 37°C with ethanol alone, (control, final concentration of ethanol 0.05%), or with 10 nM 1,25(OH)₂D₃, or 300 nM TPA. At the indicated times, cells were lysed by boiling in an SDS-containing buffer, and MARCKS was immunoprecipitated (400 μg protein incubated for 12 h at 4°C with 10 μl of polyclonal rabbit antibodies) as previously described (23). Immunoprecipitated MARCKS was back-phosphorylated for 30 min at 30°C as previously described (24), with minor modifications. Specifically, the kinase reaction (50 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 1 mM EGTA, 1.8 mM CaCl₂, 0.5 μg/μl phosphatidylserine, 0.03 μg/

μ l diolein, 0.01% BSA, 0.5% NP-40, 10 mM DTT, 25 μ M [γ - 32 P]ATP (1,500 cpm/pmol), and either 70 ng of purified rat brain PKC or 25 ng of hydroxyapatite-purified α -isoform of rat brain PKC. Reactions were stopped by the addition of 27 mg solid urea and 2.5 μ l 2-mercaptoethanol. Aliquots were resolved by SDS-PAGE and separate aliquots were separated by two-dimensional gel electrophoresis (25). 32 P incorporation into MARCKS was detected by autoradiography and quantitated by scanning densitometry or by Cherenkov counting.

Statistical analysis. Results are expressed as the mean $\pm$ SEM of n determinations. Data were analyzed using ANOVA or Student's t test for unpaired samples, as appropriate. Values of $P < 0.05$ were considered statistically significant.

Results

Rat colonocytes contain Ca^{2+} -sensitive and Ca^{2+} -insensitive PKC isoforms. The PKC isoforms expressed in vitamin D-sufficient rat colonocytes were the α , δ , ϵ , and ζ isoforms. We were, however, unable to detect PKC- β or γ , despite partial PKC purification by DEAE chromatography of the 100,000-g soluble and Triton X-100-extracted particulate fractions (13), which were then probed for these isoforms by Western blotting. Under basal conditions, PKC- α and ζ were predominantly soluble, with $\sim 70\%$ found in the soluble fraction. In contrast, PKC- δ and ϵ were largely particulate associated (Fig. 1).

PKC- α , δ , and ϵ redistribute in response to acute treatment with TPA. To examine the effects of phorbol esters on PKC isoforms in vitamin D-sufficient rat colonocytes, we investigated the time-dependent changes in PKC isoform distribution with TPA treatment. TPA (300 nM) caused translocations of soluble PKC- α , δ , and ϵ to the particulate fraction with no change in total isoform-specific PKC expression (Fig. 1). For each of these isoforms the redistribution was significant within 10 min, and by 30 min the particulate fraction of PKC- α , δ , and ϵ had increased from 29, 63, and 86% to 71, 96, and 100%, respectively (Fig. 1). PKC- ζ , in contrast, did not redistribute in response to TPA (Fig. 1). The effect of TPA was concentration dependent, with 300 and 1,000 nM, but not 10 or 100 nM, causing significant increases in the particulate associations of PKC- α , δ , and ϵ (data not shown). The higher concentrations of TPA (300–1,000 nM) required to translocate these PKC isoforms in colonocytes is consistent with the previously reported greater doses of phorbol esters necessary to cause physiological responses in intestinal-derived cells (26).

Vitamin D status modulates PKC isoform distribution, but not expression. In previous studies we have demonstrated that vitamin D deficiency abrogates a number of acute effects of 1,25(OH) $_2$ D $_3$ that occurred in response to this secosteroid in vitamin D-sufficient rat colonocytes (16). It was, therefore, of interest to examine the effect of vitamin D status on PKC isoform expression and distribution. In agreement with prior studies from our laboratory, animals raised on vitamin D-sufficient or vitamin D-deficient diets were found to have comparable body weights (13, 16). Moreover, 1,25(OH) $_2$ D $_3$ repletion was not associated with any changes in body weight (data not shown). Serum 25(OH)D $_3$ levels were, however, almost undetectable in the vitamin D-deficient group (< 5 ng/ml), and serum 1,25(OH) $_2$ D $_3$ levels (22 ± 4 pg/ml, $n = 6$) were significantly lower, compared to vitamin D-sufficient (96 ± 13 pg/ml, $n = 6$, $P < 0.05$) or 1,25(OH) $_2$ D $_3$ -repleted rats (101 ± 17 pg/ml, $n = 6$, $P < 0.05$).

Neither the qualitative nor the quantitative expression of colonic PKC isoforms was altered by vitamin D deficiency or

B

Figure 1. TPA causes time-dependent redistributions of PKC- α , δ , and ϵ , but not ζ , in rat colonocytes. Colonocytes were treated with vehicle (ethanol) or 300 nM TPA, and at the indicated times cells were rapidly cooled and soluble and particulate fractions were prepared as described in Methods. (A) Western blot of PKC- α , δ , ϵ , and ζ in the particulate fractions from a representative experiment performed in duplicate. Lanes 1 and 2, vehicle treated (0'). Lanes 3–10, TPA treated for the indicated times: lanes 3 and 4 (5'), lanes 5 and 6 (10'), lanes 7 and 8 (15'), lanes 9 and 10 (30'). (B) Particulate isoform-specific PKC is expressed as the percentage of total PKC- α , δ , or ϵ immunoreactivity in the cells incubated with vehicle alone. Values given are the mean $\pm$ SEM for three independent experiments performed in duplicate. a , $P < 0.05$; b , $P < 0.005$, compared with vehicle-treated samples. The distribution of PKC- ζ did not change in response to TPA.

vitamin D repletion (data not shown). Vitamin D deficiency did, however, cause a significant redistribution ($P < 0.005$) of PKC- α and ζ from the soluble to particulate fraction (Fig. 2). In contrast, vitamin D deficiency was accompanied by a significant translocation ($P < 0.05$) of PKC- δ from the particulate to soluble fraction (Fig. 2), with a similar trend that did not reach significance in the case of PKC- ϵ . The *in vivo* repletion with 1,25(OH) $_2$ D $_3$ caused a redistribution of PKC- α , δ , and ζ , compared with vitamin D-deficient animals, partially restoring their distributions to those in the D-sufficient condition (Fig. 2).

Vitamin D status modulates PKC- α redistribution in re-

Figure 2. Vitamin D status exerts differential effects on the distributions of PKC isoforms. Colonocytes were isolated from vitamin D-sufficient or -deficient rats, or vitamin D-deficient rats that were repleted in vivo with $1,25(\text{OH})_2\text{D}_3$ for 5–7 d as described in Methods (*D-repleted*). Subcellular fractions were prepared and assayed for PKC isoform expression as described in Methods. (A) Representative Western blots of PKC- α , - δ , - ϵ , and - ζ distributions in soluble (S) and particulate (P) fractions from vitamin D-sufficient (control), vitamin D-deficient, and vitamin D-repleted rats. (B) Quantitative effect of vitamin D status on PKC- α , - δ , - ϵ , and - ζ subcellular distributions. Data are means $\pm$ SEM of three independent experiments. *a*, $P < 0.005$; *b*, $P < 0.05$, compared with D-sufficient animals; *c*, $P < 0.005$; *d*, $P < 0.05$, compared with D-deficient animals.

sponse to acute treatment with $1,25(\text{OH})_2\text{D}_3$. We next examined the effect of $1,25(\text{OH})_2\text{D}_3$ on the subcellular distributions of the PKC isoforms in colonocytes prepared from vitamin D-sufficient, vitamin D-deficient, and vitamin D-repleted rats. In colonocytes prepared from vitamin D-sufficient rats, $1,25(\text{OH})_2\text{D}_3$ (10 nM, final concentration) caused a rapid, but transient, time-dependent translocation of PKC- α from the soluble to the particulate fraction, with the secosteroid causing a significant ($P < 0.05$) increase in particulate PKC- α from 60 to 90 s with no change in total PKC- α expression (Fig. 3). By 90 s, the particulate-associated PKC- α had increased from 29 to 57%, nearly a twofold increase, and by 120 s the distribution of PKC- α had returned to basal levels. There was no change in

Figure 3. Vitamin D status modulates the ability of $1,25(\text{OH})_2\text{D}_3$ to stimulate PKC- α redistribution. Colonocytes were prepared from vitamin D-sufficient or -deficient rats, or from animals repleted in vivo with $1,25(\text{OH})_2\text{D}_3$ for 5–7 d. After treatment with vehicle (ethanol) or 10 nM $1,25(\text{OH})_2\text{D}_3$ for the indicated times, cells were rapidly cooled and PKC isoforms were detected in the subcellular fractions as previously described. (A) Representative Western blots of PKC- α in the particulate fraction of colonocytes from D-sufficient, D-deficient, or D-repleted rats as described in Methods. Lanes 1 and 2, vehicle treated (0s). Lanes 3–10, $1,25(\text{OH})_2\text{D}_3$ treated for the indicated times: lanes 3 and 4 (30s), lanes 5 and 6 (60s), lanes 7 and 8 (90s), lanes 9 and 10 (120s), and lanes 11 and 12 (300s). (B) Particulate PKC- α from vitamin D-sufficient (■), D-deficient (□), and vitamin D-repleted rats (●); * $P < 0.05$, compared with vehicle-treated samples from appropriate D status controls. Data are expressed as the percentage of total PKC- α immunoreactivity. Values given are the mean $\pm$ SEM for three independent experiments performed in duplicate. The distributions of PKC- δ , - ϵ , and - ζ were not altered by treatment with $1,25(\text{OH})_2\text{D}_3$ in colonocytes from D-sufficient, D-deficient, or D-repleted rats.

PKC- α distribution in response to vehicle (ethanol) alone at all time points (data not shown). In contrast, PKC- δ , - ϵ , and - ζ did not redistribute in response to $1,25(\text{OH})_2\text{D}_3$ (data not shown). The effect of $1,25(\text{OH})_2\text{D}_3$ was specific, as the vitamin D₃ metabolites, $25(\text{OH})\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$, at concentrations as high as $1 \mu\text{M}$ did not influence the distributions of PKC- α , - δ , - ϵ , or - ζ (data not shown). In contrast, in colonocytes prepared from vitamin D-deficient rats, $1,25(\text{OH})_2\text{D}_3$ failed to alter the distribution of PKC- α (Fig. 3) or any of the other PKC isoforms. Repletion with $1,25(\text{OH})_2\text{D}_3$ partially, but significantly, restored responsiveness of PKC- α to this secosteroid (Fig. 3).

Treatment of intact colonocytes with TPA or $1,25(\text{OH})_2\text{D}_3$ inhibits the back-phosphorylation of immunoprecipitated MARCKS by exogenous PKC. We examined the effect of TPA or $1,25(\text{OH})_2\text{D}_3$ on the phosphorylation of MARCKS, a major substrate for PKC, using a back-phosphorylation assay pre-

Figure 4. Pretreatment of intact colonocytes with TPA or $1,25(\text{OH})_2\text{D}_3$ inhibits immunoprecipitated MARCKS back-phosphorylation by exogenous PKC. Isolated colonocytes prepared from vitamin D-sufficient rats were treated with vehicle (ethanol), 300 nM TPA, or 10 nM $1,25(\text{OH})_2\text{D}_3$ for the indicated times. The cells were lysed in SDS-containing buffer and, after immunoprecipitation and phosphorylation by exogenous rat brain PKC, MARCKS was separated by two-dimensional gel electrophoresis as described in Methods. Shown in the figure are representative autoradiograms of two independent experiments. Similar results were obtained with exogenous PKC- α purified by hydroxyapatite from rat brain.

viously described for MARCKS (24). In a back-phosphorylation assay, agonist pretreatment, which inhibits subsequent substrate phosphorylation by an exogenous kinase, is interpreted to reflect agonist-stimulated phosphorylation of the substrate in intact cells with unlabeled phosphate. Isolated colonocytes were treated with vehicle (ethanol, 0.05% final concentration), 300 nM TPA, or 10 nM $1,25(\text{OH})_2\text{D}_3$ for the indicated times, reactions were stopped, and MARCKS was isolated by immunoprecipitation as described in Methods. After back-phosphorylation, MARCKS was resolved by SDS-PAGE or two-dimensional gel electrophoresis (25, 27). The ^{32}P -labeled immunoprecipitate in these back-phosphorylation experiments was indeed colonic MARCKS as assessed by four criteria: (a) immunoprecipitation with MARCKS-specific antisera (23); (b) ability to serve as a PKC substrate (27); (c) M_r 82,000 (27); and (d) acidic pI, 4.3 (27). In three independent experiments, pretreatment with either TPA, a known activator of PKC, or $1,25(\text{OH})_2\text{D}_3$ inhibited PKC back-phosphorylation of MARCKS immunoprecipitated from rat colonocytes. Treatment with 300 nM TPA for 5 min caused a $68.7 \pm 8.6\%$ inhibition ($P < 0.005$), compared with vehicle-treated controls (Fig. 4). Treatment of intact colonocytes with 10 nM $1,25(\text{OH})_2\text{D}_3$ caused a time-dependent decrease in MARCKS back-phosphorylation that was significant by 90 s and persisted for at least 180 s (Fig. 5). By 180 s, pretreatment with $1,25(\text{OH})_2\text{D}_3$ caused a $56 \pm 10\%$ inhibition in MARCKS back-phosphorylation (Figs. 4 and 5).

Discussion

The present studies have demonstrated for the first time that rat colonocytes express the Ca^{2+} -independent PKC isoforms δ , ϵ , and ζ . Our studies confirm as well the earlier findings that, of the Ca^{2+} -dependent PKC isoforms, only PKC- α is expressed (6). Acute treatment of rat colonocytes with TPA increased the particulate association of PKC- α , $-\delta$, and $-\epsilon$ for at least 30 min. In contrast, PKC- ζ did not redistribute with TPA. The unresponsiveness of PKC- ζ to TPA treatment may reflect primary structural features in the C1 domain, the phorbol ester binding region (28). Previous studies have shown that phorbol esters, presumably through PKC activation, alter several important processes in normal rat colonic epithelium, including Na^+ and Cl^- transport (17) and particulate guanylate cyclase

activity (18). Based on our present findings, PKC- α , $-\delta$, and/or $-\epsilon$ would appear to be involved in these phorbol ester-induced events.

Previous studies from our laboratory have demonstrated that there is a redistribution of PKC biochemical activity from the soluble to the particulate fraction in colonocytes obtained from vitamin D-deficient rats, but no change in total activity (16). In the present studies we have confirmed these observations, noting that, for each of the isoforms, total expression is unchanged in colonocytes from D-deficient rats. We have found, however, isoform-specific redistributions, with predominantly soluble isoforms moving to the particulate fraction and largely particulate-associated isoforms increasing their soluble distribution in D-deficiency. In addition to the effect of vitamin D status on basal PKC distribution, vitamin D status has been found to

Figure 5. $1,25(\text{OH})_2\text{D}_3$ treatment of intact colonocytes causes a time-dependent decrease in the back-phosphorylation of MARCKS by exogenous PKC. Colonocytes from vitamin D-sufficient rats were isolated and treated with vehicle (ethanol) or 10 nM $1,25(\text{OH})_2\text{D}_3$ for the indicated times. The cells were lysed in SDS-containing buffer and, after immunoprecipitation and phosphorylation by exogenous rat brain PKC, MARCKS was separated by SDS-PAGE as described in Methods. Values are means $\pm$ SEM from three experiments. * $P < 0.05$, compared with vehicle-treated colonocytes.

influence the response of isolated colonocytes to acute treatment with $1,25(\text{OH})_2\text{D}_3$. In particular, in colonocytes obtained from D-sufficient but not D-deficient rats, $1,25(\text{OH})_2\text{D}_3$ increases diacylglycerol and intracellular calcium and causes a translocation of PKC biochemical activity from the cytosol to the membrane (13, 16). The present studies have confirmed and extended these observations, identifying PKC- α as the isoform responsive to acute treatment with $1,25(\text{OH})_2\text{D}_3$ and dependent on D status for activation by this secosteroid. Furthermore, these observations are in agreement with our recent findings that $1,25(\text{OH})_2\text{D}_3$ specifically activated PKC- α in Caco-2 cells (15). The transient effect of $1,25(\text{OH})_2\text{D}_3$ on PKC- α distribution, in contrast to the more persistent changes in the distribution of this isoform in response to TPA, is consistent with the known differences in rates of metabolism of diacylglycerol and TPA. The mechanism by which vitamin D deficiency causes a loss of PKC- α responsiveness to acute treatment with $1,25(\text{OH})_2\text{D}_3$ is uncertain. In this regard, however, the recent identification of a putative membrane receptor for $1,25(\text{OH})_2\text{D}_3$ in basal lateral membranes of chick intestinal epithelium suggests that vitamin D deficiency may lead to changes at the membrane receptor or postreceptor level (29).

As noted above, $1,25(\text{OH})_2\text{D}_3$ -induced increases in diacylglycerol and free intracellular calcium trigger PKC- α translocation, an event widely accepted as an indicator of PKC activation (30). PKC translocation to the membrane is not, however, necessarily required for activation (31). To further confirm that $1,25(\text{OH})_2\text{D}_3$ activated PKC, we therefore examined the effect of pretreatment of intact colonocytes with this secosteroid on the back-phosphorylation of MARCKS, a major substrate of PKC (27, 32). Phosphorylation of MARCKS is widely accepted as a useful indicator of PKC activation in intact cells (23, 27), although recent studies indicate that it serves as a substrate for proline-directed protein kinases such as microtubule-associated protein kinases and Cdk5 as well (33). Inhibition of exogenous PKC back-phosphorylation of MARCKS should be a reliable indicator of MARCKS phosphorylation by endogenous PKC, as the phosphorylation sites of MARCKS by the endogenous or exogenous kinase appear to be identical (27). Both TPA and $1,25(\text{OH})_2\text{D}_3$ inhibited MARCKS back-phosphorylation by exogenous PKC, including purified PKC- α . These results not only confirmed PKC activation by these agents but for the first time demonstrated that MARCKS is phosphorylated in intact colonocytes in response to $1,25(\text{OH})_2\text{D}_3$ and TPA. Taken together with our findings of PKC- α redistribution by $1,25(\text{OH})_2\text{D}_3$ and TPA, this suggests PKC- α mediates MARCKS phosphorylation by this secosteroid, and perhaps by TPA. Moreover, as measured by MARCKS phosphorylation, effects of PKC- α activation persist for times longer than redistribution of this isoform and are in agreement with earlier studies (34).

MARCKS is an actin-cross-linking and calmodulin-binding protein that redistributes from the plasma membrane to the cytoplasm after phosphorylation by PKC (35, 36), and it may be involved in the $1,25(\text{OH})_2\text{D}_3$ -induced morphologic changes previously described by our laboratory in Caco-2 cells (9). It is tempting to speculate that one or more of the events caused by colonocyte stimulation by $1,25(\text{OH})_2\text{D}_3$, such as alterations in $\text{Na}^+ - \text{H}^+$ exchange (16), changes in calcium entry across the plasma membrane (13), and/or effects on cellular proliferation (13), may be regulated by calcium-dependent changes in MARCKS phosphorylation and/or calmodulin binding that me-

diates changes in the cytoskeleton. In this regard, we have previously shown that $\text{Na}^+ - \text{H}^+$ exchange in Caco-2 cells is regulated by calcium and calmodulin (14). MARCKS could, therefore, mediate calcium-sensitive reversible changes in actin assembly to regulate this exchange process.

The physiologic roles of specific PKC isoforms remain poorly defined. In the case of PKC- α , this isoform is ubiquitous, suggesting an essential role in the physiology of all cells (5). PKC- α has been implicated in a number of cellular functions (15, 37, 38), including our recent demonstration in Caco-2 cells that PKC- α , activated by $1,25(\text{OH})_2\text{D}_3$, limited the secosteroid-stimulated increase in free intracellular calcium (15) and activated particulate guanylate cyclase (38). Less is known regarding the regulation and functions of the Ca^{2+} -independent PKC isoforms δ , ϵ , and ζ , but, like the better-characterized classical isoforms, their effects are cell specific. We have recently demonstrated that PKC- ζ , an atypical member of the PKC family of isozymes implicated in regulating gene expression induced by growth factors and hormones (39, 40), is constitutively expressed in the nucleus of Caco-2 cells (41).

In summary, we have demonstrated that rat colonocytes express both Ca^{2+} -dependent (PKC- α) and Ca^{2+} -independent (PKC- δ , ϵ , and ζ) isoforms of PKC, with TPA causing translocations of all but PKC- ζ . Furthermore, vitamin D deficiency was accompanied by an increase in particulate-associated PKC- α and ζ , in contrast to an increase in the soluble fraction of PKC- δ , changes that were partially reversible with 5–7 d of in vivo repletion with $1,25(\text{OH})_2\text{D}_3$. Only PKC- α , the Ca^{2+} -regulated isoform, was activated by acute treatment with $1,25(\text{OH})_2\text{D}_3$, a response abrogated by vitamin D deficiency and partially restored by in vivo D repletion. Finally, treatment with either TPA or $1,25(\text{OH})_2\text{D}_3$ stimulated MARCKS phosphorylation, a potentially important event mediating cytoskeletal changes induced by these agents.

Acknowledgments

This research was supported in part by the following grants: 5P30DK26687 (Clinical Nutrition Research Unit), P30DK42086 (Digestive Diseases Research Core Center), and DK39573 and CA36745 (T. A. Brasitus) and DK02022 (M. Bissonnette) from the U.S. Public Health Service. T. A. Brasitus is the recipient of a MERIT Award (National Cancer Institute, National Institutes of Health).

References

- Craven, P. A., J. Pfanstiel, and F. R. DeRubertis. 1987. Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation and reactive oxygen formation by bile acids. *J. Clin. Invest.* 79:532–541.
- Guillem, J. G., C. A. O'Brian, C. J. Fitzner, M. D. Johnson, K. A. Forde, P. LoGerfo, and B. Weinstein. 1987. Studies on protein kinase C and colon carcinogenesis. *Arch. Surg.* 122:1475–1478.
- Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash. DC)*. 258:607–614.
- Dekker, L. V., and P. J. Parker. 1994. Protein kinase C: a question of specificity. *TIBS (Trends Biochem. Sci.)*. 19:73–77.
- Wetsel, W. C., W. A. Khan, I. Merchenthaler, H. Rivera, A. E. Halpern, H. M. Phung, A. Negro-Vilar, and Y. A. Hannun. 1992. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J. Cell Biol.* 117:121–133.
- Craven, P. A., and F. R. DeRubertis. 1992. Alterations in protein kinase C in 1,2-dimethylhydrazine induced colonic carcinogenesis. *Cancer Res.* 52:2216–2221.
- Mukhtar, H., and M. Athar. 1988. Dietary anticarcinogens and cancer prevention. *Cleavel. Clin. J. Med.* 55:507–508.
- Minghetti, P. P., and A. W. Norman. 1988. $1,25(\text{OH})_2$ -vitamin D₃ receptors:

- gene regulation and genetic circuitry. *FASEB. (Fed. Am. Soc. Exp. Biol.) J.* 2:3043–3053.
9. Halline, A. G., N. O. Davidson, S. F. Skarosi, M. D. Sitrin, C. Tietze, D. H. Alpers, and T. A. Brasitus. 1994. Effects of 1,25-dihydroxyvitamin D₃ on proliferation and differentiation of Caco-2 cells. *Endocrinology*. 134:1710–1717.
 10. Pence, B. C., and F. Buddingh. 1988. Inhibition of dietary fat-promoted colon carcinogenesis in rats by supplemental calcium or vitamin D₃. *Carcinogenesis (Oxf.)*. 9:187–190.
 11. Sitrin, M. D., A. G. Halline, C. Abrahams, and T. A. Brasitus. 1991. Dietary calcium and vitamin D modulate 1,2-dimethylhydrazine-induced colonic carcinogenesis in the rat. *Cancer Res.* 51:5608–5613.
 12. Belleli, A., S. Shany, J. Levy, R. Guberman, and S. A. Lamprecht. 1992. A protective role of 1,25-dihydroxyvitamin D₃ in chemically induced rat colon carcinogenesis. *Carcinogenesis (Oxf.)*. 13:2293–2298.
 13. Wali, R. K., C. L. Baum, M. D. Sitrin, and T. A. Brasitus. 1990. 1,25(OH)₂ vitamin D₃ stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. *J. Clin. Invest.* 85:1296–1303.
 14. Wali, R. K., C. L. Baum, M. J. G. Bolt, T. A. Brasitus, and M. D. Sitrin. 1992. 1,25-dihydroxyvitamin D₃ inhibits Na⁺-H⁺ exchange by stimulating membrane phosphoinositide turnover and increasing cytosolic calcium in Caco-2 cells. *Endocrinology*. 131:1125–1133.
 15. Bissonnette, M., X.-Y. Tien, S. M. Niedziela, S. C. Hartmann, B. P. Frawley, Jr., H. K. Roy, M. D. Sitrin, R. L. Perlman, and T. A. Brasitus. 1994. 1,25(OH)₂ vitamin D₃ activates PKC- α in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca²⁺]. *Am. J. Physiol.* 267:G465–G475.
 16. Wali, R. K., C. L. Baum, M. D. Sitrin, M. J. G. Bolt, P. K. Dudeja, and T. A. Brasitus. 1992. Effect of vitamin D status on the rapid actions of 1,25-dihydroxycholecalciferol in rat colonic membranes. *Am. J. Physiol.* 262:G945–G953.
 17. Donowitz, M., H. Y. Cheng, and G. W. G. Sharp. 1986. Effects of phorbol esters on sodium and chloride transport in rat colon. *Am. J. Physiol.* 251:G509–G517.
 18. Khare, S., D. M. Wilson, X.-Y. Tien, R. K. Wali, M. Bissonnette, and T. A. Brasitus. 1994. Protein kinase C mediates the calcium-induced activation of rat colonic particulate guanylate cyclase. *Arch. Biochem. Biophys.* 314:200–204.
 19. Brandsch, M., Y. Miyamoto, V. Ganapathy, and F. H. Leibach. 1994. Expression and protein kinase C-dependent regulation of peptide/H⁺ co-transport in the Caco-2 human colon carcinoma cell line. *Biochem. J.* 299:253–260.
 20. Khare, S., D. M. Wilson, X.-Y. Tien, P. K. Dudeja, R. K. Wali, M. D. Sitrin, and T. A. Brasitus. 1993. 1,25-dihydroxycholecalciferol rapidly activates rat colonic particulate guanylate cyclase via a protein kinase C-dependent mechanism. *Endocrinology*. 133:2213–2219.
 21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacterial phage T4. *Nature (Lond.)*. 227:680–685.
 22. Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56:502–514.
 23. Aderem, A. A., K. A. Albert, M. M. Keum, J. K. T. Wang, P. Greengard, and Z. A. Cohn. 1988. Stimulus-dependent myristoylation of a major substrate for protein kinase C. *Nature (Lond.)*. 332:362–364.
 24. Hoffman, F. J., Jr., and R. A. Janis. 1993. Effects of calcium channel antagonists on the phosphorylation of major protein kinase C substrates in the rat hippocampus. *Biochem. Pharmacol.* 46:677–681.
 25. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
 26. Weikel, C. S., C. L. Spann, C. P. Chambers, J. K. Crane, J. Linden, and E. L. Hewlett. 1990. Phorbol esters enhance the cyclic GMP response of T84 cells to the heat-stable enterotoxin of *Escherichia coli* (STa). *Infect. Immun.* 58:1402–1407.
 27. Blackshear, P. J., L. Wen, B. P. Glynn, and L. A. Witters. 1986. Protein kinase C-stimulated phosphorylation in vitro of a M_r 80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts. *J. Biol. Chem.* 261:1459–1469.
 28. Burns, D. J., and R. M. Bell. 1991. Protein kinase C contains two phorbol ester binding domains. *J. Biol. Chem.* 266:18330–18338.
 29. Nemere, I., M. C. Dormanen, M. W. Hammond, W. H. Okamura, and A. W. Norman. 1994. Identification of a specific binding protein for 1 α ,25-dihydroxyvitamin D₃ in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J. Biol. Chem.* 269:23750–23756.
 30. Kraft, A. S., and W. B. Anderson. 1983. Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond.)*. 301:621–623.
 31. Grabarek, J., and J. A. Ware. 1993. Protein kinase C activation without membrane contact in platelets stimulated by bryostatin. *J. Biol. Chem.* 268:5543–5549.
 32. Albert, K. A., A. C. Nairn, and P. Greengard. 1987. The 87-kDa protein, a major specific substrate for protein kinase C: purification from bovine brain and characterization. *Proc. Natl. Acad. Sci. USA*. 84:7046–7050.
 33. Taniguchi, H., S. Manenti, M. Suzuki, and K. Titani. 1994. Myristoylated alanine-rich C kinase substrate (MARCKS), a major protein kinase C substrate, is an in vivo substrate of proline-directed protein kinase(s). *J. Biol. Chem.* 269:18299–18302.
 34. Trilivas, I., P. M. McDonough, and J. H. Brown. 1991. Dissociation of protein kinase C redistribution from the phosphorylation of its substrate. *J. Biol. Chem.* 266:8431–8438.
 35. Thelen, M., A. Rosen, A. C. Nairn, and A. Aderem. 1991. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. *Nature (Lond.)*. 351:320–322.
 36. Hartwig, J. H., M. Thelen, A. Rosen, P. A. Janney, A. C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature (Lond.)*. 356:618–622.
 37. Balboa, M. A., B. L. Firestein, C. Godson, K. S. Bell, and P. A. Insel. 1994. Protein kinase C α mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. *J. Biol. Chem.* 269:10511–10516.
 38. Khare, S., X.-Y. Tien, D. Wilson, R. K. Wali, B. M. Bissonnette, B. Scaglione-Sewell, M. D. Sitrin, and T. A. Brasitus. 1994. The role of protein kinase-C α in the activation of particulate guanylate cyclase by 1 α ,25-dihydroxyvitamin D₃ in Caco-2 cells. *Endocrinology*. 135:277–283.
 39. Dominguez, I., L. Sanz, F. Arenzana-Seisdedos, M. T. Diaz-Meco, J.-L. Virelizier, and J. Moscat. 1993. Inhibition of protein kinase C ζ subspecies blocks the activation of an NF- κ B-like activity in *Xenopus laevis* oocytes. *Mol. Cell. Biol.* 13:1290–1295.
 40. Sanz, L., E. Berra, M. M. Municio, I. Dominguez, J. Lozano, T. Johansen, J. Moscat, and M. T. Diaz-Meco. 1994. ζ PKC plays a critical role during stromelysin promoter activation by platelet-derived growth factor through a novel palindromic element. *J. Biol. Chem.* 269:10044–10049.
 41. Frawley, B. P., Jr., X.-Y. Tien, S. C. Hartmann, R. K. Wali, S. M. Niedziela, N. O. Davidson, M. D. Sitrin, T. A. Brasitus, and M. Bissonnette. 1994. TPA causes divergent responses of Ca²⁺-dependent and Ca²⁺-independent isoforms of PKC in the nuclei of Caco-2 cells. *Biochim. Biophys. Acta*. 1222:301–305.