Characterization of phosphoinositide-specific phospholipase C in rat colonocyte membranes

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The phosphoinositide signal transduction pathway mediates important processes in intestinal physiology, yet the key enzyme, phosphoinositide-specific phospholipase C (PI-PLC), is not well-characterized in the colon. PI-PLC activity was examined in rat colonic membranes using exogenous [^3H]phosphatidylinositol 4,5-bisphosphate (PIP_2) as substrate, and β -glycerophosphate to suppress degradation of substrate or product. The activity of membrane PI-PLC increased 6-fold with the addition of alamethicin, and a further 2–3-fold enhancement was observed with 10 μM guanosine 5'-[γ -thio]triphosphate (GTP[S]), suggesting the involvement of G-protein(s). The effect of GTP[S] appeared to be specific, as up to 100 μM adenosine 5'-[γ -thio]-

triphosphate failed to stimulate PI-PLC activity, and guanosine 5'-[β -thio]diphosphate inhibited activity. The response of membrane PI-PLC to Ca^{2+} was biphasic, while $> 0.5 \text{ mM}$ Mg^{2+} was inhibitory with or without GTP[S]. Comparable total PI-PLC activities and responses to GTP[S] and Ca^{2+} were observed in purified brush-border and basolateral membranes. Western immunoblots probed with monoclonal antibodies to PLC isoenzymes PLC- $\beta 1$, $\gamma 1$ and $\delta 1$ demonstrated that these antipodal plasma membranes contain predominantly the PLC- $\delta 1$ isoform, with small amounts of PLC- $\gamma 1$ present but no detectable PLC- $\beta 1$. PLC- $\gamma 1$ was the major isoform detected in cytosol.

INTRODUCTION

There is mounting evidence that the phosphoinositide signal transduction system plays a major role in mediating important events in intestinal physiology. Processes such as ion transport and secretion, regulation of intracellular pH [1–6], cellular proliferation and carcinogenesis [7,8] have been associated with some of the key steps in the phosphoinositide cascade. In addition, our laboratory and others have reported evidence that phosphoinositide signal transduction in the intestine is activated by 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$] [9–13]. This phenomenon is dependent on the developmental age [13] and the vitamin D status [10] of the animal.

Phosphoinositide-specific phospholipase C (PI-PLC) is the key enzyme in the initiation of phosphoinositide signal transduction. By catalysing the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), it causes the intracellular release of inositol trisphosphate (IP_3) and diacylglycerol. These second messengers result respectively in an increased intracellular calcium concentration, and activation and translocation of cytosolic protein kinase C. Multiple isoenzyme forms of PI-PLC, with different properties and subcellular localizations, have now been identified in a variety of tissues [14,15]. PI-PLC isoform $\beta 1$ has been shown to be activated specifically by the G-protein isoform G_q [16], an example of the high degree of signalling specificity which is obtainable.

Investigations of PI-PLC in the colon have been limited to the study of cultured colonic cancer cell types [17,18]. To our knowledge, PLC isoenzymes have not been systematically investigated in normal colonic mucosa, nor has PI-PLC activity been assessed.

In this paper, we report for the first time detailed studies of PI-PLC activity in rat colonocyte microsomes, brush-border membranes (BBM) and basolateral membranes (BLM) using exogenous PIP_2 as substrate. In addition, we characterize the isoenzymes of PI-PLC present in colonic antipodal membranes and subcellular fractions, using immunologically distinct monoclonal antibodies to PLC- $\beta 1$, $\gamma 1$ and $\delta 1$.

MATERIALS AND METHODS

Materials

The following were purchased: [$\text{inositol-2-}^3\text{H}(\text{n})$] PIP_2 (5 $\mu\text{Ci}/\text{mmol}$) and IP_3 radioreceptor assay kit NEK-064 from New England Nuclear (Boston, MA, U.S.A.); phosphatidylinositol (PI) from Serdary Research Laboratories (Port Huron, MI, U.S.A.); ammonium salts of PIP_2 and phosphatidylinositol 4-phosphate (PIP), lithium salts of guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[β -thio]diphosphate (GDP[S]), sodium cholate and octyl β -D-glucoside from Boehringer Mannheim (Indianapolis, IN, U.S.A.); prestained molecular mass markers, Bio-Rad Protein Assay Kit and Tween-20 from Bio-Rad (Richmond, CA, U.S.A.); EGTA from Fluka (Ronkonkoma, NY, U.S.A.); Immobilon-P from Millipore (Bedford, MA, U.S.A.); Budget-Solve from RPI (Mount Prospect, IL, U.S.A.); monoclonal antibodies to PLC isoforms from UBI (Lake Placid, NY, U.S.A.); ECL Western Blotting Detection System from Amersham (Arlington Heights, IL, U.S.A.); 0.1 M calcium standard and calcium-sensitive electrode from Orion Research (Boston, MA, U.S.A.); 1,25($\text{OH})_2\text{D}_3$ from Steroids, Ltd. (Chicago, IL, U.S.A.); buffer salts from BRL (Gaithersburg, MD, U.S.A.) or Sigma (St. Louis, MO, U.S.A.); and adenosine

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; IP_3 , *myo*-inositol 1,4,5-trisphosphate; IP_2 , *myo*-inositol 1,4-bisphosphate; IP_1 , *myo*-inositol monophosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PI-PLC, phosphoinositide-specific phospholipase C; PMSF, phenylmethanesulphonyl fluoride; BBM, brush-border membranes; BLM, basolateral membranes; 1,25($\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol; TBS, Tris-buffered saline.

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5'-[γ -thio]triphosphate (ATP[S]) lithium salt, β -glycerophosphate, phenylmethanesulphonyl fluoride (PMSF), bethanechol, alamethacin, sodium deoxycholate and 1 M MgCl_2 solution from Sigma.

Preparation of colonic microsomal membranes, BBM and BLM

Male Sprague-Dawley rats weighing approx. 250 g were routinely used for these studies. The animals were fasted overnight, with access to water, and killed the following morning. The colons of three to six rats were removed and colonic epithelial cells, relatively devoid of goblet cells, were obtained using a technique involving mild mechanical dissociation and bivalent cation chelation as previously described [19]. The cells were then homogenized and used as starting material for the isolation and purification of microsomal membranes [9], BBM [20] and BLM [19]. The purity of the antipodal plasma membrane preparations was assessed by assaying appropriate marker enzymes as described [20]. The specific activity ratios [(purified membrane)/(original homogenates)] for cysteine-sensitive alkaline phosphatase, a BBM marker [20], ranged from 10 to 12 in BBM preparations; whereas those of Na^+ - K^+ -ATPase, a BLM marker [20], ranged from 9 to 13 in BLM preparations. In agreement with our previous studies, BBM and BLM were found to be minimally cross-contaminated by the other plasma membranes, i.e. BBM Na^+ - K^+ -ATPase specific activity ratios ranged from 0.5 to 1.5, and BLM cysteine-sensitive alkaline phosphatase activity ratios ranged from 0.6 to 1.3. Moreover, each of these antipodal plasma membrane preparations was also found to be minimally contaminated by microsomal and mitochondrial membranes, as assessed by the specific activity ratios of NADPH-cytochrome *c* reductase and succinic dehydrogenase respectively, which ranged from 0.6 to 1.5 in each of these preparations.

Membrane fractions were suspended in 50 mM mannitol and 2 mM Hepes, pH 7.0, saturated with PMSF, and adjusted to a protein concentration in the range 1–5 mg/ml. Protein content was measured by the Bio-Rad (Bradford) assay using BSA as a standard, and aliquots were frozen at -80°C . No loss of PLC activity occurred with freezing for several months. Aliquots of membrane suspensions used for Western immunoblotting were boiled for 3 min in Laemmli SDS sample buffer and frozen until use.

Assay for PI-PLC activity

PI-PLC activity was assayed by measuring the formation of total radiolabelled inositol phosphates generated from [^3H]PIP $_2$. The standard assay was as follows: 60 μg of alamethicin in 4 μl of ethanol was added to 75 mm $\times$ 12 mm polyethylene tubes, and the ethanol was allowed to evaporate. A membrane suspension of 5 μg in 0.02 ml, with or without GTP[S] (0–200 μM), was added to the tubes followed by 0.04 ml of 2.5 $\times$ concentrated buffer (final assay concentrations: 1 mM EGTA, 10 mM Hepes, 12 mM LiCl, 88 mM KCl, 0.5 mM MgCl_2 , 25 mM β -glycerophosphate and the indicated concentrations of calcium; the pH was precisely adjusted to 7.000 ± 0.005 with KOH or HCl). After preincubation of enzyme and buffer at 32°C for 10 min in a shaking water bath, the reaction was initiated by the addition of 0.04 ml of 2.5 $\times$ concentrated substrate (final assay concentration of 25–50 μM PIP $_2$ containing ~ 20000 d.p.m. of [^3H]PIP $_2$). Substrate was prepared fresh by mixing the radiolabelled PIP $_2$ in solvent carrier with an aqueous solution of

the ammonium salt of PIP $_2$, followed by repeated vortex-mixing under a gentle stream of argon to drive off the solvent. Ca^{2+} in the buffer was adjusted as described by Bers [21], which was validated for our assay conditions using an Orion 811 meter and a Ca^{2+} -sensitive electrode. In the test solutions for calcium measurements, 12 mM LiCl was replaced by 12 mM KCl, since Li interferes with the signal stability of the Ca^{2+} -sensitive electrode. The inclusion of 25 mM β -glycerophosphate, up to 200 μM GTP[S] or ATP[S] and up to 5.0 mM MgCl_2 did not appreciably alter the Ca^{2+} concentration in the system, provided that the pH was strictly controlled. Following timed incubations, usually 10 min at 32°C in a shaking water bath, the reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1, v/v) and 0.5 ml of 1 M HCl. The tubes were capped, vigorously vortex mixed, and the phases separated by centrifugation. A 1 ml portion of the top layer (aqueous phase), containing [^3H]inositol phosphates, was transferred to a scintillation vial. Budget-Solve scintillation cocktail (10 ml) was added, and the radioactivity (in d.p.m.) was measured in a Packard TriCarb scintillation system. Total counts in reagent blanks and samples were measured and used in the computation of PLC activity, which was expressed as pmol of [^3H]inositol phosphates produced/min per μg of protein. The reaction was linear under standard assay conditions with respect to protein concentration and time (results not shown).

In selected assays, using ~ 80000 d.p.m. of [^3H]PIP $_2$ (25 μM PIP $_2$) per reaction, separation and quantification of IP $_3$, *myo*-inositol 1,4-bisphosphate (IP $_2$) and *myo*-inositol monophosphate (IP $_1$) partitioning in the aqueous phase was achieved by anion-exchange chromatography as previously described [9]. The integrity of the substrate [^3H]PIP $_2$ during the course of the assay was evaluated by t.l.c. of the organic phase, using methods reported by our laboratory [9]. Assay conditions were varied in these experiments to include low and high Ca^{2+} concentrations (0.18 and 100 μM), 0 or 10 μM GTP[S], 0 or 5 mM Mg^{2+} , 2 or 5 μg of microsomal protein, and a 5 or 10 min reaction time. Reagent blanks containing no added membranes were also analysed, and blank values subtracted where appropriate. Alamethicin (60 μg) was present in all assays.

In separate experiments, production of IP $_3$ by PI-PLC from endogenous PIP $_2$ in colonic membranes was assayed by a radio-competitive binding kit, after extraction of IP $_3$ from membranes by the method of Palmer et al. [22]. Assay conditions were as described above, except that exogenous PIP $_2$ was omitted and the amount of protein was increased 4–6-fold. Reactions were initiated by the addition of buffer/cofactor solutions to the membrane sample, with subsequent transfer from ice to a 37°C shaking water bath.

Immunocharacterization of PI-PLC Isoenzymes

Rat colonocyte homogenates, cytosol, microsomal membrane, BBM and BLM fractions were prepared. Aliquots of each fraction were boiled for 3 min in Laemmli SDS sample buffer. The sucrose concentration in the BBM and BLM fractions was decreased by dialysis against additional SDS buffer, and protein was assayed by the method of Schaffner and Weissmann [23]. Protein (30 μg) was resolved by SDS/PAGE (7.5% resolving gel). Prestained molecular mass markers were run in an adjacent lane to permit molecular mass determinations, and rat brain homogenate was used as a reference source of the PLC isoenzymes. Proteins were electroblotted at 4°C to Immobilon-P membranes in 25 mM Tris/HCl, pH 8.3, 192 mM glycine and 20% methanol at 50 mA for 12 h by the method of Towbin et al. [24]. The membranes were subsequently stained with 0.05% India ink to confirm equal protein loading and transfer. The

membranes were incubated for 2 h at room temperature in Tris-buffered saline (TBS) containing 150 mM NaCl and 50 mM Tris/HCl, pH 7.5, and 0.05 % Tween-20, which had been brought to 5 % (w/v) non-fat dry milk to block non-specific antibody binding. After four washes in TBS, the blots were incubated for 15 h at 4 °C with isoenzyme-specific monoclonal anti-PLC antibodies to PLC- β 1, PLC- δ 1 or PLC- γ 1. The antibodies (0.125 mg/ml) were diluted 1:4000 in TBS containing 1 % BSA prior to incubation. The blots were washed in TBS containing 0.5 M NaCl, and then washed three times in TBS. After washing, the blots were incubated with horseradish peroxidase-coupled sheep anti-mouse antibodies (1 mg/ml) diluted 1:3000 in TBS prior to incubation. Antigen was detected with xerography using enhanced chemiluminescence following the procedure recommended by the manufacturer. Antigen was quantified by scanning densitometry of the xerograms. The chemiluminescence reaction was linear with respect to protein loading in the range 10–50 μ g, and was found to give comparable results to detection with 125 I-Protein A, but was more sensitive.

Statistical analyses

Minitab Statistical Software (State College, PA, U.S.A.) was used to perform Student *t*-test comparisons, analysis of variance and Dunnett's multiple comparisons, as appropriate.

RESULTS

Integrity of substrate and nature of product

After exposure of the substrate, [3 H]PIP $_2$ (25 μ M), to a variety of assay conditions, more than 85 % of the radiolabelled phosphoinositides chromatographed as PIP $_2$, 7–11 % as PIP and < 3.2 % as PI. Product inositol phosphates were 63–84 % IP $_3$, 16–37 % IP $_2$ and < 0.5 % IP $_1$ depending on assay conditions, as described in the Materials and methods section. Taken together, these data indicate that the substrate PIP $_2$ remained largely intact over the range of experimental conditions employed in our studies. In addition, the predominant reaction product was IP $_3$, with a subsequent minor conversion to IP $_2$. To further validate our assay for PI-PLC using exogenous substrate, we measured directly the IP $_3$ generated from endogenous PIP $_2$ in colonic membrane preparations. Since β -glycerophosphate, which is required to suppress lipid phospholipases and phosphatases, interfered with the competitive binding assay, assay sensitivity was reduced. Nevertheless, the assay of PI-PLC activity using endogenous substrate paralleled that employing exogenous substrate. At 0.182 μ M Ca $^{2+}$, IP $_3$ production was barely detectable (40 pmol/5 min per mg of protein). At 42 μ M Ca $^{2+}$, IP $_3$ production was 75 pmol/5 min per mg of protein, and this increased to 86 pmol/5 min per mg of protein on addition of 10 μ M GTP[S].

PI-PLC activity in colonic microsomal membranes, BBM and BLM

Effect of alamethicin and bile acids

In order to expose latent enzyme activity and to demonstrate a response to stable analogues of GTP, a number of investigators have added various types of detergents to their assays of PI-PLC [25]. For colonic membrane preparations we found that the pore-forming peptide alamethicin proved to be most useful. Preincubation of colonic microsomes with alamethicin (60 μ g)

increased PI-PLC activity by up to 6-fold, with no further increase at higher concentrations (Figure 1). This amount was used in subsequent assays. PI-PLC activity in colonic membranes could also be enhanced by the bile acid detergents cholic acid and deoxycholic acids; activity increased by 2.5- and 10.6-fold respectively when these bile acids were present at 0.01–1.0 mM. The detergent CHAPS was mildly stimulatory at 100 μ M, while up to 1.5 % octyl β -D-glucoside had no effect (results not shown). Only in the case of alamethicin (see below) did GTP[S] further stimulate PI-PLC activity.

Figure 1 Effect of alamethicin on PI-PLC activity in rat colonic microsomes

Alamethicin at the indicated concentrations was preincubated with 5 μ g of microsomal protein and 2.5 $\times$ concentrated buffer for 10 min prior to the addition of substrate. Assays contained 0.182 μ M Ca $^{2+}$, 0.5 mM Mg $^{2+}$, 10 μ M GTP[S] and 25 μ M PIP $_2$, with other conditions as described in the Materials and methods section. Each point is the mean of duplicate determinations.

Figure 2 GTP[S] stimulation and GDP[S] inhibition of PI-PLC activity in rat colonic microsomes

Microsomes were incubated with the indicated concentrations of GTP[S] in an assay containing 5 μ g of protein, 0.182 μ M Ca $^{2+}$, 25 μ M PIP $_2$ and 60 μ g of alamethicin. Assays were performed under the conditions described in the Materials and methods section, either alone (●) or in the presence of 200 μ M GDP[S] (□). Each point is the mean of duplicate determinations.

Table 1 Effects of non-hydrolysable analogues of guanine and adenine nucleotides on membrane PI-PLC activity

Rat colonic microsomes were incubated as described in the Materials and methods section, with the assay containing 25 μM PIP_2 , 5 μg of protein, 60 μg of alamethicin, 0.5 mM Mg^{2+} , 0.182 μM Ca^{2+} and the indicated concentration of nucleotide. Data are means $\pm$ S.D. of four experiments. *Significant difference from 'no addition' value at $P < 0.001$; **significant difference from 'no addition' value at $P < 0.05$, using one-way ANOVA and Dunnett's comparisons.

Addition	Inositol phosphates produced (pmol/min per μg of protein)
None	4.17 ± 0.24
GTP[S] (10 μM)	$9.14 \pm 0.59^*$
ATP[S] (10 μM)	3.69 ± 0.14
ATP[S] (100 μM)	$2.91 \pm 0.11^{**}$

Figure 3 Effect of Mg^{2+} on PI-PLC activity in rat colonic microsomes

A series of $2.5 \times$ concentrated buffers were prepared with the indicated concentrations of Mg^{2+} , and the pH of each was adjusted to 7.000 ± 0.005 prior to addition to microsomes. The assays contained 5 μg of protein, 0.182 μM Ca^{2+} , 25 μM PIP_2 and 60 μg of alamethicin. Assays were performed under the conditions described in the Materials and methods section, either alone (□) or in the presence of 10 μM GTP[S] (●). Each point is the mean $\pm$ S.D. of quadruplicate determinations. *Significant differences from controls (no Mg^{2+}) at $P < 0.001$ by one-way ANOVA and Dunnett's comparisons.

Effect of guanine and adenine nucleotides

The stable GTP analogue GTP[S] causes extended activation of G-proteins, and has been used by many investigators to demonstrate G-protein regulation of PI-PLC activity [15,25,26]. GTP[S] stimulated PI-PLC activity in colonic microsomes, with half-maximal stimulation occurring at 50 nM and maximal stimulation (> 2.5 -fold over control) occurring at 10 μM (Figure 2). No further increase in activity was observed with up to 200 μM GTP[S] (results not shown).

Further evidence for G-protein involvement was demonstrated by the specificity of the enhancing effect of GTP[S]. In the presence of 200 μM GDP[S], a GTP[S] antagonist, microsomal PI-PLC activity was inhibited at all levels of GTP[S] tested

Figure 4 Effect of free Ca^{2+} , alone or in the presence of alamethicin or alamethicin plus GTP[S], on microsomal PI-PLC activity

Rat colonic microsomes were incubated under conditions described in the Materials and methods section with the assay containing 50 μM PIP_2 , 0.5 mM Mg^{2+} and 2.5 μg of protein in the absence (▲) or presence of 30 μg of alamethicin alone (○) or 30 μg of alamethicin plus 100 μM GTP[S] (■). Each point is the mean of duplicate determinations.

(Figure 2). Although 10 μM GTP[S] more than doubled PI-PLC activity, an equal amount of ATP[S] was without effect, and at 0.1 mM ATP[S] was slightly inhibitory (Table 1).

Effect of bivalent cations

Bivalent cations are reported to influence the activity of PI-PLC in membranes from many cell types [15,25]. We examined the effect of Mg^{2+} on the PLC activity of colonic microsomes preincubated with alamethicin alone or in the presence of 10 μM GTP[S]. Mg^{2+} at 0.05 mM caused slight stimulation of PI-PLC activity in the presence of GTP[S], but at concentrations greater than 1 mM, Mg^{2+} (with or without GTP[S]) inhibited PI-PLC activity (Figure 3). We chose to use 0.5 mM Mg^{2+} in subsequent assays.

Figure 4 illustrates the response of PI-PLC activity to Ca^{2+} concentration in colonic microsomes in the presence and absence of alamethicin and GTP[S]. Under all assay conditions, the response of PI-PLC activity to Ca^{2+} concentration was biphasic, with maximal activity occurring at $\sim 42 \mu\text{M}$ and significant inhibition at 1 mM. Over the Ca^{2+} concentration range tested, the rank order of activation of PI-PLC was alamethicin + GTP[S] $>$ alamethicin alone $>$ no additives.

Comparison of PI-PLC activities in the antipodal plasma membranes

PI-PLC activities in colonic BBM and BLM were compared with respect to their responses to different concentrations of Ca^{2+} and GTP[S]. Both BBM and BLM activities showed responses to Ca^{2+} that were similar to those of the microsomal preparation (Figure 4), and were indistinguishable in the range of physiological Ca^{2+} concentrations. The activity of PI-PLC in BBM or BLM showed closely parallel responses to increasing concentrations of GTP[S], similar to that of the microsomal preparation (Figure 2).

We attempted to demonstrate coupling of PI-PLC activity to membrane receptor(s). For these studies, the agonists

Figure 5 Immunoreactivity of PLC isoenzymes in rat colon

Homogenate (H), cytosol (C), BBM and BLM fractions were prepared from rat colon as described in the Materials and methods section. Blots were incubated with isoform-specific monoclonal antibodies to PLC- $\delta 1$ (a) or PLC- $\gamma 1$ (b). The molecular mass standards are shown to the left.

bethanechol (0.1 mM) and 1,25(OH) $_2$ D $_3$ (10–100 μ M) were tested in colonic microsomes and antipodal plasma membrane fractions, alone or in the presence or absence of alamethicin and GTP[S]. We were unable, however, to demonstrate an effect of bethanechol or 1,25(OH) $_2$ D $_3$ on PI-PLC activity under any of the conditions tested (results not shown).

Immunocharacterization of PI-PLC isoenzymes

Using monoclonal antibodies to the immunologically distinct PI-PLC isoenzymes, we detected the presence of PLC- $\gamma 1$ and PLC- $\delta 1$, but not PLC- $\beta 1$, isoforms in the homogenate and subcellular fractions from rat colonocytes (Figure 5). In a control experiment all three isoforms were detected in a rat brain homogenate (results not shown). PLC- $\delta 1$, with an apparent molecular mass of 86.5 kDa, was present in the crude homogenate and in the antipodal membrane fractions, but very little was found in the cytosol. More than 90% of colonocyte PLC- $\delta 1$ was membrane-associated, which includes both plasma and intracellular membranes. In contrast, PLC- $\gamma 1$, with an apparent molecular mass of 141 kDa, was found predominantly in the cytosolic fraction: more than 95% of PLC- $\gamma 1$ was recovered in the cytosol. Detectable but small amounts of PLC- $\gamma 1$ were also found in the BLM and BBM fractions. For equal amounts of protein loaded, the immunoblot intensities for PLC- $\delta 1$ and - $\gamma 1$ were 2.7-fold and 3.7-fold greater respectively for BLM than for BBM.

DISCUSSION

In this paper we have partially characterized PI-PLC in rat colonocyte membranes, demonstrating Ca $^{2+}$ - and Mg $^{2+}$ -sensitivity and regulation by guanine nucleotides. In addition, the subcellular distribution of isoenzyme forms of PI-PLC in the cytosol and antipodal plasma membranes was determined.

Similar to the experience of Vaandrager et al. [27] who used membranes from rat small intestine, we found that a reliable assay of PI-PLC activity in rat colonic membranes required the inhibition of resident phosphatases, including alkaline phosphatase. These enzymes are very effective in metabolizing inositol phosphates at a physiological pH [28,29]. Under the conditions of our assay, we were able to show that the substrate PIP $_2$ remained predominantly intact, that the major reaction product was IP $_3$, and that the production of inositol phosphates was linear with respect to time and protein concentration.

An interesting finding in the development of this assay was that alamethicin, alone among the detergents tested, allowed GTP[S] to enhance PI-PLC activity. Alamethacin is a mixture of structurally related antibiotic peptides which, at higher concentrations such as were used in the present experiment, act as a detergent [30]. These amphiphilic helical peptides can also aggregate to form large transmembrane channels in proteins [31]. Mastoparan is another amphiphilic helical peptide, and is thought to influence PI-PLC activity through its interaction with G-protein(s), possibly by mimicking the regulatory domain of G-protein-coupled membrane receptors [26,32]. Whether alamethicin shares this property with mastoparan deserves further investigation.

PI-PLC in rat colonic membranes share many characteristic properties with this enzyme in membranes from a variety of other tissues. The response of PI-PLC activity to increasing concentrations of GTP[S] was sigmoidal, similar to that reported for PI-PLC in rat liver plasma membranes [33] and rabbit and bovine brain membranes [34–36]. In agreement with the studies of PLC in membranes from brain [36,37] and rat liver [33], we found that PI-PLC activity was inhibited in the presence of GDP[S]. Further evidence for nucleotide specificity was demonstrated in our experiments by the failure of ATP[S] to enhance PI-PLC activity, a finding also reported by Taylor and Exton [33] using rat liver membranes.

Comparable to the data reported by others for membranes from a variety of cell types [33,37–39], we found that Ca $^{2+}$ was a potent regulator of rat colonic membrane PI-PLC activity. In contrast to Ca $^{2+}$, the effect of Mg $^{2+}$ on membrane-associated PI-PLC activity appears to vary with cell type [33,36–38]. The inhibition of rat colonic PI-PLC activity by Mg $^{2+}$ seen in the present experiment most closely resembled the situation observed in renal cortical membranes [38]. Studies using highly purified PI-PLC and/or G-proteins have suggested that Mg $^{2+}$ has a neutral or inhibitory effect on PI-PLC activity when no G-proteins are present [25,39–41], whereas Mg $^{2+}$ has been reported to variably enhance GTP binding to G-proteins [16,42].

Our studies also showed that BBM and BLM from rat colon contain comparable amounts of PI-PLC activity, expressed per unit protein, with similar responses to Ca $^{2+}$ and GTP[S]. These results are in agreement with two other studies of PI-PLC activity in rat antipodal plasma membrane preparations. In an assay of PI-PLC in small intestinal BLM and BBM, Vaandrager et al. [27] found that the capacities of these prelabelled membranes to release [3 H]IP $_3$ in response to Ca $^{2+}$ or GTP[S] were comparable. Using exogenous [3 H]PIP $_2$ in an assay similar to ours, Felder et al. [38] demonstrated little difference in PI-PLC activities between renal tubular BBM and BLM under either basal or agonist-stimulated conditions. Taken together, these studies suggest that there is little regional variation in the total amount of PI-PLC activity present in rat plasma membranes prepared from several polarized cell types.

Using Western blotting experiments, we showed that both BBM and BLM contain largely PLC- $\delta 1$. In brain tissue, in contrast, this PLC isoform has been found in the cytosol [14]. The specific feature of PLC- $\delta 1$ that determines its targeting to membranes or cytosol in different cell types is unknown. In these experiments, quantitative densitometry of the immunoblot for PLC- $\delta 1$ and - $\gamma 1$ indicated that the BLM fraction was somewhat enriched over the BBM fraction for both of these isoforms. Since the total enzyme activity was comparable in both membrane preparations, this suggests that other, as yet unidentified, PLC isoforms are present in these plasma membranes. Supporting this possibility is the work of Hiramatsu et al. [43], who demonstrated that rat parotid gland plasma membranes contain measurable

PI-PLC activity but do not immunoblot with antibodies to PLC- $\beta 1$, $\gamma 1$ or $\delta 1$.

PLC- $\gamma 1$ was predominantly cytosolic, although $\sim 5\%$ was detected in purified BLM and BBM. This isoform is unique in that it possesses Src homology (SH) domains 2 and 3 [15]. These SH domains are thought to allow PLC- $\gamma 1$ to associate with other proteins in the plasma membrane or cytoskeleton, via affinity to tyrosine-phosphorylated regions of growth factor-activated receptor(s) [15,44]. Subsequent phosphorylation and activation of PLC- $\gamma 1$ may then occur by the action of receptor tyrosine kinases. Our laboratory is currently investigating the role of agonists such as $1,25(\text{OH})_2\text{D}_3$ in eliciting translocation of PLC- $\gamma 1$ to colonic antipodal plasma membranes.

The question of how specificity is achieved in antipodal plasma membrane phosphoinositide signal transduction continues to be a challenging one. We have recently shown that $1,25(\text{OH})_2\text{D}_3$ stimulates phosphoinositide turnover when applied to the basolateral surface, but not to the brush-border surface, of Caco-2 cells when these cells are grown as tight polarized monolayers [12]. Asymmetry in the antipodal distributions of cell surface receptors is likely to be of prime importance, but as yet we cannot rule out a component of specificity that is due to antipodal differences in the distribution of isoenzyme forms of PI-PLC or associated G-proteins.

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