Asymmetric signal transduction in polarized ileal Na^+-absorbing cells: carbachol activates brush-border but not basolateral-membrane PIP_2–PLC and translocates PLC-γ_1 only to the brush border

Seema KHURANA,* Sawsan KREYDIYYEH,† Anna ARONZON,* W. Alexandra HOOGERWERF,* Sue Goo RHEE,‡ Mark DONOWITZ*§ and Michael E. COHEN*

*Departments of Medicine and Physiology, G. I. Division, Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A., and ‡Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institute of Health, Bethesda, MD 20892, U.S.A.

In ileal Na^+ absorptive cells, carbachol inhibits NaCl absorption and its component brush-border Na^+/H^+ exchanger, acting via basolateral membrane (BLM) receptors. This carbachol effect involves brush-border but not BLM protein kinase C. In the present work we describe another asymmetric aspect of signal transduction in these epithelial cells, this time involving phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C (PLC). Thirty seconds and 1 min after carbachol treatment, brush-border PIP_2-specific PLC activity increased, returning to control levels by 2.5 min. Involvement of brush-border tyrosine kinase(s) in this effect was suggested by inhibition of the carbachol effect on NaCl absorption by the tyrosine kinase inhibitor genistein, added to the mucosal but not the serosal surface. Luminal genistein pretreatment also prevented the carbachol-induced increase in brush-border PLC activity. In contrast, carbachol exposure did not change the BLM PIP_2-specific PLC activity. Western analysis and immunoprecipitation demonstrated that PLC-γ_1 is present in the brush border and that carbachol increases the PLC-γ_1 amount in the brush border. Both the brush border and BLM contain PLC-β_3 and a small amount of PLC-δ_1 but no PLC-β_1, whereas BLM lacks detectable PLC-γ_1. No change in PLC-β_3 or PLC-δ_1 amount in the brush border occurred with carbachol exposure. No change in tyrosine phosphorylation of brush-border PLC-γ_1 occurred with carbachol treatment. The Ca^{2+} ionophore A23187 did not alter PIP_2-specific PLC activity in either the brush border or the BLM. These studies demonstrate that carbachol but not Ca^{2+} ionophore effects on brush-border NaCl absorption are associated with increases in brush-border but not BLM PIP_2-specific PLC activity and in the amount of brush-border PLC-γ_1, and involve tyrosine phosphorylation. This asymmetric aspect of epithelial signal transduction, together with the previous demonstration of localization of high-sensitivity IP_3 stores to the apical membrane area in intestinal epithelial cells, shows that different aspects of signal transduction occur at the apical and basolateral membranes in epithelia and requires studies in both domains to define mechanisms of intracellular signalling.

INTRODUCTION

In the many cell types studied, patterns of signal transduction after receptor binding of the cholinergic agonist carbachol to odd-numbered cholinergic receptor subtypes (M_1, M_3, M_5) are associated with an increase in diacylglycerol (DAG) content and elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which activate protein kinase C [1–6]. In at least one intestinal cell type (T84 cells), carbachol elevates inositol 1,4,5-trisphosphate (IP_3) content [7] as well as $[Ca^{2+}]_i$ [3].

Adding to the complexity of signal transduction in epithelial cells is the presence of two separate plasma membrane domains, the apical or brush-border and basolateral membranes. These domains could potentially perform different aspects of signal transduction. For instance, whether the IP_3 source in T84 cells exposed to carbachol is the basolateral membrane (BLM), the site of the carbachol receptor, has not been addressed [7]. Some hints of such differences in apical and basolateral membrane events in signal transduction come from studies in which carbachol binding to rabbit ileal Na^+-absorptive cell BLM was shown to inhibit brush-border NaCl absorption and Na^+/H^+ exchange [8–10]. This inhibition was due to an asymmetric increase in brush-border protein kinase C activity and DAG content [9]. This increase was asymmetric because carbachol did not increase BLM protein kinase C activity.

Here we describe further carbachol-induced ileal Na^+ absorptive cell-signal transduction events that accompany the elevation in brush-border kinase C and demonstrate an asymmetric increase in ileal phospholipase C (PLC) activity and PLC-γ_1 amount in the brush border but not the BLM. The asymmetric, membrane-specific aspects of signal transduction in polarized epithelial cells is likely to reveal new information about signalling in non-polarized cells, which initially was not understood because the various components of the signalling pathway could not be separated.

EXPERIMENTAL PROCEDURES

Materials

Sodium orthovanadate, β-glycerophosphate, phenylalanine, phosphorhamidone, aprotinin, leupeptin, carbachol,

Abbreviations used: BBM, brush-border membrane(s); BLM, basolateral membrane(s); $[Ca^{2+}]_i$, intracellular free calcium; DAG, 1,2-diacylglycerol; DTT, dithiothreitol; *G*, conductance; IP_3, inositol 1,4,5-trisphosphate; I_{sc}, short-circuit current; J_{ms}, mucosal-to-serosal flux; J_{sm}, serosal-to-mucosal flux; PD, potential difference; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; TCA, trichloroacetic acid.

† Present address: Department of Biology, American University of Beirut, Lebanon.

§ To whom correspondence should be addressed.

phosphatidylinositol 4,5-bisphosphate, Triton X-100, Ca^{2+} ionophore A23187 and PMSF were obtained from Sigma; Pansorbin and genistein from Calbiochem; Extracti-gel D columns from Pierce; Percoll from Pharmacia; sodium deoxycholate from Fisher; anti-PLC-β_1, -γ_1 and -δ_1 monoclonal antibodies from Upstate Biotechnology; anti-PLC-β_3 polyclonal antibodies were prepared as described earlier [12]; rabbit anti-mouse IgG was obtained from Cappel; anti-phosphotyrosine polyclonal antibody from Zymed; [^{3}H]IP_3 from New England Nuclear; prestained protein molecular mass standards from Biorad (high molecular mass range); Rainbow molecular mass standards from Amersham; enhanced chemiluminescence reagents from DuPont; and calf intestine alkaline phosphatase from Boehringer Mannheim.

Methods

Distal ileum from New Zealand white male rabbits (2–2.5 kg) was used for all experiments. Rabbits were killed by nembutal overdose. Ileum was then removed, rinsed with 0.9 % saline, and exposed *in vitro* to carbachol either as sheets of tissue or isolated villus absorptive cells. For measurement of active Na^+ and Cl^- transport and preparation of brush-border membranes (BBM), tissue was used in most studies, although some studies used isolated villus cells. For preparation of BLM, isolated villus cells were always used.

Electrolyte transport *in vitro*

The methods used to measure active ileal electrolyte transport have been described previously [10]. In brief, ileal mucosa with muscularis propria removed was mounted as a flat sheet between two Lucite modified Ussing chambers having an aperture of 1.13 cm^2, oxygenated and maintained at 37 °C. Transmural potential difference (PD), short-circuit current (I_{sc}), conductance (G), and unidirectional fluxes of Na^+ and Cl^- were determined. An automatic voltage clamp corrected for fluid resistance between the PD-sensing bridges and provided continuous short-circuiting of the tissue. Unidirectional fluxes of Na^+ and/or Cl^- were measured 20–100 min after addition of isotope by using ^{22}Na and ^{36}Cl on tissue matched to differ in conductance by not more than 25 %. In ion flux experiments, a negative sign indicates net secretion; a positive sign, net absorption.

Usually six pieces of ileum from a single animal were studied simultaneously. Unless specified, the bathing solution consisted of Ringer's/HCO_3 composed (in mM) of 115 NaCl, 25 $NaHCO_3$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$; the pH was 7.4 after gassing with 95 % O_2/5 % CO_2. Glucose (10 mM) was added to the serosal and 10 mM mannitol to the mucosal bathing fluids at the time of mounting the tissue. The effect of carbachol (1 μM) added to the serosal surface was determined over two 20 min flux periods in otherwise untreated ileum or tissue exposed to genistein (100 μM) added to the ileal mucosal or serosal surface for 40 min before carbachol addition.

Membrane preparation

The tissue or cells were preincubated for 10 min at 37 °C gassed with 95 % O_2/5 % CO_2, in Ringer's/HCO_3 containing 10 mM glucose and 1 μM indomethacin to inhibit prostaglandin synthesis. Carbachol (1 μM) was then added to one set of tissues and the incubation was continued for the indicated times. The tissues were then chilled, and control and carbachol-treated samples were processed in parallel.

Preparation of BBM

At the end of the above incubation, ileal sheets were chilled on iced Petri dishes and mucosa was lightly scraped off with glass slides. The tissues were homogenized in 10 volumes of 20 mM Tris buffer, pH 7.5, containing 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM phenylalanine, 0.28 mM PMSF, 25 μg/ml aprotinin, 0.01 % leupeptin, 1.006 mM $CaCl_2$ and 1 mM EGTA (free $[Ca^{2+}] = 10\ \mu M$) (buffer A) with a polytron homogenizer at a setting of 5 with ten 10 s bursts with 20 s cooling intervals. The homogenate was centrifuged at 1000 ***g*** and the supernatant discarded. The pellets were then resuspended in buffer A with 20 strokes of a Dounce homogenizer (B pestle) and washed four times by centrifugation at 1000 ***g***. The pellets were resuspended in buffer A containing 250 mM sorbitol (buffer B) and centrifuged at 20000 ***g*** for 20 min. The pellets were resuspended in buffer B by homogenization for 1 min with a Teflon–glass homogenizer at top speed. $MgSO_4$ was added to a final concentration of 10 mM and the suspension allowed to stand for 10 min. Pellets were collected by centrifugation at 5000 ***g*** for 10 min and discarded. The supernatant was spun at 32000 ***g*** for 20 min. The pellets were resuspended in buffer B with a motor-driven Teflon–glass homogenizer and the $MgSO_4$ precipitation steps were repeated. The final BBM pellets were resuspended in 20 mM Tris buffer, pH 7.5, containing 250 mM sorbitol and 1 mM sodium orthovanadate, with five strokes of a Teflon–glass homogenizer. Purification, judged by enrichment of sucrase specific activity compared with the homogenate, was 15–17-fold, similar to that previously reported [11].

In one series of studies, BBM were prepared by exposing isolated ileal villus cells (prepared as described below) to carbachol.

Preparation of BLM

BLM were prepared from isolated villus cells instead of scraped mucosa, because the BLM of an enterocyte is indistinguishable from the plasma membrane of other cells, and the ileum is composed of more than one cell type. BLM were prepared from isolated villus cells obtained by a modification of the method of Weiser [13] as previously reported [9] with the modification that ileum was initially everted. Distal ileum was rinsed with 0.9 % saline, followed by PBS, everted with a glass rod, filled with PBS and the ends clamped so that the loop was tense. The everted intestinal segments were incubated in 800 ml of 20 mM sodium citrate, pH 7.5, containing 96 mM NaCl, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4 and 1 mM DTT, gassed with 95 % O_2/5 % CO_2, at 37 °C for 10 min. The tissues were then transferred to 800 ml of 140 mM NaCl, 8 mM Na_2HPO_4, 2.8 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM DTT, 1.5 mM Na_2EDTA, pH 7.4, at 37 °C with gassing and shaking for 27 min. In parallel studies, isolated ileal epithelial cells were prepared exactly as described, except that cells were separately collected sequentially for 4, 4, 7, 5, 7, 15 and 8 min and cell fractions characterized for sucrase specific activity and [^{3}H]thymidine incorporation. As shown in Figure 1, the 27 min collection is equivalent to fractions 1–5 [9] and, on the basis of marker enzymes, represents cells from upper villus, mid-villus and lower villus. The everted intestine was removed from the resulting cell suspension and $CaCl_2$ was added to a total final concentration of 2 mM. The cells were collected by centrifugation at 770 ***g*** for 10 min. They were resuspended in Ringer's/HCO_3 containing 10 mM glucose, 1 μM indomethacin and 10 units/ml DNAse I, divided into two aliquots and preincubated at 37 °C for 10 min. Carbachol (1 μM) was then added to one of the suspensions and the suspensions

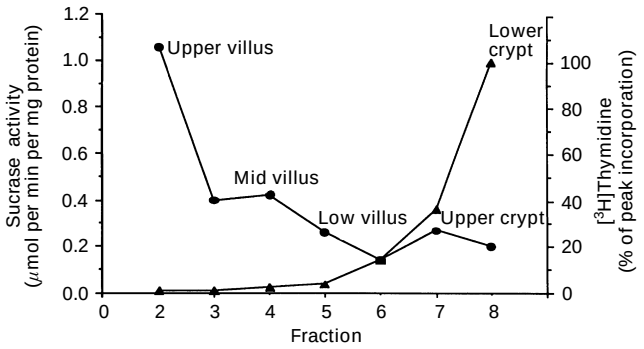

Figure 1 Crypt/villus separation of ileal epithelial cells

Rabbit ileal epithelial cells were prepared by a modification of the Weiser technique [9] using everted loops with analysis of seven cell fractions, shown on the *X*-axis, with fraction 2 representing villus tip epithelial cells and fraction 8 low crypt cells. Each fraction was studied for sucrase-specific activity and [^{3}H]thymidine incorporation. The specific activities are shown for each cell fraction. The sucrase activity is expressed as μmol glucose per min per mg of protein and the thymidine kinase activity as percentage of the fraction with the highest activity. Results are the means for three separate ileal cell separations.

were incubated at 37 °C for the indicated times. At the end of the incubations, the cells were chilled by swirling the flask in a solid-CO_2–ethanol slurry for 1 min. Cells were then collected by centrifugation at 1200 ***g*** for 10 min.

BLM were then prepared by slight modification of a method previously described [9]. Cells were homogenized in 20 volumes of buffer B with 20 strokes of a Dounce homogenizer (B pestle). The suspension was centrifuged at 1000 ***g*** for 10 min and the pellet discarded. The supernatants were spun at 26890 ***g*** for 30 min. Pellets were resuspended in buffer B with five strokes of a Dounce homogenizer, and Percoll was added to a final concentration of 14 %. The membrane suspensions were centrifuged at 47800 ***g*** for 30 min. Gradient fractions (2.6 ml) were collected and fractions 2, 4, and 5 from the top, containing BLM and no contaminating BBM, were pooled, brought to 0.9 % NaCl and centrifuged at 230000 ***g*** for 1 h. The BLM pellets were resuspended in 20 mM Tris buffer, pH 7.5, containing 250 mM sorbitol and 1 mM sodium orthovanadate.

Because Na/K-ATPase, the marker enzyme for BLM, is inhibited by sodium orthovanadate, preliminary preparations were performed with vanadate omitted to determine which gradient fractions contained BLM, and to determine the degree of purification. Purification, as judged by enrichment of Na/K-ATPase specific activity compared with the homogenate, was 10–14-fold for the pooled fractions; these membrane fractions were essentially free of BBM, as measured by sucrase specific activity (results not shown).

Marker enzyme assay

Marker enzymes for measurement of membrane purification were sucrase [14] for apical membranes, and Na/K-ATPase [15] for basolateral membranes. Protein was measured by the Bradford procedure with ovalbumin as the standard [16]. [^{3}H]Thymidine incorporation was assayed as described [17].

Measurement of PIP_2-specific PLC activity

PLC specific activity was assayed as generation of IP_3 by addition of 2.5 μg of BBM or BLM protein to 250 μl of a reaction mixture containing 2 mM DTT, 10 mM LiCl, 500 μM ATP, 0.08 % sodium deoxycholate, 20 μM phosphatidylinositol bisphosphate (PIP_2), 6 mM $MgCl_2$, 3 mM EGTA, and 20 mM Hepes, pH 7.0, and various amounts of $CaCl_2$ (unless stated, free [Ca^{2+}] used in the assay was 10 μM). Free Ca^{2+} was calculated by a computer program as described [18]. The reaction was performed at 25 °C for 10 min and stopped by addition of trichloroacetic acid (TCA) to a final concentration of 5 % (w/v). Insoluble material was removed by centrifugation at 23000 ***g*** for 10 min. Little IP_3 could be detected if the membranes were incubated at 37 °C, probably because of increased breakdown of IP_3. Activity was normalized per μg membrane protein assayed.

When no exogenous PIP_2 was added to the reaction mixture, IP_3 formation was very close to background levels (less than 4 pmoles). With the addition of exogenous PIP_2 (20 μM), IP_3 formation was linear with respect to time (0–20 min) and membrane protein. Although equivalent basal PLC activity was found in the absence of detergent and in the presence of 0.05 % Triton X-100, 0.05 % Triton X-100 plus 0.15 % octyl glucoside, or 0.04–0.1 % sodium deoxycholate, the maximum carbachol-induced increase in PLC activity occurred when sodium deoxycholate was used. Thus the assay used contained 2.5 μg membrane protein and included exogenous PIP_2 (20 μM) and 0.08 % sodium deoxycholate.

PLC activity was also assayed in the absence of exogenous substrate under conditions where the assay was linear for protein (150 μg of membrane protein, 4 °C, 10 min). The units for the IP_3 generated under these conditions are different from those used for the assay of PLC in the presence of exogenous PIP_2 to emphasize the small amounts of IP_3 generated in the absence of exogenous PIP_2.

IP_3 assay

IP_3 was measured with the competitive binding assay of Bredt et al. [19]. TCA was removed from the samples by extracting six times with 3 volumes of diethyl ether. An aliquot of sample was mixed with [^{3}H]IP_3 and incubated with membranes from rat cerebellum containing IP_3-binding protein. The membranes were centrifuged at 23000 ***g***, the supernatant was discarded, and the pellets were dissolved in 100 % formic acid and counted in a Beckman liquid-scintillation counter. A standard curve containing 1–50 pmol of unlabelled IP_3 was studied simultaneously with the samples. A blank assay tube containing no brush border or BLM gave less than 1 pmol of IP_3 in the assay.

Immunoprecipitation of PLC-γ_1

BBM (500 μg protein) were extracted with a solution containing 1 % Triton X-100, 20 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF and 1 mM PMSF for 15 min at 4 °C. PLC-γ_1 was immunoprecipitated from the soluble extracts as described earlier [20] by using monoclonal antibodies (05-163, UBI). Immunoprecipitated proteins were separated by SDS/PAGE (7 % gel, w/v). The separated proteins were transferred to nitrocellulose (Costar) for 1 h at 100 V with a Bio-Rad TransBlot apparatus. After blocking with 5 % (v/v) non-fat milk in TBS (PBS, 13 mM Tris, 0.02 % Triton X-100), the nitrocellulose filters were probed with the same monoclonal antibodies against PLC-γ_1. Immunostained proteins were revealed with the enhanced chemiluminescence (ECL) (DuPont) detection system according to the instructions of the supplier. Molecular masses were determined by using simultaneously studied Biorad and Rainbow high-range prestained molecular mass standards.

Immunoprecipitation of tyrosine-phosphorylated proteins

Tyrosine-phosphorylated proteins were immunoprecipitated from BBM essentially as described for PLC-γ_1, except that polyclonal antibodies to phosphotyrosine (61-5800, Zymed) were used.

Immunoblot analysis of PLC-γ_1, PLC-β_1, PLC-β_3 and PLC-δ_1

BBM or BLM containing various amounts of protein were separated by SDS/PAGE (7 % gel). The proteins were transferred to nitrocellulose as described above and probed with monoclonal antibodies to PLC-β_1 (05-164, UBI), PLC-γ_1 (05-163, UBI), PLC-δ_1 (05-165, UBI) or polyclonal antibodies to PLC-β_3 [12]. Measurement of PLC concentration was by computerized densitometry (Loats Assoc., Westminster, MD, U.S.A.) with ECL under conditions whereby the PLC signal was not saturated.

Removal of detergent from extracts of PLC-γ_1 immunoprecipitates

BBM were extracted in a buffer containing 1 % (w/v) Triton X-100 and PLC-γ_1 was immunoprecipitated from the soluble extracts. Residual PIP_2-specific PLC activity was measured in the soluble extracts after PLC-γ_1 immunoprecipitation. To remove the Triton X-100, which interferes with the IP_3 assay, 1 ml Extracti-gel D columns were used. In preliminary standardization, over 70 % of the applied PLC activity was recovered in the void volume. PLC activity was measured in BBM fractions in the absence of Triton X-100, in the presence of 1 % Triton X-100 and in Triton-containing fractions after elution from the column. Triton X-100 inhibited the PLC activity by approx. 75 %. In contrast, the PLC activity in eluted fractions after passing through the Extracti-gel D column was not significantly different from the total BBM PLC activity measured before the addition of Triton X-100 (results not shown). The column therefore did not interfere with the PLC activity measurement. The column was equilibrated with 100 mM NaCl, 10 mM Tris/HCl, pH 7.4, and the proteins were eluted with the same buffer.

Preparation of IP_3-binding protein and PLC standards from rat brain

Male Sprague–Dawley rats were killed, and intact brain or cerebellum was removed immediately.

IP_3 binding protein [19]

The cerebella were homogenized in 30 volumes of 50 mM Tris buffer, pH 7.7, containing 1 mM EDTA and 1 mM 2-mercaptoethanol, with a Polytron homogenizer (setting of 8; 10 s). Membranes were centrifuged at 20000 ***g*** and resuspended in 30 volumes of the same buffer by Polytron homogenization and washed four times. The final membrane pellet was resuspended in 15 volumes of 50 mM Tris buffer, pH 7.7, containing 1 mM EDTA and 1 mM 2-mercaptoethanol and stored at −70 °C.

Preparation of PLC standards [12]

Intact rat cerebrum was homogenized in a Teflon–glass homogenizer for 1 min in a buffer containing 20 mM Tris, pH 6.8, 1 mM EDTA, 1 mM PMSF, 10 g/ml leupeptin, 10 μg/ml aprotinin. The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant was used as a standard for PLC-γ_1, PLC-β_1 and PLC-δ_1. The supernatants were stored at −70 °C and used within 30 days of preparation. Purified recombinant PLC-β_3 expressed in Sf9 insect cells was used as a standard for PLC-β_3 (S. G. Rhee, unpublished work).

RESULTS

Carbachol increases the PIP_2-specific PLC activity in BBM

Both BBM and BLM contain PIP_2-specific PLC activity. BBM had approx. 4–5-fold higher PLC specific activity than BLM (Table 1). This was true regardless of whether BBM were prepared from isolated villus cells or from scraped villus mucosa.

Carbachol exposure to sheets of ileal mucosa *in vitro* caused a rapid but short-lived increase in brush-border PLC specific activity (Table 2). The increase in specific activity occurred by 0.5 min after carbachol exposure (which is as quickly as ileal mucosa could be obtained after carbachol exposure), peaked at 1 min and returned to control values by 2.5 min after carbachol exposure, with no further change for 10 min (Table 2). A similar increase in brush-border PLC specific activity was seen when isolated ileal villus cells were first exposed *in vitro* to carbachol for 1 min before BBM preparation (49.9 ± 13.9 compared with 67.9 ± 17.9 pmol/min per μg protein for control and carbachol-exposed cells respectively; $n = 5$, $P < 0.05$).

Endogenous plasma membrane substrate for PIP_2-specific PLC activity is complexed in a manner (with profilin [22]) that makes it unavailable to inactive PLC-γ. Consequently, the above study with exogenous PIP_2 did not demonstrate that there was an

Table 1 Ileal villus cells have higher PIP_2-PLC specific activity in the BBM than BLM

Brush border and BLM were prepared from ileal villus epithelial cells and assayed for PLC activity as described in the Methods section. Isolated villus cells were used for preparation of BLM and for some BBM, whereas scraped ileal mucosa was used for the preparation of the other BBM. *n*, Number of membrane preparations studied; *P*, comparison between BLM activity and each of the BBM activities (unpaired *t*-test).

Sample	PLC specific activity (pmol per min per μg of protein)	*P* compared with BLM
BLM	11.8 ± 2.3 ($n = 10$)	–
BBM		
From scraped mucosa	56.5 ± 7.7 ($n = 14$)	< 0.0001
From isolated cells	49.9 ± 13.9 ($n = 5$)	< 0.0004

Table 2 Time course of effect of carbachol on PIP_2-PLC specific activity in ileal BBM

Ileal tissue was incubated in Ringer's/HCO_3 at 37 °C containing 1 μM carbachol or untreated control tissue for indicated times. At the end of the incubation, the tissue was chilled, mucosa removed by scraping, BBM were prepared and assayed for PLC activity as described in the Methods section (free $[Ca^{2+}] = 10$ μM). Control and carbachol-treated tissue were always processed in parallel. *n*, Number of separate matched carbachol/control BBM preparations studied; *P*, comparison of carbachol and simultaneously studied control (paired *t*-test).

Time (min)	PLC specific activity (pmol per min per μg of protein) Control	Treated	
0.5	69.1 ± 20.5	81.7 ± 17.1	$n = 5$, $P < 0.08$
1.0	66.9 ± 8.3	99.4 ± 2.2	$n = 4$, $P < 0.0008$
2.5	59.8 ± 11.8	40.0 ± 6.2	$n = 3$, n.s.
10.0	42.6 ± 0.6	39.0 ± 3.8	$n = 3$, n.s.

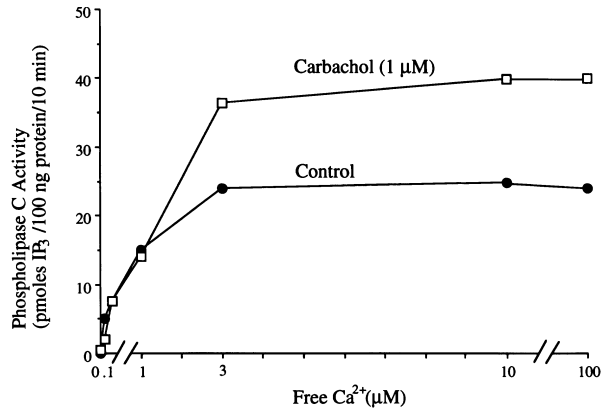

Figure 2 Effect of Ca^{2+} concentration on PLC specific activity *in vitro* in brush-border membranes from control and carbachol-exposed ileum

BBM were prepared from ileum incubated in the presence of 1 μM carbachol or from simultaneously studied control tissues. Membranes were then assayed for PLC activity as described in the Methods section. For each Ca^{2+} concentration the assay mixture contained 3 mM EGTA plus $CaCl_2$ to give the indicated free Ca^{2+} concentration. This is a representative experiment of six similar experiments.

increase in brush-border PIP_2-specific PLC activity *in vivo*. To establish that this occurred, studies were repeated with an assay of PLC performed without exogenous PIP_2. Carbachol exposure to sheets of ileal mucosa *in vitro* increased the total brush-border PIP_2-specific PLC activity at 1 min, as measured in the absence of exogenous substrate (21.1 ± 6.4 compared with 39.6 ± 6.1 pmol per 10 min per 10 μg protein in control and carbachol-treated membranes respectively; $n = 4$, $P < 0.02$). Thus the PIP_2-specific PLC activity in the brush border is elevated by carbachol.

Brush-border PLC activity was Ca^{2+}-dependent (Figure 2). There was almost no PLC activity in the absence of Ca^{2+}, a Ca^{2+}-dependent increase in activity with 0.1 μM free Ca^{2+} significantly increasing basal activity, and maximum activity observed between 3 and 10 μM free Ca^{2+}. The Ca^{2+} dose dependence of PLC activity was similar for BBM from control and carbachol-treated tissue (Figure 2). PLC activity from BBM from carbachol-treated tissues was higher than controls at Ca^{2+} concentrations greater than 1 μM. The brush-border PIP_2-specific PLC activity in control and carbachol-treated BBM was not reproducibly affected by GTP-γS (10 nM to 10 μM), AlF_4^- (10 mM NaF, 30 μM $AlCl_3$) or GDP-βS (10 nM to 10 μM) (results not shown).

That Ca^{2+} elevation did not fully explain the carbachol activation of brush-border PLC activity was further supported by studies with the Ca^{2+} ionophore, A23187. Incubation of ileal mucosa *in vitro* with A23187 at concentrations of 3 and 10 μM (sufficient to cause changes in Na^+ and Cl^- absorption in rabbit ileal Na^+-absorbing cells similar to that caused by 1 μM carbachol ([9], and results not shown) did not significantly alter brush-border PLC activity (33.0 ± 8.4 compared with 41.6 ± 11.2 pmol per min per μg protein in control and Ca^{2+} ionophore (3μM)-treated tissue, respectively, $n = 7$, n.s.).

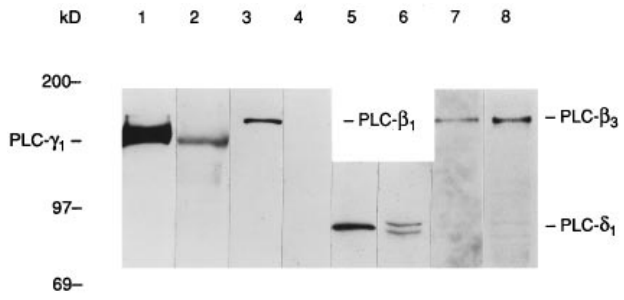

Figure 3 Ileal brush border contains PLC-γ_1 and -δ_1 and PLC-β_3 but not PLC-β_1

Immunoblot evaluating the expression of PLC isoforms γ_1, β_1, β_3 or δ_1 in rabbit ileal BBM. Rabbit ileal BBM proteins (50–500 μg) were separated by SDS/PAGE (7% gel) and probed with monoclonal antibodies to PLC-β_1, -γ_1 or -δ_1 or polyclonal antibodies to -β_3. Lanes: (1) Rat brain extract (50 μg) as a standard, showing PLC-γ_1 as a 145 kDa protein; (2) rabbit ileal BBM (50 μg protein) probed with anti-PLC-γ_1; (3) rat brain extract (50 μg protein) as a standard showing PLC-β_1 as a 150 kDa protein; (4) rabbit ileal BBM (500 μg protein) probed with anti-PLC-β_1; (5) rat brain extract (50 μg) as a standard showing the position of PLC-δ_1 as an 85 kDa protein; (6) rabbit ileal BBM (500 μg) probed with anti-PLC-δ_1; (7) PLC-β_3 standard obtained from Sf9 cells (20 ng) showing PLC-β_3 as a 152 kDa protein; (8) rabbit ileal BBM (50 μg protein) probed with anti-PLC-β_3. Molecular mass standards are shown on the left. The blot is a representative of similar results with three different membrane preparations.

Carbachol increases brush-border PLC-γ_1 amount

The PLC activity data show that PIP_2-specific PLC activity is present in the brush border and increases after exposure to carbachol. However, it provides no information about the PLC isoform involved, because all isoforms of PIP_2-specific PLC use PIP_2 as a substrate. Western blot analysis was used to identify the PLC isoforms present in the apical membrane of rabbit ileal villus epithelial cells (Figure 3). BBM proteins were separated on 7% polyacrylamide gels, transferred to nitrocellulose and probed with monoclonal antibodies to PLC-γ_1, -β_1, -δ_1 or polyclonal antibodies to PLC-β_3. BBM proteins (as little as 50 μg) demonstrated the presence of PLC-γ_1 (Figure 3). PLC-β_3 also was present in the BBM (50 μg protein; Figure 3). In contrast, PLC-β_1 was not detected in the BBM even with 500 μg membrane protein. No PLC-β_1 was detected in total cell homogenates of rabbit ileal villus cells (results not shown). A small amount of PLC-δ_1 was seen in BBM as a doublet when a large amount of membrane protein was studied (500 μg). The anti-PLC-δ_1 antibody recognized a single protein of 85 kDa in the rat brain; however, in rabbit BBM two bands between 83–87 kD were recognized. The BBM (500 μg) was treated with 25 units of alkaline phosphatase (25 °C, 5 min) to determine whether the two bands represented different phosphorylation states of the protein. Alkaline phosphatase failed to shift the mobility of the two bands.

Having established that PLC-γ_1 was the major isoform of PIP_2-specific PLC in rabbit ileal BBM, the effect of carbachol exposure on the amount of PLC-γ_1 in the BBM was determined. Rabbit ileal mucosa was treated *in vitro* with carbachol (1 μM) for 1 min, a time when PLC activity is maximally stimulated in the BBM, or 10 min, a time at which the brush-border inositol PIP_2-specific PLC activity has returned to control values. BBM were prepared from the ileal mucosa, and PLC-γ_1 was immunoprecipitated by using a monoclonal antibody. The immunoprecipitated PLC-γ_1 was separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with the same monoclonal anti-PLC-γ_1 antibody. Equal amounts of BBM protein were used from both carbachol-treated and simultaneously studied controls. Figure 4 shows the amounts of PLC-γ_1 in the BBM after 1 and 10 min of carbachol treatment, compared with their respective controls. There was an increase in the amount of PLC-γ_1 in the BBM after 1 min of carbachol treatment (385 ± 133% of control, $P < 0.05$, $n = 4$). In contrast, there was no change in the PLC-γ_1 amount compared with control after carbachol exposure for 10 min.

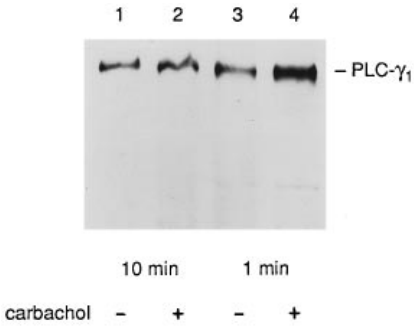

Figure 4 Carbachol increases the amount of brush-border PLC-γ_1

Immunoprecipitates of PLC-γ_1 from rabbit ileal BBM made from tissue exposed to carbachol for 1 or 10 min are shown. Rabbit ileal mucosa was treated *in vitro* with or without carbachol (1 μM) for 1 or 10 min. BBM were prepared and PLC-γ_1 immunoprecipitated from them by using a monoclonal antibody, separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with the same monoclonal antibody. Lanes: (1) BBM from control tissue incubated in Ringer's/HCO_3 for 10 min; (2) BBM from tissue incubated with carbachol for 10 min; (3) control BBM incubated in Ringer's/HCO_3 for 1 min; (4) BBM from tissue incubated with carbachol for 1 min. The position of PLC-γ_1 was identified by using a rat brain extract as standard and is shown on the right. This is a representative of seven separate experiments.

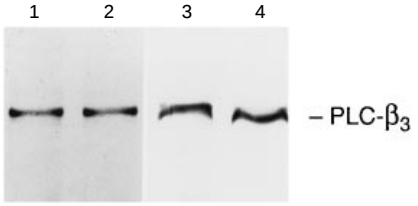

Figure 5 Carbachol does not change the amount of BBM or BLM PLC-β_3

BBM (50 μg protein) and BLM (150 μg protein) from control and carbachol-treated rabbit ileum were separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with polyclonal antibodies to PLC-β_3. Lanes: (1) BBM from control tissue; (2) BBM from tissue exposed to carbachol for 1 min; (3) BLM from control tissue; (4) BLM from tissue exposed to carbachol for 20 s. The position of PLC-β_3 was identified by using a recombinant PLC-β_3 purified from Sf9 cells. The amount of PLC-β_3 protein present in the BBM and the BLM is not comparable from this Western blot, because the two experiments were performed on separate days and the ECL exposure times were not the same. This is a representative of three separate experiments.

Carbachol exposure did not alter the amount of brush-border PLC-β_3 (Figure 5), PLC-β_1 or PLC-δ_1 (results not shown). These results suggest that the increased PIP_2-specific PLC activity in the BBM comes from an increase in the amount of PLC-γ_1 in response to carbachol.

To confirm that all the carbachol-induced increase in BBM PIP_2-specific PLC activity was due to PLC-γ_1, PLC-γ_1 was immunoprecipitated from BBM prepared from control and ileal tissue treated with carbachol for 1 min, and residual PIP_2-specific PLC activity measured in the extracts. The detergent (Triton X-100) used for the extraction of PLC-γ_1 from the membranes interferes with the binding assay for IP_3 and also inhibits PLC activity. Consequently, the detergent in the extracts was initially removed by using 1 ml Extracti-gel D columns, and PIP_2-specific PLC activity measured in the eluates. Approximately 6% of the total BBM PIP_2-specific PLC activity remained in both control (7.0 ± 0.4 compared with 115.1 ± 4.2 pmol per min per μg protein, $n = 3$) and carbachol-treated extract (11.0 ± 2.2 compared with 172.6 ± 5.7 pmol per min per μg protein, $n = 3$) after immunoprecipitating PLC-γ_1 from the BBM extracts. This demonstrates that PLC-γ_1 is the major isoform in brush border of both control and carbachol-exposed cells and shows that the increases in PIP_2-specific PLC activity after carbachol exposure come from an increase in PLC-γ_1 activity. Because 94% of the BBM PLC activity in control and carbachol-treated membranes came from PLC-γ_1, these results indicated that PLC-β_3 was unlikely to contribute significantly to the brush-border PIP_2-specific PLC activity in control and carbachol-treated tissue.

Carbachol fails to alter BLM PIP_2-dependent PLC activity

PLC activity was measured in ileal villus cell BLM at short periods after carbachol exposure, because any biological signal relevant to regulation of brush-border PLC activity should have preceded PLC changes in the BBM. Exposure of ileal cells to carbachol for 20 s produced no change in BLM PLC activity (6.7 ± 2.3 compared with 6.9 ± 2.3 pmol per min per μg protein in control and carbachol-treated respectively, $n = 3$, n.s.). No carbachol-induced changes in BLM PLC activity were observed whether 1 mM sodium orthovanadate was added to (12.3 ± 2.2 compared with 13.2 ± 3.1 pmol per min per μg protein in control and carbachol-treated respectively, $n = 4$, n.s.) or omitted from (data above) the tissue incubation solution. Also direct addition of carbachol (1 μM) to the buffers used to prepare the membranes in addition to initial exposure to tissue, or direct addition of carbachol to the PLC assay mixture, had no effect on PLC activity. Neither PLC-γ_1 nor PLC-β_1 was identified when up to 500 μg of BLM of control and carbachol-treated (20 s) BLM were studied by Western blots. A small amount of PLC-δ_1 was detected in the BLM only when 500 μg or more of membrane protein was studied (results not shown). PLC-β_3 was present in the BLM (Figure 5), although less than in the BBM, and the amount did not change with 20 s of carbachol exposure. Also PIP_2-specific PLC activity measured in the BLM was unaffected by the presence of GTP-γS, or AlF_4^- in control and carbachol-exposed BLM (results not shown).

Basolateral and brush-border membranes contain similar 5-phosphatase activities

To determine whether the BBM and the BLM contain differing amounts of 5-phosphatase activities that would degrade IP_3 to IP_2 and therefore underestimate the PLC activity, the following experiment was performed. The PLC specific activity was measured in BBM and BLM in the absence of exogenous PIP_2. Under these conditions, the amount of IP_3 generated by 2.5 μg of BBM or BLM protein is between 1 and 3 pmoles. The membranes were then treated with 20 pmoles of unlabelled IP_3, and the amount of IP_3 was measured as in the PLC assay described in the Methods section. The amount of IP_3 in the BBM and BLM was not significantly different in both control and carbachol-treated membranes (20.5 ± 0.8 compared with 21.7 ± 2.1 pmol per min per μg protein in control and carbachol-treated BBM, respectively, $n = 3$; and 22.8 ± 2.1 compared with 22.9 ± 0.6 pmol per min per μg protein in control and carbachol-treated BLM, respectively, $n = 3$). Thus differences in basal and carbachol-activated PLC activity are not due to differences in 5-phosphatase activity in the two membranes.

Effects of carbachol on active ileal electrolyte transport involve a tyrosine kinase

As previously reported [8], carbachol (1 μM) added to the ileal serosal surface inhibited active Na^+ and Cl^- absorption as indicated by the $^{22}Na/^{36}Cl$ flux studies (Figure 6, middle and bottom panels: note carbachol-induced inhibition of mucosal-

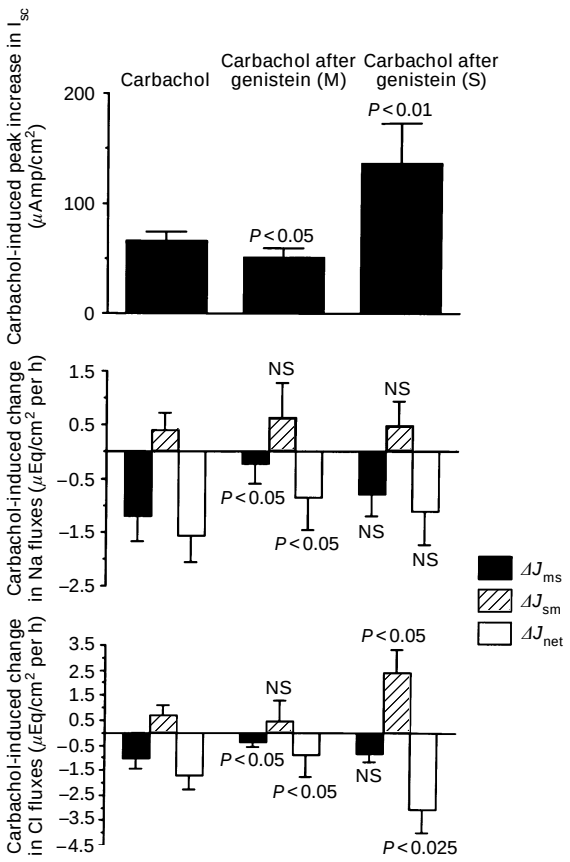

Figure 6 Mucosal but not serosal genistein inhibits the carbachol stimulation of Cl^- secretion and inhibition of Na^+ and Cl^- absorption

Ileal mucosa was exposed under voltage-clamped conditions to 1 μM carbachol on the serosal surface and the effect determined over two 20 min flux periods with determination of mucosal-to-serosal flux (J_{ms}) and serosal-to-mucosal flux (J_{sm}) of ^{22}Na and ^{36}Cl (net fluxes represent $J_{ms} - J_{sm}$). Studies were performed in the absence of genistein and in tissue pretreated for 40 min with genistein (100 μM) on the mucosal or serosal surfaces. In the top panel is shown the peak increase in I_{sc} after carbachol, which represents electrogenic Cl^- secretion. In the middle panel is shown the effect of carbachol on Na^+ fluxes (average of two 20 min flux periods in the presence of carbachol minus two 20 min flux periods in the same tissue before carbachol addition). In the bottom panel is shown the effect of carbachol on Cl^- fluxes obtained simultaneously with Na^+ fluxes. Tissue from five animals was studied. *P* values represent comparison of effects of carbachol in the absence and in the presence of genistein (paired *t*-test).

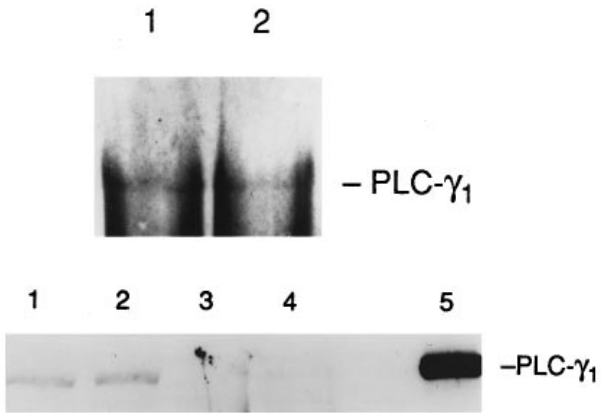

Figure 7 Carbachol does not increase tyrosine phosphorylation of PLC-γ_1

Upper panel: PLC-γ_1 was immunoprecipitated from rabbit ileal BBM made from control and tissue exposed to carbachol for 1 min by using a monoclonal antibody against PLC-γ_1 as described in the Methods section. The immunoprecipitated proteins were separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with a polyclonal antibody against phosphotyrosine. Lanes: (1) BBM from control tissue incubated in Ringer's/HCO_3 for 1 min; (2) BBM from tissue incubated with carbachol for 1 min. This is a representative of six separate experiments. Lower panel: Tyrosine-phosphorylated proteins were immunoprecipitated from rabbit ileal BBM made from control and tissue exposed to carbachol for 1 min by using a polyclonal antibody against phosphotyrosine. The supernatant from the immunoprecipitate, containing proteins that were not precipitated with the anti-phosphotyrosine antibody and the immunoprecipitated proteins, each were separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with monoclonal antibodies against PLC-γ_1. Lanes: (1) supernatant from control tissue, containing proteins that are not tyrosine-phosphorylated; (2) supernatant from carbachol-treated tissue, containing proteins that are not tyrosine-phosphorylated; (3) BBM from control tissue immunoprecipitated with anti-phosphotyrosine; (4) BBM from tissue incubated with carbachol for 1 min, immunoprecipitated with anti-phosphotyrosine; (5) rat brain standard showing the position of PLC-γ_1. This is a representative of three separate experiments.

to-serosal and net fluxes of Na^+ and Cl^-) and caused electrogenic Cl^- secretion as indicated by the increase in short-circuit current (Figure 6, top panel). Pretreatment with genistein (100 μM) added to the mucosal surface (Figure 6, middle and lower panels) significantly inhibited both the carbachol inhibition of Na^+ and Cl^- absorption (in the presence of mucosal genistein, carbachol caused a smaller decrease in mucosal-to-serosal and net Na^+ and Cl^- fluxes) and the increase in short-circuit current (top panel). In contrast, genistein on the serosal surface (Figure 6, middle and bottom panel) did not alter the carbachol effect on Na^+ and Cl^- absorption (in the presence of serosal genistein, carbachol caused a similar decrease in mucosal-to-serosal Na^+ and Cl^- fluxes and net Na^+ fluxes) but increased Cl^- secretion. Thus a tyrosine kinase inhibitor causes different effects on carbachol-induced changes in active ileal electrolyte transport when added to the ileal mucosal and the ileal serosal surfaces. Further studies pursued the mechanism of the mucosal genistein effect. Owing to the presence of multiple cell types affected with serosal addition of genistein, the mechanism of the serosal genistein effect was not pursued further.

The catalytic activation of PLC-γ_1 has been suggested to occur through tyrosine phosphorylation [21,22]. We studied the tyrosine phosphorylation of BBM PLC-γ_1 after treatment of ileal tissue with carbachol (Figure 7). PLC-γ_1 was immunoprecipitated from control BBM and BBM from carbachol-treated tissue. The immunoprecipitated proteins were separated by SDS/PAGE (7% gel), transferred to nitrocellulose and probed with polyclonal antibodies to phosphotyrosine. No significant change was seen in the tyrosine phosphorylation of PLC-γ_1 in carbachol-treated BBM compared with control BBM (Figure 7a). In a separate study, tyrosine-phosphorylated proteins were immunoprecipitated from BBM of control and carbachol-treated ileum for 1 min by using an anti-phosphotyrosine polyclonal antibody, and both the immunoprecipitates and supernatants (containing proteins that were not tyrosine phosphorylated) were separated on a 7% polyacrylamide gel, transferred to nitrocellulose and probed with monoclonal antibodies to PLC-γ_1. Figure 7b shows that only a very small fraction of the total PLC-γ_1 translocated to the BBM was tyrosine phosphorylated.

To demonstrate that a tyrosine phosphorylation event is nevertheless essential for the increase in the brush-border PIP_2-specific PLC activity after carbachol treatment, rabbit ileal loops were exposed *in vivo* to 100 μM genistein on the luminal surface for 1 h and the subsequent effect of 1 μM carbachol exposure *in vitro* to this tissue for 1 min was determined on BBM

PLC activity and PLC-γ_1 amount. BBM were prepared from simultaneously studied control and carbachol-treated ileum, all initially exposed *in vivo* to genistein. PLC-γ_1 was immunoprecipitated from these membranes for determination of amount, and PIP_2-specific PLC activity was separately measured. In the presence of genistein, carbachol exposure did not have an effect on the BBM PLC-γ_1 amount (results not shown) or PLC specific activity (57.1 ± 6.5 pmol per min per μg protein in control compared with 59.3 ± 10.6 pmol per min per μg protein in carbachol-treated, $n = 3$, n.s.). The carbachol-induced increase in brush-border PLC activity, measured in the absence of exogenous substrate, was also inhibited by the addition of genistein (28.8 ± 10.0 compared with 23.3 ± 10.3 pmol per min per μg protein in control and carbachol-treated membrane, respectively, $n = 4$, n.s.). These data suggest that a tyrosine kinase-mediated event is essential for both the increase in BBM PLC-γ_1 amount and PLC activity after carbachol treatment.

DISCUSSION

This study shows that carbachol-initiated signal transduction, which inhibits NaCl absorption in intestinal epithelial cells, is highly asymmetrical. It was previously known that carbachol acts on intestinal epithelial cells via BLM receptors currently classified as M_3 [23]; these are linked to inhibition of NaCl absorption and of brush-border Na^+/H^+ exchange, which is part of this Na^+ absorptive process [8–10]. Brush-border protein kinase C mediates this inhibition of NaCl absorption and Na^+/H^+ exchange [9]. Previously recognized steps in carbachol-initiated signal transduction at the ileal brush border include brush-border translocation of protein kinase C along with an increase in brush-border DAG content by 1 min after carbachol exposure. The asymmetrical aspect of signal transduction previously recognized was that there was no increase in BLM protein kinase C after carbachol treatment [9]. Because DAG and elevated cytosolic $[Ca^{2+}]$ (usually released from IP_3-sensitive stores) are necessary for most stimulation of protein kinase C, we hypothesized that a brush-border PLC might play a role in this signal transduction pathway. In fact in another intestinal cell, the Cl^- secretory cell line T84, carbachol elevates IP_3 [7].

In this study we demonstrate that carbachol causes a rapid but short-lived increase in brush-border but not BLM PIP_2-specific PLC activity, with an increase as early as the villus cells could be obtained (30 s), a peak at 1 min and a return to baseline by 2.5 min. This increase in PIP_2-specific PLC activity was accompanied by an increase in the amount of PLC-γ_1 in the brush border, suggesting that at least part of the increase in activity is due to translocation of PLC-γ_1 to the apical membrane. The mechanism for the increased PLC activity and the increase in PLC-γ_1 amount in brush border from cells exposed to carbachol seems to involve a tyrosine kinase because mucosal exposure to the tyrosine kinase inhibitor genistein prevents the carbachol-induced increase in brush-border PLC activity and PLC-γ_1 amount. A similar observation of dependence on tyrosine phosphorylation of plasma membrane location of PLC-γ was made by Nakanishi et al. [25]. They found that genistein caused the PLC-γ_1 and phosphoinositide-specific PLC activity to transfer from the membrane to the cytosolic fraction. The involvement of brush-border tyrosine phosphorylation in the carbachol effect on ileal NaCl absorption is further supported by the inhibition of carbachol regulation of NaCl absorption by apical exposure to genistein, whereas genistein did not inhibit the carbachol effect on Na^+ and Cl^- absorption when exposed to the serosal surface (Figure 6). However, whether this observation involves a direct tyrosine phosphorylation of PLC-γ_1 is unknown but unlikely because we could not demonstrate an increase in BBM PLC-γ_1 tyrosine phosphorylation after carbachol treatment. Thus the mechanism by which a tyrosine phosphorylation event regulates BBM PIP_2-specific PLC activity is unknown. For instance, PLC-γ_1 could have been recruited via its SH2 domains to the apical membrane via tyrosine phosphorylation of another protein. Along a similar line is the recent suggestion that epidermal growth factor-mediated tyrosine phosphorylation of PLC-γ in hepatocytes seems to be an insufficient signal for activation of PIP_2-dependent PLC activity [24].

PLC-γ_1 is a Ca^{2+}-dependent enzyme, and carbachol increases ileal cytosolic free $[Ca^{2+}]$ in intestinal epithelial cells [3]. However, elevated $[Ca^{2+}]_i$ is not the only signal needed to increase brush-border PLC activity. This is shown by the failure of Ca^{2+} ionophore to increase brush-border PLC activity when used under conditions that caused changes similar to those caused by carbachol in ileal NaCl transport and brush-border protein kinase C translocation [9,10]. This observation is further supported by the Ca^{2+} dose response of brush-border PLC activity *in vitro*, which shows that PLC activity from brush border of carbachol-treated tissues remains higher than PLC of BBM from control tissue, even at high Ca^{2+} concentrations. If elevated $[Ca^{2+}]$ were the only signal causing the increase in PLC activity, it would be expected that the PLC activity in control and carbachol-treated membranes would converge as the Ca^{2+} concentrations were increased. However, with regard to the last point, it cannot be excluded that the effect of Ca^{2+} on substrate hydrolysis *in vitro* is related (at least in part) to lipid presentation. These results indicate that the carbachol-induced increase in brush-border PLC activity and amount occur at least partly at a step in signal transduction before the increase in $[Ca^{2+}]_i$.

This delineation of apical membrane events in ileal Na^+-absorbing cells almost certainly applies to several other epithelial cells that respond to carbachol. In pancreatic acinar cells, carbachol triggers an increase in $[Ca^{2+}]_i$ in the apically located secretory granule area, which then spreads to the BLM area where the receptors for the agonists are located [26,27]. In similar studies done with hepatocytes, lachrymal gland cells and MDCK cells (a renal cell line), carbachol also elevates $[Ca^{2+}]$ initially at the apical membrane, with waves spreading over the rest of the cell [28]. This phenomenon has been attributed in pancreatic acinar cells to the presence of high-affinity IP_3 receptors in the secretory granule area and lower-affinity receptors in the rest of the cell [29–31]. In intestinal cells (the colon cancer cell line HT-29) a high-sensitivity IP_3-responsive intracellular Ca^{2+} pool (called type 3 receptors and which have been suggested as being epithelial specific), is localized near the apical membrane [29]. In immunocytochemical localization studies done on adult rat jejunum, type 3 IP_3 receptors were prominently stained in the cytoplasm adjacent to the brush border [29]. We suggest that the asymmetric, localized activation of PLC-γ_1 described here in the brush border may act on this apically localized IP_3-sensitive pool to further create an asymmetrical $[Ca^{2+}]_i$ signal centreing on the apical membrane. Furthermore the assumption that carbachol-induced apical elevation of $[Ca^{2+}]_i$ was due to diffusion of BLM IP_3 to apical membrane, which seemed inefficient, needs to be reexamined. The steps in carbachol-related signal transduction proposed here incorporate not only the local Ca^{2+} stores but also the creation of a high local accumulation of IP_3, coming from the brush border, rather than requiring IP_3 diffusion from the BLM.

The data presented in this paper suggest that brush-border PIP_2-specific PLC may be involved in the initial stimulation of brush-border PKC, but not the prolonged PKC elevation and effects on NaCl absorption that occur after carbachol treatment [9]. Both brush-border PIP_2-specific PLC and PKC are elevated

at 1 min after carbachol treatment, but PKC activity remains elevated for at least 40 min after carbachol treatment [9] whereas PLC activity returned to control levels by 2.5 min after carbachol. This suggests that another enzyme may be involved in the prolonged effects of carbachol. In fibroblasts, thrombin causes an initial increase in PIP_2-specific PLC followed by a prolonged elevation of phosphatidylcholine-dependent PLC [32]. Recently, phospholipase D involvement in prolonged Ca^{2+}-related signal transduction has been emphasized [33]. Our previous ileal studies with carbachol showed that once PKC had translocated to the BBM, Ca^{2+} was no longer needed to keep the enzyme on the membrane, so only a brief spike of elevated Ca^{2+} might be needed [9]. In fact the ileal apical localization of the increase in PLC-γ_1, and the apical presence of high-sensitivity IP_3 stores, could explain the asymmetric rise in PKC acitivity seen with carbachol. A brief localized apical increase in PLC and apical calcium release would be expected to activate Ca^{2+}-dependent enzymes at the apical membrane and not at the basolateral membrane.

The PIP_2-specific PLC isoform involved in ileal carbachol-related signal transduction is PLC-γ_1, with no evidence of involvement of other isoforms. PLC-γ_1 makes up approx. 94% of brush-border PLC activity as assayed in both control and carbachol-exposed brush border. Although PLC-δ_1 and PLC-β_3 but not PLC-β_1 (PLC-β_2 was previously shown not to be present in rat small intestine [34]) are present in the ileal apical and BLM, the former occurs in only small amounts and neither PLC-δ_1 nor PLC-β_3 change in amount with carbachol exposure in the apical or BLM. Further, while PLC-β_3 is present in both the BBM and BLM, no G-protein-stimulatable PIP_2-specific PLC activity could be measured in either BBM or BLM in control conditions or after carbachol treatment, suggesting that PLC-β_3 is not the isoform accounting for the increase in brush-border PLC activity.

Others have reported that the intestinal carbachol receptor is coupled to phosphoinositide metabolism, although there was no evidence of a physical link between the carbachol receptor and PLC [35]. However, those studies only measured IP_3 accumulation in intact cells minutes after carbachol treatment and did not test whether the relationship between carbachol binding and phosphatidylinositol (PI) metabolism was direct or indirect. The linkage of a muscarinic receptor and PLC-β_1 was demonstrated by reconstitution of receptor-catalysed PLC activity with incorporation of M1 receptor, PLC-β_1 and the heterotrimeric G-protein Gq/11 [36]. In the present study we have shown that increased IP_3 production occurs in a different compartment from the carbachol receptor and the link between the carbachol receptor and PI metabolism is indirect. Relevant to this study, ileal BLM contains the carbachol receptor but does not contain PLC-γ_1 or -β_1, and probably not -β_2 (and has only PLC-δ_1 and PLC-β_3). No linkage of carbachol receptors to PLC-δ_1 has yet been reported. How carbachol receptors in the BLM signal the apical membrane to recruit kinase(s) activity, increase PIP_2-specific PLC activity and eventually inhibit NaCl absorption via protein kinase C is not known. The signal is not BLM PIP_2-specific PLC. Also the fact that BLM PLC basal activity is so much lower than that in the apical membrane suggests that apical PLC activity is more important in signal transduction. In a study done by Vaandrager et al. [37] in rat jejunum, a similar observation was made that BBM contained higher amounts of PIP_2 and PLC activity than BLM. The carbachol-induced basolateral signal is not elevated $[Ca^{2+}]_i$. Ca^{2+} originating at the basolateral membrane would not be expected to reach the apical membrane, and A23187, which presumably elevates $[Ca^{2+}]_i$ throughout the cell, did not duplicate the activation of brush-border PLC. The signal also does not seem to be a basolateral increase in tyrosine phosphorylation because basolateral genistein did not inhibit the carbachol effect on Na^+ and Cl^- absorption. However, there are multiple other possibilities for the method by which the BLM communicates with the apical membrane after carbachol treatment. Some possibilities include: (1) lipid movement along the inner plasma membrane leaflet, but phosphatidic acid and arachidonic acid are unlikely to be involved because they fail to affect ileal NaCl transport when added from either the ileal mucosal or serosal surface and cause no effect on brush-border Na^+/H^+ exchange (M. Donowitz, M. E. Cohen and G. W. G. Sharp, unpublished work); (2) mitogen-activated protein kinase cascade or other protein kinase cascades; (3) cytoskeletal rearrangement, although there is no effect of the microfilament or microtubule inhibitors cytochalasin D, phalloidin or colchicine on the carbachol-induced changes in ileal NaCl absorption (M. Donowitz, M. E. Cohen and G. W. G. Sharp, unpublished work); (4) activation of brush-border tyrosine kinase(s) and/or tyrosine phosphatase(s). Although the latter is likely to be an immediate upstream event responsible for PLC-γ_1 activation, the identity of the brush-border tyrosine kinase/tyrosine phosphatase involved is not known. Nor is it known how the basolateral carbachol receptor causes an increase in brush-border tyrosine phosphorylation. However, these studies suggest how important it is to define both brush-border and BLM events to understand signal transduction in polarized cells.

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