The regulation of protein kinase C in the regulation of Na+/K+/Cl- cotransport was investigated in cultured HT29 human colonic adenocarcinoma cells. We have demonstrated previously the presence of a Na+/K+/Cl- cotransport pathway in HT29 cells (Kim, H. D., Tsai, Y.-S., Franklin, C. C., and Turner, J. T. (1989) Biochim. Biophys. Acta 946, 397-404). Treatment of cells with the phorbol esters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) caused an increase in membrane-associated protein kinase C activity that was accompanied by a concomitant decrease in cytosolic protein kinase C activity. PMA also produced a rapid transient increase in cotransport to 137% of control values by 5 min followed by a progressive decrease to 19% of control values by 2 h. To determine the underlying mechanism for the reduction in Na+/K+/Cl- cotransport, changes in cotransporter number and/or affinity were determined in radioligand binding studies using [3H]bumetanide. PMA and PDBu produced essentially identical time- and dose-dependent decreases in specific [3H]bumetanide binding that were similar to the observed decreases in cotransport. Analysis of saturation and competition binding data indicated that the decrease in binding was due to a lowered Bmax with no change in affinity. Both the decrease in binding and the changes in cotransport elicited by PMA were prevented by the protein kinase inhibitor H7. These findings suggest that phorbol esters cause a decrease in the number of cotransporters in HT29 cells, resulting in a reduction in Na+/K+/Cl- cotransport activity.

Na+/K+/Cl- cotransport has been well characterized in a variety of cells, is thought to play an important role in the transcellular movement of ions, and may be involved in the regulation of cell volume (2-4). We have shown previously the presence of a Na+/K+/Cl- cotransport pathway in HT29 human colonic adenocarcinoma cells, which is stimulated by increases in cyclic AMP and inhibited by divalent cations and by decreases in ATP levels (1).

Recently, phorbol esters and some mitogens have been shown to affect an ouabain-insensitive K+ transport pathway that exhibits many characteristics of a Na+/K+/Cl- cotransport system. Phorbol esters have been reported to alter cotransport activity in a differential manner depending on cell type and time of exposure. Phorbol 12-myristate 13-acetate (PMA), a potent phorbol ester, stimulates cotransport in hamster fibroblasts (5) while inhibiting cotransport in vascular smooth muscle (6) and Balb/c 3T3 cells (7, 8). Although these responses have been well characterized with respect to the kinetic properties of Na+/K+/Cl- cotransport, the molecular mechanisms by which stimulation or inhibition is achieved are poorly understood.

Tumor-promoting phorbol esters mimic the effects of the endogenous protein kinase C activator, diacylglycerol, in both intact cells (9, 10) and cell-free systems (11). Stimulation of protein kinase C activity in intact cells by phorbol esters is characterized by a rapid translocation of the protein kinase C enzyme from the cytosol to a stabilized membrane-bound form (9, 10). Phorbol esters modulate a variety of transport systems (12, 13) including hexose transport (14, 15), leucine transport (16, 17), phosphate transport (18), and chloride secretion (19, 20). Of particular interest is the stimulation of an amiloride-sensitive Na+/H+ exchange system, which produces cytoplasmic alkalization by extrusion of intracellular H+ (21). An increase in intracellular pH is a relatively consistent phorbol ester-induced response and is thought to play a role in the initiation of cell proliferation in response to phorbol esters and some mitogens (21). Efflux of H+ ions is accompanied by increased Na+ influx, leading to an increase in ouabain-sensitive K+ influx (21). The binding characteristics and number of putative Na+/K+/Cl- cotransporters in several tissues have been determined in binding studies utilizing radiolabeled loop diuretics. Binding of tritiated loop diuretics to intact cells (22) and cell membranes (23-25, 41) closely parallels Na+/K+/Cl- cotransport with respect to inhibition by loop diuretics and dependence on Na+, K+, and Cl-, suggesting that the sites labeled in binding studies are the sites mediating inhibition of cotransport.

In this study we report the results of our investigation on the regulation of Na+/K+/Cl- cotransport in HT29 cells by phorbol esters. [3H]Bumetanide binding assays were used to establish the correlation between changes in cotransport and in the number of putative cotransporters in response to phorbol ester treatment. Our results indicate that protein kinase C-activating phorbol esters produce concomitant decreases in [3H]bumetanide binding site density and Na+/K+/Cl- cotransport in HT29 cells.

The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; HT, 1-(5-isoquinolylsulfonyl)-2-methylpiperazine; PDBu, phorbol 12,13-dibutyrate; 4-O-methyl-PMA, phorbol 12-myristate 13-acetate 4-O-methyl ether; DMEM, Dulbecco’s modified Eagle’s medium; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; iC50, concentration at which 50% inhibition is observed.

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EXPERIMENTAL PROCEDURES

Cell Culture—HT29 cells, derived from a human colonic adenocarcinoma (28), were grown as described previously (1) in DMEM supplemented with 5% fetal bovine serum and 5% newborn bovine serum in 75-cm² tissue culture flasks in a humidified atmosphere of 5% CO₂:95% air. Confluent cultures were subcultured with 0.01% trypsin, 0.01% EDTA into either 60-mm culture dishes or 16-mm 24-well culture plates. Cells were seeded in DMEM supplemented with 2% fetal bovine serum and 5% newborn bovine serum at a density of 5 × 10⁶ cells/cm² and grown to confluence with the medium being replaced every other day. Confluent cultures were used on days 5–9 of growth with the final medium replacement being no longer than 24 h prior to use.

Protein Kinase C Assay—Cells in 6-mm dishes were washed and treated as stated in the figure legends in DMEM containing 20 mM HEPES, pH 7.4 (DMEM/HEPES). Cells were washed, scraped, and homogenized with a Tissuemiser (Tekmar Co., Cincinnati, OH) three times for 10 s at setting 90 in ice-cold lysis buffer (2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM β-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5, at 30°C). The cell lysate was centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected and designated as the cytosolic fraction. The resulting pellets were washed, preincubated, and/or treated as stated in the figure legends in DMEM/HEPES at 25°C. The pellets were washed, preincubated, and/or treated as stated in the figure legends in DMEM/HEPES at 25°C. The pellets were then incubated with 120 mM sodium gluconate, 25 mM potassium gluconate, 5 mM KCl, and 10 mM Tris-HCl, pH 7.4 and incubated at 25°C in 0.3 ml of binding buffer containing the indicated concentration of [3H] bumetanide (20–50 nM) unless otherwise stated. The absence or presence of unlabeled bumetanide or bumetanide. Binding was terminated by rapidly washing the cells three times with ice-cold 0.15 M NaCl. The cells were harvested as previously described, and bound radioactivity was quantitated by liquid scintillation spectroscopy (7/3 efficiency). Specific binding was calculated as the difference between binding in the absence (total binding) and presence (nonspecific binding) of 100 nM unlabeled bumetanide. Nonspecific binding was 10–30% of total binding. All assays were performed in duplicate or triplicate.

The equilibrium dissociation constant (Kd) and the number of binding sites (Bmax) for [3H]bumetanide were determined by saturation binding analysis. Cells were washed and treated as described above, then incubated for 45 min at 25°C in 0.2-0.3 ml of binding buffer containing various concentrations of [3H]bumetanide (0.01–1 μM) in the absence or presence of 100 μM unlabeled bumetanide. The Kd and Bmax of [3H]bumetanide binding were calculated by nonlinear regression of the untransformed data (bound versus added [3H] bumetanide) using the curve-fitting program GraphPAD (SD Software, Philadelphia, PA). Data were transformed and plotted by the method of Rosenthal (32).

For competition binding assays cells were washed and treated as stated above, and binding of 20–50 nM [3H]bumetanide was determined in the absence or presence of the indicated concentrations of unlabeled bumetanide or furosemide. The equilibrium dissociation constants (Kd) of unlabeled bumetanide and furosemide were calculated from Kd = IC50/(1 + F/Kd), where F is the concentration of [3H] bumetanide added, Kd is the equilibrium dissociation constant of [3H] bumetanide as determined by saturation binding analysis, and IC50 values are the concentrations of unlabeled loop diuretic that inhibited 50% of specific [3H] bumetanide binding as determined using the curve-fitting program BDA (EMF Software, Baltimore, MD). In the label and unlabeled loop diuretics were added in the presence of 5 μM ouabain and 5 μM furosemide. Values were calculated from the competition binding data from Bmax = (B0 × IC50)/F, where B0 is the amount of [3H] bumetanide bound/mg of protein.

Materials—[3H]Bumetanide (approximately 21 Ci/mmol) was the gracious gift of Dr. John Cuppoletti of the University of Cincinnati, OH. [3H]Furosemide (168 Ci/mmol) and [3H]PDPBu (10–20 Ci/ mmol) were purchased from Du Pont-New England Nuclear, and [3H]ATP (450 Ci/mmol) was obtained from International Chemical and Nuclear Radiochemicals (Irvine, CA). Furosemide was purchased from Aldrich Chemical Co., and bumetanide was a gift from Dr. Peter Sorter of Hoffman-LaRoche. Sera, trypsin, and 24-well culture plates were purchased from Gibco Laboratories, and DMEM was purchased from KC Biologics, Inc. (Lexena, KS). DEAE-cellulose (DE52 resin) was obtained from Whatman LabSales, Ltd. (Hillaboro, OR), and nitrocellulose filters were obtained from Millipore Corp. (Bedford, MA). The following chemicals and compounds were obtained from Sigma: phenylmethylsulfonyl fluoride, histone type III-S phosphatidylinerine, diolein, oleic acid, Triton X-100, PMA, 4-O-methyl-PMA, H7, 6.5 mM, NaOH, harvested, and neutralized with 1 M HCl. After the addition of 4 ml of Buffer-Solvent scintillation mixture cell-associated radioactive activity was quantitated by liquid scintillation spectrophotometry. Kᵢ influx was calculated as previously described (30) and expressed as nmol/(min × mg of protein). Protein was measured by the method of Lowry et al. (31). All experiments were performed in duplicate.

RESULTS

Redistribution and Depletion of Protein Kinase C in Response to Phorbol Esters—Stimulation of protein kinase C activity by phorbol esters in intact cells can be readily detected by a characteristic redistribution of enzyme activity from the cytosol to a membrane-bound fraction (3, 10). Treatment of HT29 cells with 1 μM PMA at 25°C stimulated a time-dependent increase in membrane-associated protein kinase C activity.
activity and a concomitant decrease in cytosolic activity (Fig. 1A, closed and open circles, respectively). A quantitatively similar redistribution of protein kinase C activity was seen in response to a 30-min treatment with 10 μM PDBu, whereas treatment with the biologically inactive PMA analog, 4-O-methyl-PMA (1 μM), had no effect on the translocation of protein kinase C activity (Fig. 1A, bars).

Although PMA elicited a rapid redistribution of protein kinase C activity, total cellular protein kinase C activity did not change appreciably upon exposure to PMA for up to 2 h at 25 °C (Fig. 1B). The stability of total cellular protein kinase C activity in response to a 2-h PMA treatment correlates well with the sum of the cytosolic and membrane-bound activities measured over the same period of time (Fig. 1A). Further treatment with PMA beyond 2 h at 37 °C, however, caused a progressive decrease in total cellular protein kinase C activity. Treatment for 12, 24, or 48 h caused a reduction in protein kinase C activity to 43, 18, and 10%, respectively, of the protein kinase C activity in untreated cells (Fig. 1B).

Effects of Phorbol Esters on \( \ce{Na+/K+/Cl-} \) Cotransport Activity—Ouabain-resistant bumetanide-sensitive \( \ce{^36Rb} \) influx has been characterized previously as \( \ce{Na+/K+/Cl-} \) cotransport activity in HT29 cells (1). Since biologically active phorbol esters stimulate protein kinase C activity in intact HT29 cells, it was of interest to determine whether increased protein kinase C activity influenced \( \ce{Na+/K+/Cl-} \) cotransport in HT29 cells. The effects of phorbol esters on \( \ce{Na+/K+/Cl-} \) cotransport are shown in Fig. 2. Incubation of cells with 1 μM PMA caused a rapid (within 5 min) transient increase in cotransport activity from 5.4 ± 0.6 nmol/(min × mg of protein) \((n = 9)\) in control cells to 7.3 ± 1.4 nmol/(min × mg of protein) \((n = 7)\) (Fig. 2A, closed circles). The transient increase in cotransport was followed by a progressive decrease to 3.59 ± 0.59 nmol/ (min × mg of protein) \((n = 8)\) after 30 min and to 1.04 ± 0.24 nmol/(min × mg of protein) \((n = 5)\) after 2 h of exposure to PMA. Addition of the protein kinase inhibitor, H7 (300 μM), to the pretreatment medium prevented both the transient stimulation and prolonged inhibition of cotransport elicited by PMA (Fig. 2A, open circles). H7 at 300 μM has been shown to completely inhibit the \textit{in vitro} protein kinase C activity isolated from both HT29 cells (data not shown) and rat brain (33). Treatment with the more hydrophilic phorbol ester, PDBu, over the same time period (30–120 min) produced results similar to those of PMA (Fig. 2A, triangles).

As shown in Fig. 2B, PMA and PDBu decreased \( \ce{Na+/K+/Cl-} \) cotransport in a dose-dependent manner. PMA inhibited cotransport with an \( IC_{50} \) of 170 ± 40 nM \((n = 4)\), whereas the \( IC_{50} \) for PDBu was 260 ± 150 nM \((n = 5)\). The inactive PMA analog, 4-O-methyl-PMA, which did not stimulate protein kinase C translocation in HT29 cells (Fig. 1A), had no effect on cotransport at concentrations up to 10 μM (Fig. 2B, squares).

Characteristics of \([3H]Bumetanide Binding to Intact HT29 Cells—\textit{Preliminary experiments indicated that maximal [3H]bumetanide binding to intact HT29 cells was obtained using a low Cl\textsuperscript−}, high K\textsuperscript+ binding buffer (15 mM Cl\textsuperscript−, 30 mM K\textsuperscript+, 120 mM Na\textsuperscript+) with isotonicity being maintained by replacement with gluconate. A representative time course of \([3H]bumetanide binding is shown in Fig. 3A. Specific \([3H]bumetanide binding (closed squares) reached steady state by 30 min and remained at this level for 2 h. An incubation of 45 min was used in all subsequent binding experiments. As shown in Fig. 3B, bumetanide and furosemide inhibited \([3H]bumetanide binding in intact HT29 cells with calculated \( K_i \) values of 0.13 ± 0.01 μM \((n = 5)\) and 3.1 ± 0.3 μM \((n = 3)\).
Both saturation and competition binding assays were used to determine the number and affinity of \([^3H]\)bumetanide binding sites in control and PMA-treated HT29 cells. For the saturation experiments, cells were treated with 1 \(\mu\)M PMA for either 30 min or 2 h, and specific \([^3H]\)bumetanide binding as a function of \([^3H]\)bumetanide concentration was determined. The results of a representative experiment are shown in Fig. 5A. While the affinity of \([^3H]\)bumetanide binding was unchanged, the number of binding sites decreased to 74 and 53% of the control value after exposure to 1 \(\mu\)M PMA for 30 min and 2 h, respectively (see Table I). PMA had no effect on nonspecific \([^3H]\)bumetanide binding (data not shown). Rosenthal plots of the saturation binding data were linear (Fig. 5B), suggesting that in both control and PMA-treated cells \([^3H]\)bumetanide labeled a single population of binding sites over the range of \([^3H]\)bumetanide concentrations used (0.01-1 \(\mu\)M).

Results from competition binding experiments performed on control and PMA-treated cells confirmed the results from the saturation binding experiments. A representative experiment is shown in Fig. 6. PMA caused a decrease in specific binding, whereas no change was observed in nonspecific binding. Again the binding affinity was not significantly altered, while the number of binding sites decreased to 73 and 46% of the control value in response to 30-min and 2-h PMA treatment, respectively (see Table I). Taken together, the results from the saturation and competition binding experiments clearly indicate that PMA induces a down-regulation of \([^3H]\)bumetanide binding sites, while not affecting binding affinity.

Reversibility of Phorbol Ester-induced Reductions in \(Na^+/K^+/Cl^-\) Cotransport and \([^3H]\)Bumetanide Binding—To determine whether the phorbol ester-induced effects were reversible, the recoveries of \(Na^+/K^+/Cl^-\) cotransport and \([^3H]\)bu-
98% of cell-associated [3H]PDBu from HT29 cells. The representative experiment performed in duplicate. Absence free [3H]bumetanide concentration. Data shown are from a representative experiment performed in duplicate. Kd and Bmax values were calculated as described under “Experimental Procedures” and are summarized in Table I. B, the saturation binding data presented in A are plotted by method of Rosenthal (32). The linearity of the transformed data indicates binding to a single population of binding sites.

**Table I**

| Treatment | Saturation analysis | Competition analysis |
|-----------|---------------------|----------------------|
|           | Bmax (pmol/mg) | Kd (nM) | Bmax (pmol/mg) | Kd (nM) | Bmax (pmol/mg) | Kd (nM) |
| Control   | 14.8 ± 0.17       | 106 ± 12 (7) | 1.54 ± 0.16 | 132 ± 15 (5) | 1.13 ± 0.20 | 184 ± 21 (5) |
| 30-min PMA| 1.10 ± 0.15       | 201 ± 62 (4) | 1.13 ± 0.20 | 184 ± 21 (5) | 1.13 ± 0.20 | 184 ± 21 (5) |
| 2-h PMA   | 0.78 ± 0.17       | 200 ± 53 (6) | 0.71 ± 0.13 | 206 ± 52 (5) | 0.71 ± 0.13 | 206 ± 52 (5) |

*Values are statistically different from control (p < 0.02, Wilcoxon rank sum test).

Bumetanide binding in phorbol ester-pretreated cells were determined. Due to its lipophilicity, PMA is difficult to remove from cells by washing. In an attempt to monitor the recoveries of cotransport and binding, the more hydrophilic compound, PDBu, was utilized. As shown above, PDBu and PMA produced similar effects on protein kinase C redistribution, Na+/K+/Cl- cotransport, and [3H]bumetanide binding (Figs. 1A, 2, and 4, respectively).

As shown in Fig. 7A, the wash procedure utilized removed >98% of cell-associated [3H]PDBu from HT29 cells. The recovery of Na+/K+/Cl- cotransport and [3H]bumetanide binding could thus be measured in cells pretreated with PDBu, then washed and maintained for the indicated time periods.
HT29 cells have been shown previously to possess a Na+/K+/Cl- cotransport pathway that is sensitive to divalent cations and intracellular cyclic AMP and ATP levels (1). In this study we examined the possible involvement of protein kinase C in the regulation of Na+/K+/Cl- cotransport in HT29 cells. The results presented here suggest that biologically active phorbol esters cause a decrease in the number of cotransporters in HT29 cells, resulting in a reduction in Na+/K+/Cl- cotransport activity.

Translocation of protein kinase C activity from the cytosol to a membrane fraction is characteristic of the initial response seen upon activation of protein kinase C in intact cells (9, 10). Approximately 80% of the protein kinase C activity in untreated HT29 cells was recovered in a cytosolic enzyme fraction. Phorbol esters shown in other systems to activate and translocate protein kinase C (PMA and PDBu, but not 4-O-methyl-PMA) induced a shift of protein kinase C activity to the membrane fraction of HT29 cells, thus demonstrating the presence of a phorbol ester-sensitive protein kinase C activity in these cells.

PMA caused a rapid (within 5 min) transient increase in Na+/K+/Cl- cotransport activity, followed by a time-dependent decrease in both cotransport and [3H]bumetanide binding site density. Long-term (>2 h) treatment of HT29 cells with PMA also produced a characteristic depletion of total cellular protein kinase C activity similar to that observed in 3T3 cells (34). However, it is important to note that Na+/K+/Cl- cotransport and [3H]bumetanide binding site density were both markedly reduced within 30 min, whereas no appreciable change in total cellular protein kinase C activity could be detected for up to 2 h (Fig. 1). These findings suggest that a stimulation of protein kinase C activity and not a down-regulation or desensitization of protein kinase C activity is involved in the phorbol ester-induced effects.

The biphasic effects of PMA on cotransport described here are consistent with previous reports of both a short time stimulation of cotransport in hamster fibroblasts (5) and a prolonged inhibition of cotransport in A7r5 smooth muscle cells (6) and Balb/c 3T3 cells (7, 8), although an immediate (within 5 min) rather than a delayed inhibition of cotransport was observed in Balb/c 3T3 cells (7). In this study we extend those results by demonstrating the involvement of protein kinase C in both the stimulation of cotransport and subsequent reductions in cotransport and [3H]bumetanide binding seen in response to PMA. An increase in protein kinase C activity, as detected by the translocation of protein kinase C activity, is apparently required for these responses in that 4-O-methyl-PMA, which did not cause a redistribution of protein kinase C activity (Fig. 1), was also ineffective in altering cotransport and [3H]bumetanide binding. The PMA effects on both cotransport (Fig. 2A) and binding (Fig. 2A) were also prevented by H7. Taken together these results indicate that a stimulation of protein kinase C activity is necessary for the phorbol ester-induced responses described here in HT29 cells.

While it appears that PMA decreases Na+/K+/Cl- cotransport in HT29 cells and other cell types, the molecular mechanisms by which inhibition is achieved are poorly understood. Pretreatment with phorbol esters causes the down-regulation and/or desensitization of a variety of receptor-second messenger systems (35-40). In HT29 cells, PMA and other protein kinase C activators desensitize the vasoactive intestinal peptide-receptor adenylate cyclase system in a dose-dependent manner (35). The recently available radioligand [3H]bumetanide has allowed examination of whether or not alterations in the number and/or binding affinity of putative Na+/K+/Cl- cotransporters were involved in the PMA-induced reduction in cotransport activity.

[3H]Bumetanide binding to intact HT29 cells was inhibited by loop diuretics with a rank order potency (bumetanide > furosemide, Fig. 3B) characteristic for inhibition of Na+/K+/Cl- cotransport in HT29 cells (1) and other cell types (2). A close correlation also exists between [3H]bumetanide binding site number and cotransport in mutant MDCK cells (41) and vascular smooth muscle cells from spontaneously hypertensive rats (43), both of which exhibit reduced Na+/K+/Cl- cotransport activity and [3H]bumetanide binding site density. Coordinate regulation of cotransport and binding site number has also been reported in response to both cell shrinkage and norepinephrine treatment of nucleated red blood cells (22), ATP depletion in MDCK cells (42), and 8-bromo-cyclic AMP treatment of vascular smooth muscle cells (43). Taken together, these findings suggest that specific [3H]bumetanide binding represents binding to the Na+/K+/Cl- cotransporter and can be used as an indicator of cotransporter number. Thus it seems reasonable that the PMA-induced decrease in cotransport in HT29 cells may be the direct result of a reduction in the number of cotransporters present as detected by the down-regulation of [3H]bumetanide binding sites.

Turnover numbers for the Na+/K+/Cl- cotransporter were calculated with the assumption of a single [3H]bumetanide binding site/cotransporter. Turnover numbers of 60, 56, and 26 K+ ions/(site x s) were calculated using Bmax values of 1.48, 1.06, and 0.67 pmol/mg protein (see Table I) and cotransport values of 5.36, 3.59, and 1.04 nmol/(min x mg of protein) for control HT29 cells and cells treated with PMA for 30 min and 2 h, respectively. The similarity of turnover numbers in control cells and cells treated with PMA for 30 min indicates that cotransporters in these cells differ only in number and not in function. Treatment with PMA for 2 h decreased the calculated turnover number by greater than 50%. However, caution must be taken in the interpretation of this reduction, as the inherent errors associated with this calculation are relatively large due to the low levels of cotransport and specific [3H]bumetanide binding after incubation with PMA for 2 h. The possible reduction in turnover number upon prolonged exposure to PMA suggests another, as yet undefined, inhibitory mechanism.

The time course of recoveries of Na+/K+/Cl- cotransport and [3H]bumetanide binding in PDBu-treated HT29 cells is similar to that of agonist-induced down-regulated β-adrenergic receptors in confluent 3T3 cells (44) and irreversibly blocked α1-adrenergic receptors in HT29 cells (45), both of which are thought to involve the synthesis of new receptors. Studies utilizing cycloheximide were performed to determine whether protein synthesis was involved in this recovery process in HT29 cells. However, cycloheximide was found to inhibit both cotransport and [3H]bumetanide binding in untreated HT29 cells. While the involvement of protein synthesis and/or cell growth in the recovery of Na+/K+/Cl- cotransport and [3H]bumetanide binding has not been documented, the phorbol ester-induced decreases are not readily reversible.

The results presented here suggest that phorbol esters decrease Na+/K+/Cl- cotransport in HT29 cells by reducing the number of cotransporters present. This study also further substantiates the involvement of protein kinase C in both the stimulatory and inhibitory phases of cotransport regulation in response to PMA. While it is still unclear how protein kinase C might reduce cotransporter number, an attractive

2. C. C. Franklin, J. T. Turner, and H. D. Kim, unpublished observation.
hypothesis would include the functional inactivation of the Na⁺/K⁺/Cl⁻ cotransporter by a protein kinase C-mediated phosphorylation reaction. This inactivation process could involve either a reduction in the synthesis or insertion of new transporters into the membrane or an increased degradation of the cotransporters present. In that several transport proteins have been shown to be substrates for protein kinase C phosphorylation both in vivo and in vitro (13), studies are currently in progress to determine whether the putative cotransporter protein, a 150-kDa protein (46), may be a substrate for protein kinase C phosphorylation. The possible regulation of cotransporter number and function through stimulation of protein kinase C activity has important implications with regard to the biological effects seen in response to various hormones, growth factors, and mitogens that stimulate the phospholipase C/protein kinase C signal transduction pathway.

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