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Research Article

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Carbachol- and Elevated Ca^{2+}-induced Translocation of Functionally Active Protein Kinase C to the Brush Border of Rabbit Ileal Na$^+$ Absorbing Cells

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Abstract

Protein kinase C is involved in mediating the effects of elevated Ca^{2+} in ileal villus Na$^+$ absorbing cells to inhibit NaCl absorption. The present studies were undertaken to understand the mechanism by which this occurs. The effects of carbachol and the calcium ionophore A23187, agents which elevate intracellular Ca^{2+} and inhibit NaCl absorption in ileal villus cells, were studied. Carbachol treatment of villus cells caused a rapid decrease in protein kinase C activity in cytosol, with an accompanying increase in microvillus membrane C kinase. Exposure of the villus cells to calcium ionophore also caused a quantitatively similar decrease in cytosol C kinase and increase in C kinase activity in the microvillus membrane. This increase caused by carbachol and Ca^{2+} ionophore was specific for the microvillus membrane. In fact, 30 s and 10 min after exposure of the cells to carbachol, basolateral membrane protein kinase C decreased, in a time-dependent manner; whereas 10 min of Ca^{2+} ionophore exposure did not alter basolateral C kinase. Exposure of villus cells to Ca^{2+} ionophore or carbachol caused similar increases in microvillus membrane diacylglycerol content. As judged by the ability to inhibit Na$^+$/H$^+$ exchange measured in ileal villus cell brush border membrane vesicles, the protein kinase C which translocated to the microvillus membrane was functionally significant. Inhibition of Na$^+$/H$^+$ exchange required ATP and was reversed by the protein kinase C antagonist H-7. In conclusion, the effect of carbachol and Ca^{2+} ionophore in regulation of ileal NaCl absorption is associated with an increase in microvillus membrane diacylglycerol content and functionally active protein kinase C. The effects of both carbachol and Ca^{2+} ionophore are different on brush border and basolateral membrane distribution of protein kinase C. (*J. Clin. Invest.* 1991. 88:855–863.) Key words: Ca^{2+} • protein kinase C • Na$^+$/H$^+$ exchange • Na$^+$ absorption

Introduction

Calcium plays a major regulatory role in Na$^+$ absorption by the rabbit ileum (1). Drugs or hormones which raise cytosol free

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Ca^{2+} levels ([Ca^{2+}]$_i$) in ileal Na$^+$ absorbing epithelial cells inhibit NaCl absorption, whereas those which lower [Ca^{2+}]$_i$ stimulate NaCl absorption (1). It is believed that the NaCl absorptive process is made up of two neutral exchangers, Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$, acting in parallel in the brush border membrane of ileal villus epithelial cells. Calcium regulates NaCl absorption by affecting the activity of the Na$^+$/H$^+$ exchanger (2). The regulation by Ca^{2+} is somewhat complex. Basal Na$^+$ absorption is regulated by an endogenous brush border membrane Ca^{2+}/calmodulin-dependent protein kinase, which is a form of CaM kinase II; however, the effects of elevating [Ca^{2+}]$_i$ above basal are not blocked by calmodulin inhibitors, including the naphthalenesulfonamide W13. Rather, the effects are mediated via protein kinase C and are reversed by the protein kinase C inhibitor, the isoquinolenesulfonamide H-7 (3).

The purpose of the present studies was to characterize the mechanism by which protein kinase C mediates the effect of elevated [Ca^{2+}]$_i$ on ileal NaCl absorption and Na$^+$/H$^+$ exchange. Activation of protein kinase C has been shown to be associated in some cells with translocation from cytosol to membranes (for examples, see references 4–7). In the present work, we investigated the effect of carbachol and the calcium ionophore A23187, two agents which inhibit ileal NaCl absorption by elevating [Ca^{2+}]$_i$, on protein kinase C translocation.

Methods

Distal ileum from 2–2.5-kg New Zealand White male rabbits was used for all experiments. Unless otherwise indicated, all procedures were carried out at 0–4°C. Rabbits were sacrificed by nembutal overdose. Ileum was removed and flushed free of its contents with 0.9% saline, followed by 0.9% saline containing 50 μg/ml phenylmethylsulfonylfluoride (PMSF), 25 μg/ml aprotinin, and 1 μM indomethacin. In most studies, ileal sheets were exposed in vitro to secretagogues, with biochemical studies done on lightly scraped ileal mucosa. In a few studies, isolated ileal epithelial cells were initially obtained and then exposed to secretagogues.

Scraped ileal mucosa. Ileal segments of ~ 20 cm were cut lengthwise and incubated for the indicated times in 200 ml of Ringer's-bicarbonate gassed with 95% O$_2$/5% CO$_2$ at 37°C, containing 1 μM indomethacin. The incubation medium contained either 1 μM carbachol, 1 μM calcium ionophore A23187, or an equivalent volume of Ringer's-bicarbonate (control). After incubation in Ringer's-bicarbonate the ileal segments were chilled, and mucosa was removed by lightly scraping with a glass slide. This procedure yields predominantly villus cells. Homogenization was with a polytron homogenizer in 10 vol of 2 mM DTT, 1 mM EGTA/1.006 mM CaCl$_2$ (free Ca^{2+} = 10 μM), 50 μg/ml PMSF, 1 μg/ml phosphoramidone, 25 μg/ml aprotinin, 0.01% leupeptin, 20 mM Tris, pH 7.5. The homogenization consisted of 10 10-s bursts with 20-s cooling intervals between bursts. The homogenate was centrifuged at 1,900 g for 10 min in a model SS-34 rotor (Sorvall Instru-

ments, Newton, CT). The pellet was used to prepare microvillus membranes by a modification of a previously described method (8) and the supernatant was used to prepare cytosol.

The pellets from the first centrifugation were resuspended in 2 mM DTT, 1 mM EGTA/1.006 mM CaCl₂, 20 mM Tris, pH 7.5, by homogenization with a Dounce homogenizer (20 strokes, loose pestle), and centrifuged at 1,075 g for 10 min. The pellets were resuspended by vortexing in the same buffer and washed three times at 1,075 g for 10 min before being resuspended in 250 mM sorbitol, 2 mM DTT, 1 mM EGTA/1.006 mM CaCl₂, 20 mM Tris, pH 7.5, and centrifuged at 25,000 g for 20 min. They were then resuspended in the same buffer by homogenization with a motor-driven glass-Teflon homogenizer for 1 min. MgSO₄ was added, with stirring, to the membrane suspension to a final concentration of 10 mM and the mixture was allowed to stand for 10 min and then centrifuged at 5,000 g for 10 min. The pellets were discarded and the supernatants were centrifuged at 25,000 g for 20 min. The pellets were then resuspended in the sorbitol buffer and the magnesium precipitation steps were repeated. The final pellets, representing microvillus membrane, were resuspended in 10% glycerol, 2 mM DTT, 20 mM Tris, pH 7.5, and stored at −80°C.

The supernatants from the initial centrifugation were centrifuged at 171,000 g for 1 h in a model 70Ti rotor (Beckman Instruments, Inc., Fullerton, CA). The pellets were discarded, and glycerol was added to the supernatants to a final concentration of 10%, and this cytosol fraction was stored at −80°C.

Purified basolateral membranes were prepared by a modification of the method of Scalera et al. (9). 4 g of scraped ileal mucosa were homogenized in 80 ml of 2 mM DTT, 1 mM EGTA, 1.006 mM CaCl₂, 50 mg/ml PMSF, 1 mg/ml phosphorhamidone, 0.01% leupeptin, 25 μg/ml aprotinin, 20 mM Tris, pH 7.5, with a polytron homogenizer (two 10-s bursts at top speed, six 10-s bursts at 70% of top speed). The homogenate was centrifuged at 750 g for 10 min and the pellets discarded. The supernatant was centrifuged at 23,400 g for 20 min. The fluffy layer of the pellets was resuspended in 250 mM sucrose, 2 mM DTT, 1 mM EGTA, 1.006 mM CaCl₂, 20 mM Tris, pH 7.5 (buffer A) by gentle swirling and homogenized with five strokes of a Dounce homogenizer. The suspension was again centrifuged at 23,400 g for 20 min and the fluffy layer of the pellet resuspended in buffer A containing 14% Percoll. This suspension was centrifuged at 47,800 g for 38 min in a Sorvall Instruments model SS-34 rotor and fractionated from the top by pumping 60% (wt/vol) sucrose to the bottom of the tube. 2 vol of buffer A containing 0.9% NaCl were added to each gradient fraction and the fractions centrifuged at 246,800 g for 1 h. Pellets were resuspended in 2 mM DTT, 10% glycerol, 20 mM Tris, pH 7.4, and stored at −80°C. The basolateral band was located by assaying each fraction for Na/K ATPase.

Isolated ileal villus cells. Because basolateral membranes prepared from scraped ileal mucosa could be contaminated with plasma membranes from nonepithelial cells, isolated villus cells were initially prepared and then both microvillus membranes and basolateral membranes purified. The dissociated ileal villus cells were prepared as we have described (10), using a modification of the citrate/distention technique of Weiser (11). The first three fractions were utilized, which correspond to the fractions having highest sucrase activity (10). The dissociated cells were pelleted by centrifugation at 1,200 g, 10 min, 4°C, resuspended in Ringer's-bicarbonate and preincubated with gassing with 95% O₂/5% CO₂ for 15 min. Carbachol was then added for 30 s or 10 min. The incubation was stopped by rapidly chilling the cells, then centrifuging the cells at 1,200 g, 10 min, 4°C, and resuspending the cells in the same homogenization buffer described for the scraped ileal mucosa studies. Microvillus and basolateral membranes were prepared from dissociated cells by the same methods used for scraped ileal mucosa, described above, except in preparation of basolateral membranes a Dounce homogenizer with loose pestle (20 strokes) was used instead of a polytron and the homogenization solution was pH 8.3.

Partial purification of protein kinase C. Leupeptin was added to aliquots of each subcellular fraction to a final concentration of 0.01% and Triton X-100 was added to a final concentration of 0.3%. The

mixtures were vortexed and incubated at 0°C for 30 min, and then centrifuged at ∼ 12,000 g in a microfuge for 10 min. The pellets were discarded and the supernatants were mixed with DE-52 cellulose (Whatman Inc., Clifton, NJ), by vortexing for 10 min. The gel slurries were centrifuged in the microfuge for 5 min and the supernatants discarded. The DE-52 fractions were washed three times with 0.5 mM EGTA, 5 mM EDTA, 50 mM DTT, 20 mM Tris, pH 7.5. The washed gel was then mixed with the same buffer containing 100 mM NaCl. The gel slurry was vortexed for 10 min and then centrifuged at approximately 12,000 g in a microfuge for 5 min. The gel was discarded and the supernatant used as the partially purified C kinase.

Protein kinase C assay. Protein kinase activity was determined by a histone IIIS phosphorylation assay and, in several studies, the amount of protein kinase C also was determined by phorbol dibutyrate binding.

Histone IIIS phosphorylation assay. The assay mixture contained 10 mM MgCl₂, 2 mg/ml histone IIIS, 1.1 mM CaCl₂, 1.0 mM EGTA (free Ca²⁺ = 100 μM), 40 μg/ml phosphatidylserine (PS), 10 μM phorbol dibutyrate, 5 μM γ[³²P]ATP (6.25 Ci/mol), 2 mM DTT, 0.01% leupeptin, 20 mM Tris, pH 7.5 in a total volume of 100 μl. The reaction was started by addition of the partially purified enzyme and allowed to proceed for 10 min at 30°C before termination by addition of 1 ml of 10% TCA, 0.2% sodium pyrophosphate, and boiling for 10 min. The precipitates were collected on Whatman Inc. GF/A filters and washed with 24 ml of 10% TCA, 0.2% sodium pyrophosphate, and radioactivity determined in a scintillation counter. C kinase activity was defined as the difference in [³²P]ATP incorporated in the presence and absence of phorbol dibutyrate and phosphatidylserine (PS), with Ca²⁺ present throughout. Protein kinase C activity was expressed in picomoles ³²P incorporated into histone per milligram membrane protein. Protein kinase C activity of the subcellular fractions was stable for at least 2 wk and assays were performed within 5 d of preparation.

Phorbol binding assay. Protein kinase C in microvillus membranes was also quantified by [³H]phorbol dibutyrate binding (12). Reaction tubes contained 100 μg of membrane protein from control or carbachol treated tissues, 4 × 10⁻⁸ M [³H]phorbol dibutyrate (12.4 Ci/mol), 75 mM Mg (acetate)₂, 5 mM CaCl₂, 1.2 mg/ml BSA, 96 μg/ml PS, and 20 mM Tris, pH 7.5, in a total volume of 500 μl. The reaction mixture was incubated at 0°C for 18 h and then filtered through Whatman Inc. 934-AH filters and washed four times with 8-ml aliquots of iced 0.15 M NaCl, 0.05 M Tris, pH 7.5. [³H]Phorbol dibutyrate in the samples was then measured in a liquid scintillation counter. Nonspecific phorbol binding was determined with parallel samples containing phorbol dibutyrate (3 μM) in addition to the [³H]phorbol dibutyrate. Specific phorbol binding was calculated as the difference in total and nonspecific binding. Duplicates of all samples were studied. Phorbol binding was expressed in picomoles per milligram membrane protein.

Measurement of diacylglycerol (DAG)¹ content in microvillus membrane. Total DAG in the microvillus membrane was measured as previously described (13): lipids were extracted from microvillus membrane prepared from control tissue or from tissue exposed to 1 μM carbachol or 1 μM Ca²⁺ ionophore for 10 min, by mixing 4 vol of chloroform/methanol (2/1:vol/vol) with 1 vol of membrane suspension with vortexing. The samples were centrifuged and the organic phase was dried under nitrogen. The resulting lipid samples were applied in parallel lanes to thin layer chromatography plates (prepared with a slurry of Silica Gel 60 H in H₂O, spread to a thickness of 0.25 mm on a 20 × 20-cm glass plate, air dried for at least 1 h, and activated before use by heating to 120°C for 1 h). The plates were developed in a solvent system containing heptane/diethyl ether/formic acid (90/40/3:vol/vol/vol). Lipid spots were located with I₂ vapor. This chromatographic technique separated 1,2-diolein or 1,2-distearin from the phospholipids, 1,3-diolein, monolein, triolein, and cholesterol, all of which remained at the origin. Natural 1,2-DAG prepared by phospholipase C treatment of egg phosphatidylcholine migrated similarly to 1,2-diolein

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and 1,2-distearin. In each experiment, standards containing 0, 1, 3, 5, and 10 nmol of either 1,2-diolein or 1,2-distearin were run in lanes parallel to the experimental samples.

For quantitation, 1,2-DAG spots from experimental samples and standards were each scraped into clean glass test tubes. In addition, the origin from each lane was scraped into another glass test tube and total phospholipid phosphorus (Pi) was determined as described (14). To each tube containing 1,2-DAG, 200 μl of hydrolysis solution (ethanol, 1 N NaOH, 1.0/0.6:vol/vol) was added. These were then incubated at 70°C for 90 min. The resultant free glycerol was separated from the silica by alternate washing and centrifugation with 200 μl of ethanol three times using a microfuge. The pooled ethanol washes were then dried under N_2. The glycerol content was determined in each sample by measuring fluorometrically the conversion of NAD to NADH by glycerol dehydrogenase. The quantity of 1,2-DAG for each experimental sample was then determined from the standard curve and values were expressed as nanomoles of 1,2-DAG per micromole of total membrane Pi or per milligram membrane protein.

Marker enzyme assays. Marker enzymes for quantitation of membrane purification were: sucrase (15), for microvillus membranes; Na/K ATPase (16), for basolateral membranes; cytochrome c reductase (17), for endoplasmic reticulum; and monoamine oxidase (18), for mitochondria. Protein was measured using the Bradford procedure with ovalbumin as the standard (19).

Na^+/H^+ exchange in villus cell brush border membrane vesicles. Rabbit ileal brush border vesicles were prepared by differential centrifugation and Mg precipitation as described (20). Tissue was incubated for 10 min in the absence or presence of 1 μM carbachol in Ringer's-bicarbonate at 37°C as described for measurement of protein kinase C, including the presence of 1 μM indomethacin. All subsequent procedures in the membrane preparation were performed at 4°C except where noted. After incubation, the ileal villus cells were removed by light scraping with a glass slide. 10 g of each tissue (control and carbachol treated) were homogenized in 30 ml of a buffer containing: 60 mM mannitol, 2.4 mM Tris HCl, pH 7.1, 1 mM EGTA, 2.0 mM Ca(gluconate)₂, 2 mM dithiothreitol (DTT), 0.01% leupeptin, 0.32 mM PMSF, 0.003 mM phosphoramidone and 0.003 TIU/ml aprotinin. Homogenization consisted of 10 10-s bursts followed by 20-s cooling intervals with a polytron homogenizer. The homogenates were treated with 10 mM $MgCl_2$ for 15 min and then centrifuged at 12,000 g for 15 min. The pellets were discarded and the supernatants were centrifuged at 27,000 g for 30 min. The pellets were resuspended in 25 ml of a buffer containing: 60 mM mannitol, 5 mM EGTA, 6.0 mM Ca(gluconate)₂, 10 mM Tris, pH 7.1, 1 mM DTT, and 0.01% leupeptin and homogenized with 10 strokes in a glass Teflon homogenizer. The 10 mM $MgCl_2$ precipitation steps were repeated except the membrane was brought to 23°C for 15 min, and the pellets homogenized in: 200 mM mannitol, 5 mM Mg(gluconate)₂, 2 mM EGTA, 1 mM DTT, 0.01% leupeptin, and 40 mM Mopso/Tris, pH 6.5. These suspensions were centrifuged at 27,000 g for 40 min. The final two pellets, control and carbachol treated, were needle homogenized with a 1-ml syringe and 25-gauge needle in the same buffer and the suspensions brought to a protein concentration of 5 mg/ml.

For transport studies, 350-μl aliquots of the vesicles were mixed with 36 μl of the following (in final concentration): (*a*) 45 mM mannitol, 45 μM W13, (*b*) 45 mM mannitol, 45 μM W13, 542.4 μM Ca(gluconate)₂ (1.5 μM free Ca^{2+}), 1 μM phorbol dibutyrate; (*c*) 45 μM W13, 542.4 μM Ca(gluconate)₂ (1.5 μM free Ca^{2+}), 1 μM phorbol dibutyrate, 5.0 mM ATP, 26 U/ml creatine kinase; (*d*) 45 μM W13, 542.4 μM Ca(gluconate)₂ (1.5 μM free Ca^{2+}), 1 μM phorbol, 5 mM ATP, 26 U/ml creatine kinase, and 200 μM H-7; (*e*) 45 μM W13, 200 μM H-7. The vesicles then were frozen in liquid nitrogen immediately after preparation. The following day, $^{22}Na^+$ uptake or [³H]glucose uptake/equilibrium studies were performed. On the day of the experiment the samples were removed from the liquid N_2 in a staggered fashion and allowed to thaw in an ice slurry for 60 min. 10 mM creatine phosphate was added to those samples containing creatine kinase and ATP and allowed 17 min at 4°C to equilibrate before starting $^{22}Na^+$ or [³H]glucose uptake

measurements, which were terminated by rapid filtration on nitrocellulose (45 μm) filters as previously described (20). Uptake of Na^+ was measured over 8 s after mixing the membranes with transport buffer, which is during the period when $^{22}Na^+$ uptake, with and without a pH gradient, is linear with time in these vesicles (2). [³H]Glucose uptake was measured at 60 s and 90 min.

Results shown are mean±SEM. Statistical analyses were by Student's t tests.

Results

Initial experiments were all performed starting with lightly scraped ileal mucosa. Characterization of subcellular fractions are summarized in Table I. Microvillus membranes of control tissues were purified 16-fold, and contained ~ 6% of the total homogenate sucrase activity; these membranes had up to 0.9% of cytochrome c reductase, 0.8% monamine oxidase, and 1.2% of Na/K ATPase activity. Carbachol exposure did not affect either the degree of purification or the yield of the microvillus membranes.

The basolateral membranes were purified 14-fold. Their characterization included: *Specific enrichment in activity of basolateral membrane relative to homogenate:* Na/K ATPase, 14.4-fold; sucrase, 0.9-fold; monoamine oxidase, 3.0-fold; cytochrome c reductase 1.7-fold. *Yield:* Na/K ATPase, 3.2%; sucrase, 0.8%; monoamine oxidase, 0.5%; cytochrome c reductase, 0.3%.

Effect of carbachol and Ca^{2+} ionophore A23187 on ileal protein kinase C activity and distribution. Microvillus membranes, basolateral membranes, and cytosol all contain protein kinase C activity under basal conditions (Figs. 1 and 2 and Table I, and as reported previously [21]). In Figs. 1–3 the C kinase activity after 10 min exposure to carbachol and the time control are shown for homogenate, cytosol, microvillus membrane, and basolateral membrane. Protein kinase C activity was decreased in the cytosol and increased in the microvillus membranes by exposure to carbachol (Figs. 1 and 2). No
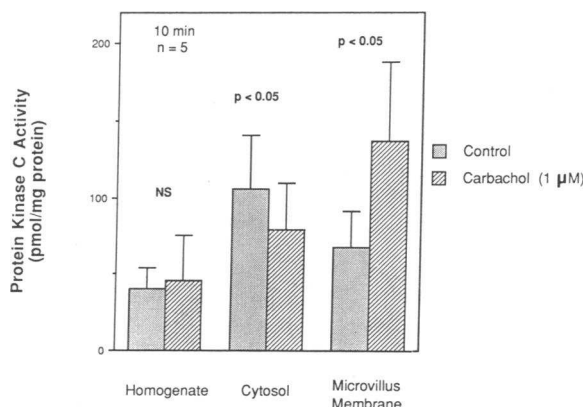
Figure 1. Effect of 10 min exposure to carbachol (1 μM) on protein kinase C activity in homogenate, cytosol, and microvillus membranes of ileal villus cells. Ileal mucosa was exposed in vitro to carbachol and then predominantly villus cells were scraped off and subcellular fractionation performed. Protein kinase C was partially purified with DE-52 before protein kinase C activity determination in each fraction. Protein kinase is expressed as picomoles ^{32}P incorporated per milligram membrane or cytosol protein. The data represent the mean±SEM of five experiments. P values above the bars are for the comparisons of control and carbachol exposed fractions (paired t test).
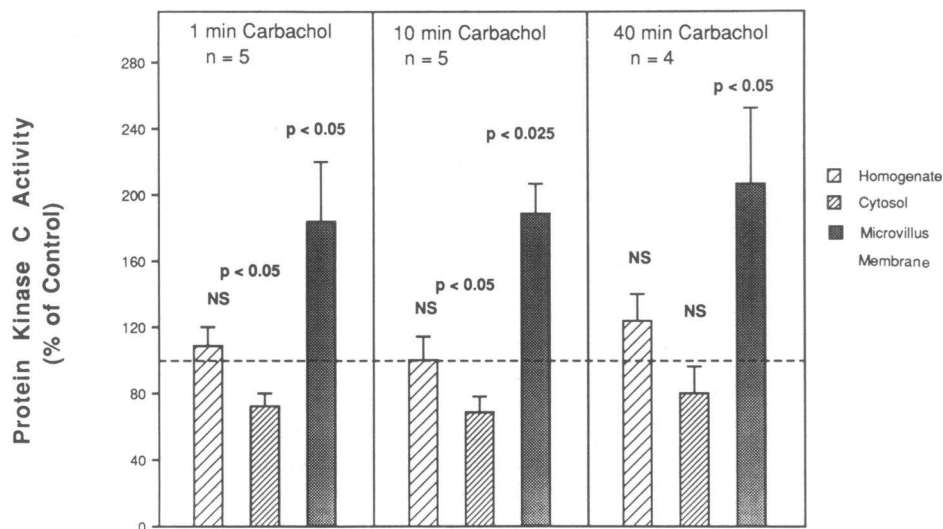
Figure 2. Effect of 1-, 10-, and 40-min exposures to carbachol (1 μM) on protein kinase C activity in homogenate, cytosol, and microvillus membranes of ileal villus cells. The data are presented as percent of control, with the control set at 100% for each fraction in each experiment (*horizontal dashed line*). Mean control values in picomoles per milligram protein were, homogenate, 74±19; cytosol, 162±44; microvillus membrane, 113±34. Results are the mean±SEM. *n*, number of experiments. *P* values are for the comparison of carbachol exposed tissue with control for each subcellular fraction (paired *t* test).

changes occurred in the homogenates. In Fig. 2, time course studies are shown for the changes in homogenate, cytosol, and microvillus membrane protein kinase C activity after exposure of ileal mucosa to carbachol for 1, 10, and 40 min. The results for each experiment are normalized to its time control and presented as percent changes. The effects of carbachol on protein kinase C activity in homogenate, cytosol, and microvillus membranes were similar at 1, 10, and 40 min (Fig. 2). Microvillus C kinase activity increased significantly between 84 and 105% above control. Cytosol C kinase activity decreased significantly by 25–30% below control values after 1 and 10 min of exposure to carbachol, but the changes were no longer significant at 40 min. Homogenate C kinase activity did not change. These results, obtained by measuring protein kinase C *activity* by histone IIIS phosphorylation and which showed an increase in C kinase activity in microvillus membranes were supported by measurements of the *amount* of protein kinase C by [^{3}H] phorbol binding. 10 min exposure to 1 μM carbachol increased the amount of [^{3}H] phorbol binding in microvillus membranes by 51±23% ($n = 5$, $P < 0.05$). Thus, the increased activity present in these membranes appears to be due to C kinase translocation.

Fig. 3 shows similar studies performed with a highly purified basolateral membrane preparation, which was obtained by Percoll density gradient centrifugation. As shown in Fig. 3, 20 s and 1 min of exposure to carbachol caused no significant changes in basolateral membrane protein kinase C activity, whereas 10 min of exposure caused a significant decrease in protein kinase C activity to 48±11% of control. Therefore, the increase in microvillus membrane C kinase activity after carbachol treatment of the cells appears to be specific for that membrane fraction.

Because basolateral membranes prepared from scraped ileal mucosa could be contaminated with plasma membranes from nonepithelial cells, similar experiments were repeated, starting with isolated ileal villus epithelial cells. Both microvillus membranes and basolateral membranes were prepared from these dissociated cells. Microvillus membranes showed an increase in sucrase specific activity of 25-fold with a total yield of 16.5%. The characterization of the basolateral membranes included: *Specific enrichment and activity of basolateral membrane relative to homogenate:* Na/K ATPase, 12.8-fold; sucrase, 0.8-fold; cytochrome *c* reductase, 1.7-fold. *Yield:* Na/K ATPase, 2.2%; sucrase, 0.2%; cytochrome *c* reductase, 2.7%.

Table I. Effect of Carbachol on Purification and Recovery of Marker Enzymes in Ileal Organelles

	Homogenate				Microvillus				Crude basolateral			
	Control		Carbachol		Control		Carbachol		Control		Carbachol	
	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA
Sucrase	11.8	0.03	12.7	0.03	0.75	0.48	0.75	0.49	0.16	0.05	0.10	0.02
(microvillus membrane)					(6.4%)	(×16)	(5.9%)	(×16)	(1.4%)	(×1.7)	(0.8%)	(×0.7)
Na/K ATPase	1032	2.27	1180	2.80	12	3.95	6	2.82	101	5.70	110	7.00
(basolateral membrane)					(1.2%)	(×1.7)	(0.5%)	(×1.0)	(9.8%)	(×2.5)	(9.3%)	(×2.5)
Monoamine oxidase	65.3	0.16	58.8	0.14	0.52	0.18	0.46	0.16	0.21	0.03	0.36	0.06
(mitochondria)					(0.8%)	(×1.1)	(0.8%)	(×1.1)	(0.3%)	(×0.2)	(0.6%)	(×0.4)
Cytochrome *c* reductase	15.8	0.04	13.8	0.03	0.15	0.07	0.16	0.08	2.5	0.16	2.07	0.12
(endoplasmic reticulum)					(0.9%)	(×1.8)	(1.2%)	(×2.7)	(16%)	(×4.0)	(15%)	(×4.0)

The results are given in units for total activity (TA) or in units per milligram protein for specific activity (SA). One unit is defined as enzyme activity hydrolyzing 1 μmol of substrate per min. Numbers in parentheses under TA represent percent of total homogenate marker enzyme recovered in each fraction; numbers in parentheses under SA represent ratio of SA in fraction/SA in homogenate.
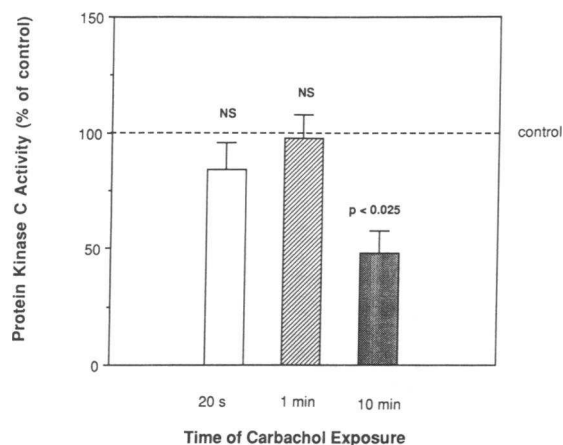
Figure 3. Effect of 20 s, 1 and 10 min exposure to carbachol (1 μM) on protein kinase C activity in purified basolateral membranes from scraped ileal villus cells. Protein kinase C activity from carbachol exposed ileal mucosa is plotted as percent of control±SEM, with the control set at 100% for each experiment (*horizontal dashed line*). Mean control values were 394±82 pmol/mg protein. Four to five separate experiments were performed at each time. The *P* value represents comparison of control and carbachol exposed tissue.

An increase in microvillus membrane protein kinase C activity and a decrease in basolateral membrane protein kinase C activity was observed in membranes prepared from dissociated cells, very similar to that found with scraped ileal mucosa. In ileal villus cells exposed for 10 min to carbachol, microvillus membrane protein kinase C activity was increased 91±31% (n = 4, $P < 0.05$) compared with control; and basolateral membrane protein kinase C activity was decreased by 58±13% compared with control (n = 3, $P < 0.05$). Similar studies analyzing basolateral membrane were performed after exposure of isolated ileal villus cells to carbachol for 30 s. Ileal basolateral membranes in carbachol exposed cells were significantly decreased by 11±3% compared with control, n = 4, $P < 0.025$.

Similar studies examined the effect of exposure of ileal mucosa to the Ca^{2+} ionophore A23187 for 10 min, a period over which the ionophore decreases active ileal NaCl absorption. Exposing tissue to 1 μM Ca^{2+} ionophore A23187 for 10 min, produced effects on cytosol and microvillus membrane similar to those observed with carbachol. There was a 70±21% increase in microvillus membrane protein kinase C activity and a 22±10% decrease in cytosol protein kinase C activity. No significant changes occurred in whole homogenate C kinase. However, in contrast to the carbachol-induced decrease in C kinase activity in the basolateral membranes, 10 min of Ca^{2+} ionophore exposure did not alter basolateral membrane protein kinase C activity (Fig. 4).

Because elevating ileal $[Ca^{2+}]_i$ caused protein kinase C translocation to microvillus membranes, the requirement for the continued presence of Ca^{2+} to retain the translocated protein kinase C was studied. Microvillus membranes made from tissue exposed for 10 min to carbachol, Ca^{2+} ionophore, or control conditions were extracted with 5 mM EGTA and 5 mM EDTA before extraction with Triton X-100 and the C kinase activity extractable by both treatments was compared (Fig. 5). Little enzyme activity could be extracted from the membranes with divalent cation chelators indicating that al-

though elevated Ca^{2+} plays a role in translocation of C kinase from cytosol to microvillus membranes, the effect is not reversed simply by removing Ca^{2+}.

Effect of carbachol and Ca^{2+} ionophore on ileal microvillus membrane diacylglycerol (Fig. 6). Measurements of DAG (expressed as nanomoles per micromole total membrane phospholipid) were determined on microvillus membranes after 10 min exposure to control conditions, carbachol (1 μM) or Ca^{2+} ionophore A23187 (1 μM). Both carbachol and Ca^{2+} ionophore caused significant and similar increases in apical membrane DAG (microvillus DAG in nanomoles per micromole total Pi in control tissue, 4.2±1.4 (n = 4); after exposure to carbachol, 8.9±3.7 (n = 3, P compared to control < 0.05); and after Ca^{2+} ionophore, 8.3±2.1 (n = 4, P compared with control < 0.05). Similar conclusions were reached when the microvillus DAG was expressed per milligram microvillus membrane protein (data not shown). Total microvillus membrane phospholipid per milligram membrane protein was not significantly altered by carbachol or ionophore exposure (micromoles microvillus membrane total phospholipid per milligram membrane protein was 1.2±0.1; 1.2±0.2; 1.0±0.1 in membranes of control, carbachol- and ionophore-treated ileum, respectively).

Functional significance of translocated protein kinase C: effect on brush border Na^+/H^+ exchange. Protein kinase C is present on the apical membranes of control and carbachol exposed ileal Na^+ absorbing cells. Studies were undertaken to determine the functional significance of the protein kinase C present on the apical membrane in control cells and after carbachol exposure. This was done by exposing ileum, in vitro, to carbachol for 10 min as described for the protein kinase C translocation studies, and then preparing right-side-out brush border membrane vesicles from control and carbachol exposed ileal villus cells. The vesicles were resuspended to contain the combinations described in the methods of 1.5 μM free Ca^{2+}; ATP, creatine phosphate, creatine kinase; phorbol dibutyrate; and the protein kinase C antagonist, H-7. All vesicles contained $MgCl_2$ and the Ca^{2+}/calmodulin (CaM) inhibitor W13 (45 μM), the latter to be certain that any effect seen was not due to the endogenous Ca^{2+}/CaM-dependent protein kinase present in the vesicles (2). Na^+ uptake was measured in brush border vesicles from control and carbachol treated tissues. It can be calculated from the results shown in Table II, that addition of ATP, phorbol dibutyrate and 1.5 μM Ca^{2+} to vesicles from carbachol-treated tissue caused a 26% decrease in Na^+/H^+ exchange (n = 4, $P < 0.05$) and a 17% decrease in Na^+ uptake into the vesicles in the presence of an inside pH gradient ($P < 0.05$), whereas there was no effect on Na^+ uptake without a pH gradient. The presence of Ca^{2+}, phorbol, and ATP together were necessary to observe this effect in carbachol-exposed tissue; Ca^{2+} plus phorbol dibutyrate in the absence of exogenous ATP did not alter Na^+ uptake. In the presence of 200 μM H-7, Ca^{2+}/phorbol/ATP did not alter Na^+/H^+ exchange in vesicles from carbachol-treated tissue (in studies done with the same vesicles as those used for the carbachol data shown in Table II, Na^+/H^+ exchange in vesicles exposed to phorbol/Ca^{2+}/ATP plus H-7 was 211±15 pmol/mg protein-8 s, n = 4; and in vesicles exposed to H-7 alone was 208±14 pmol/mg protein-8 s, n = 4). The data indicate that the carbachol-stimulated increase in C kinase activity in microvillus membranes has a functional role in regulation of apical membrane Na^+/H^+ exchange.

It is important to note that despite the presence of C kinase in control brush border vesicles, there was no effect in these
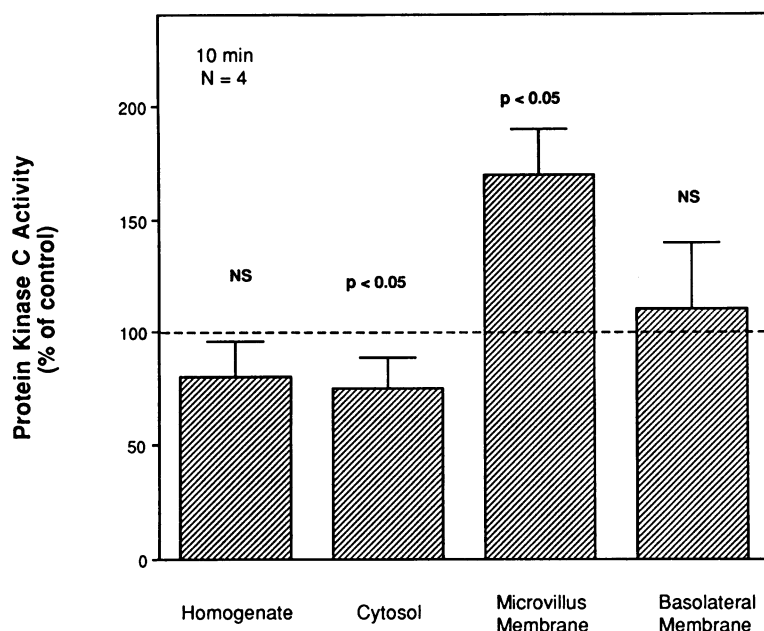
Figure 4. Effect of 10 min exposure to Ca^{2+} A23187 ionophore (1 μM) on protein kinase C activity in subcellular fractions from ileal villus cells. C kinase activity was expressed as percent of control values with controls set at 100% in each subcellular fraction of each experiment (*horizontal dashed line*). Paired control and treated tissues were studied in each experiment. Control values in picomoles per milligram protein were homogenate, 66±7; cytosol, 58±11; microvillus membrane, 88±54; basolateral membrane, 385±99. Data represent the mean±SEM of four experiments. P values above bars are for comparison of control and ionophore exposed tissue, in each subcellular fraction (paired t test).

control vesicles of Ca^{2+}/phorbol/ATP on Na^+ uptake with or without a pH gradient or on Na^+/H^+ exchange (Table II).

In contrast to the effect on Na^+/H^+ exchange, glucose overshoot values at 1 min and equilibrium values, measured at 90 min, in freeze thawed brush border vesicles were not significantly different in control and carbachol exposed tissue when treated with mannitol or with Ca^{2+}/phorbol/ATP (data not shown).

Discussion

Previous studies have suggested that the effects of ionophore A23187 and carbachol, to inhibit ileal NaCl absorption, involve protein kinase C because their effects are reversed by the isoquinolenesulfonamide H-7 under conditions in which H-7 is a relatively specific ileal villus cell protein kinase C antagonist, and not an inhibitor of villus cell cyclic AMP, cyclic GMP, or Ca^{2+}/CaM-dependent protein kinases (3). The biochemical
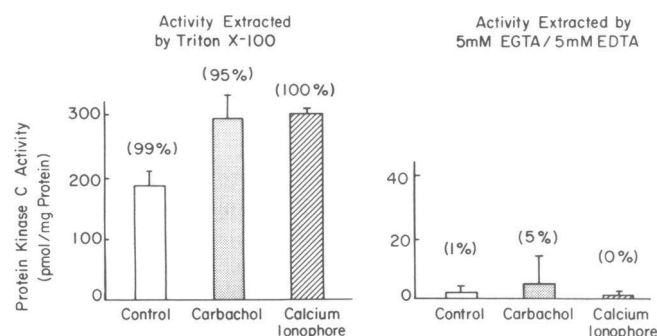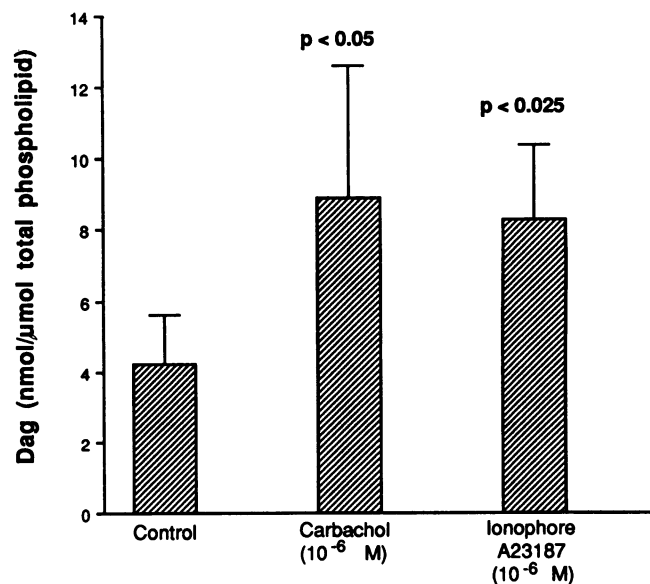

Figure 5. Lack of calcium dependence of protein kinase C already bound to microvillus membranes. Ileal mucosa was exposed to control conditions, Ca^{++} ionophore (1 μM) or carbachol (1 μM) for 10 min in vitro and microvillus membranes were prepared. After preparation, microvillus membranes from control, carbachol-, and ionophore-treated tissues were first extracted with 5 mM EDTA, 5 mM EGTA, 20 mM Tris, pH 7.5, for 30 min at 0°C, pelleted by centrifugation, and the supernatant was saved for C kinase activity determination while the pellet was extracted with 0.3% Triton X-100 for 30 min at 0°C and then assayed for C kinase. Protein kinase C in each of the extracts was partially purified with DE-52 cellulose and quantitated by histone IIIS phosphorylation. C kinase activity extracted by Triton X-100 is shown on the left and protein kinase C activity extracted by calcium chelators is shown on the right. Total C kinase activity is the sum of C kinase in the Triton X-100 fraction plus that in the divalent chelator extract of each sample. The numbers in parentheses represent the percent of total activity of each sample which was extracted by cation chelators or by Triton X-100. The data represent the mean±SEM of three experiments.

Figure 6. Carbachol (1 μM) and Ca^{2+} ionophore (1 μM) increase ileal microvillus membrane total diacylglycerol. Ileal mucosa was exposed in vitro to carbachol, A23187, or control conditions for 10 min and then predominantly villus cells scraped off, microvillus membranes prepared and total diacylglycerol determined and normalized to total membrane phospholipid. Results are mean±SEM of three experiments for carbachol and 4 for A23187. P values represent comparisons with control (paired t test).

Table II. Effect of Exposure of Ileal Mucosa In Vitro to Carbachol and Subsequent Translocation of Protein Kinase C to the Brush Border on Brush Border Na⁺ Uptake

	Na⁺ uptake at 8 s (pmol/mg protein)		
	Na⁺ uptake with pH gradient (A)	Na⁺ uptake without pH gradient (B)	Na⁺/H⁺ exchange (C = A − B)
	pmol/mg protein	*pmol/mg protein*	*pmol/mg protein*
Vesicle treatment			
Control			
Mannitol	276±31	85±21	191±26
Ca²⁺/phorbol	287±39	77±26	209±24
Ca²⁺/phorbol/ATP	260±26	76±22	184±27
Carbachol			
Mannitol	282±17	78±24	204±15
Ca²⁺/phorbol	279±21	60±18	219±18
Ca²⁺/phorbol/ATP	234±26*	83±20	151±21*

^{22}Na⁺ uptake was determined (A) with an acid inside pH gradient (pH$_{in}$6.5/pH$_{out}$ 8.0) and (B) at pH 6.5 inside and outside the vesicle; Na⁺/H⁺ exchange was defined as the difference (A minus B) in these rates of Na⁺ uptake. Ileal mucosa was exposed in vitro for 10 min to 1 μM carbachol or control, with both exposed to 1 μM indomethacin. As described in Methods, brush border vesicles were prepared, loaded by freeze thawing with Ca²⁺ (free Ca²⁺ 1.5 μM), ATP, and phorbol dibutyrate in the presence of W13, the latter to eliminate the contribution exerted by the Ca²⁺/calmodulin system. Data are means from four experiments. *$P < 0.05$ comparing mannitol control to Ca²⁺/phorbol/ATP in vesicles from carbachol exposed ileum.

mechanisms by which protein kinase C affects Na⁺ absorption had not been determined. This study has shown that the changes in NaCl absorption caused by elevation of Ca²⁺ by both Ca²⁺ ionophore and carbachol are associated with the increases in DAG and protein kinase C in the apical membranes of villus cells. It is likely these apical membrane changes are involved in regulation of NaCl absorption because the effect on brush border Na⁺/H⁺ exchange caused by activation of protein kinase C was reversed by a protein kinase C antagonist. Another reason to suspect that it is the apical membrane protein kinase C which is relevant to regulation of transport is that carbachol and ionophore cause similar changes in NaCl absorption and apical membrane C kinase translocation, but have different effects on basolateral membrane C kinase activity.

The facts that no detectable change in total tissue C kinase activity was observed to accompany the carbachol- and A23187-induced decrease in cytosol and increase in apical membrane protein kinase C activity, and that similar changes in microvillus membrane protein kinase C were found by measuring enzyme activity and by measuring the amount of phorbol ester binding after carbachol exposure, suggest that the two secretagogues caused a redistribution of C kinase rather than a change in the total amount or activity. Prostaglandins do not appear to be involved in the mechanism of this translocation because the tissues were exposed to indomethacin, a cyclooxygenase inhibitor, during the secretagogue treatment.

An unusual aspect of the translocation of protein kinase C in these polar epithelial cells is that the movement of the en-

zyme appears to be specifically to the microvillus membrane, because no increase in the basolateral membrane activity occurred. In fact, by 30 s (using isolated villus epithelial cells) and 10 min after carbachol treatment, a significant decline in basolateral protein kinase C activity was found, whereas ionophore exposure for 10 min did not alter basolateral C kinase. The failure to detect C kinase movement to the basolateral membrane is relevant because the receptors for acetylcholine are located in this membrane (22). Whereas movement of protein kinase C to the plasma membrane is associated with signal transduction in many cell types, the concept of translocation to specific membranes and focal sites has only recently been suggested (23). The source of the protein kinase C which appears on the apical membrane has not been definitely established, because both cytosol and basolateral membrane C kinase decreased with carbachol exposure (see Table III). We favor cytosol being the source, as the increase in brush border C kinase activity is maximum within 1 min of exposure to carbachol, a time at which the cytosol decrease is also maximum, but a time at which there is only a minimal change in basolateral membrane C kinase activity. Also, carbachol and Ca⁺⁺ ionophore A23187 cause similar increases in brush border and decreases in cytosol protein kinase C activity, but only carbachol significantly alters basolateral C kinase activity. Not determined in these studies is the meaning of the decrease in basolateral C kinase activity 10 min after carbachol exposure. The balance sheet in Table III shows that this decrease in basolateral C kinase activity is associated with the appearance of unaccounted for C kinase activity; which suggests that the C kinase may be translocating to other intracellular compartments.

After translocation to the microvillus membrane, the pro-

Table III. Protein Kinase C Activity per Gram Wet Weight

	Control		10 min Carbachol	
	pmol	*%*	*pmol*	*%*
Homogenate	5095	(100)	5511	(100)
Microvillus membrane	587	(11.5)	1118	(20.3)
Basolateral membrane	1544	(30.3)	1030	(18.7)
Cytosol	2822	(55)	2446	(44.4)
Not accounted for		(3.2)		(16.6)

Balance sheet of protein kinase C activity in control conditions and 10 min after carbachol exposure. Shown are total protein kinase C activity in each fraction: homogenate, plasma membrane, and cytosol fractions per gram tissue wet weight; in parentheses are percent of total C kinase in each fraction. Each fraction was assayed for protein kinase C activity in picomoles incorporated into histone per milligram protein. To calculate total activity, specific activity was multiplied by the total protein for each fraction. To calculate total homogenate and cytosol protein per gram wet weight of mucosa, the protein concentration of homogenate or cytosol (milligrams per milliliter) was multiplied by the total volume of homogenate or cytosol, and divided by the wet weight of the mucosa used. To calculate total microvillus or basolateral membrane protein, the percentage marker enzyme recovered was first calculated, using sucrase and Na-K-ATPase, respectively. Then total microvillus or basolateral membrane protein per gram wet weight mucosa was calculated by dividing the total microvillus or basolateral membrane protein by the percentage recovery, and this divided by wet weight of mucosa. Data shown represent the mean result of three preparations.

tein kinase C did not require Ca^{2+} for it to remain in the membrane, because treatment with EGTA and EDTA failed to extract it. These studies were performed on sheets of microvillus membrane which would have allowed detached C kinase to be lost (8). Thus, the prolonged increased C kinase activity in the membrane after elevation of Ca^{2+} is not simply due to a Ca^{2+}-dependent absorption of enzyme to membrane, and additional factors other than Ca^{2+} must be involved in the binding. It has been shown by Wolfe et al. (24) that phorbol dibutyrate prevents the release of protein kinase C from red blood cell membranes in the absence of Ca^{2+}. Also, they suggested that an ATP-dependent phosphorylation reaction might be involved in the release of the enzyme from the membrane. In view of our results, in which DAG was found to increase in the apical membrane after exposure to carbachol and A23187, it would seem that the most likely additional factor is DAG. Of note, is a previous report of a Ca^{2+} ionophore elevating hepatic membrane DAG (25). Therefore, our data best fits an elevation in intracellular Ca^{2+} causing protein kinase C to move to the microvillus membrane. However, it is likely that protein kinase C remains there after Ca^{2+} is no longer elevated due to the generation of DAG.

The changes in C kinase activity with carbachol exposure were similar at 1, 10, and 40 min, showing that the translocation of C kinase is both rapid and persistent, in line with persistent carbachol-induced changes in NaCl absorption which are seen in intact ileal tissue, in which inhibition of NaCl absorption is present by 10 min of exposure to carbachol with the effects persisting for at least 40 min (22, and Donowitz, M., and M. E. Cohen, unpublished observations). The time course of the carbachol-induced translocation of protein kinase C, which is reported here, is different from that reported to occur in chicken small intestine in response to phorbol dibutyrate (26). In the latter, the phorbol ester treatment caused a transient (90 s) increase in microvillus protein kinase C activity which returned to control levels by 15 min (26). These differences could be due to the different species studied or to the different agonists used.

The C kinase translocated to the apical membrane of ileal Na^+ absorbing cells was functional, because addition of phorbol dibutyrate, ATP, and Ca^{2+} to carbachol-treated but not control vesicles inhibited Na^+ uptake and Na^+/H^+ exchange. The effect was specific because there was no effect of the translocated C kinase, with or without ATP, on Na^+ uptake in the absence of a pH gradient or on Na^+-dependent glucose uptake. These data show that the newly translocated C kinase plays a role in the regulation of ileal villus cell Na^+ absorption and Na^+/H^+ exchange but that the existing C kinase in membranes from control tissues does not have such a role. This difference between C kinase in control membranes, which is nonfunctional with respect to Na^+/H^+ exchange, and the functional, translocated enzyme could be explained if the C kinase which is translocated is a different isoform from that which is already present in the membrane. Alternatively, the C kinase present in the membrane under control conditions may not be in the correct location or configuration to affect the Na^+/H^+ exchanger. It seems less likely, although possible, that a simple quantitative difference between the amounts of C kinase present in the membranes under control and stimulated conditions could provide an explanation.

The inhibition of brush border Na^+/H^+ exchange by translocated protein kinase C, in the presence of Ca^{2+}, phorbol ester,

and ATP, is the most direct demonstration that protein kinase C is involved in the control of ileal Na^+ absorption by inhibiting the brush border Na^+/H^+ exchanger. Initially, protein kinase C was suggested as stimulating all Na^+/H^+ exchangers. Now, with few exceptions (27), protein kinase C has been found to inhibit both intestinal and renal apical membrane Na^+/H^+ exchangers but to stimulate their basolateral Na^+/H^+ exchangers (3, 26, 28–30). Whether it is the location in the plasma membrane, the molecular nature of the Na^+/H^+ exchangers, or the subtype of protein kinase C involved which accounts for this differential regulation remains unknown.

Acknowledgments

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