Early signalling mechanism in colonic epithelial cell response to gastrin

Rihab R. YASSIN* and Kevin M. LITTLE

Division of Gastroenterology, Department of Medicine, Medical College of Pennsylvania and Hahnemann University, Philadelphia, PA 19102, U.S.A.

The hormone gastrin exerts a growth-promoting effect on gastrointestinal cells. The molecular mechanisms by which colonic epithelial cells respond to gastrin are still poorly understood. In this study, we demonstrate a novel feature of the action of gastrin on normal colonic cells, namely the rapid phosphorylation on tyrosine of phospholipase $C\gamma_1$ (PLC γ_1). Tyrosine phosphorylation of PLC γ_1 , elicited by gastrin, was transient, concentration-dependent, and was abrogated by pretreating the colonic cells with the gastrin-receptor antagonist proglumide, the tyrosine kinase inhibitor genistein, and by removal of the tyrosine phosphatase inhibitor orthovanadate from the isolation buffer. Tyrosine phosphorylation of PLC γ_1 correlated with the time- and

concentration-dependent decrease in the mass of membrane phosphatidylinositol 4,5-bisphosphate (PIP $_2$) and the increase in the epithelial concentration of inositol 1,4,5-trisphosphate (IP $_3$). Likewise, the stimulated increase in IP $_3$ was also prevented by proglumide and genistein. Gastrin induced a definite but transient increase in the intracellular concentration of free Ca $^{2+}$ [Ca $^{2+}$] $_i$, and increased membrane-translocation of immunoreactive α - and β -protein kinase C. The data thus indicate that gastrin elicits at least one signalling cascade, through rapid tyrosine phosphorylation of PLC γ_1 , leading to the activation of a PIP $_2$ -specific PLC pathway.

INTRODUCTION

Gastrin has been shown to exert a growth-promoting effect on gastrointestinal cells [1,2]. Continuous infusion of exogenous gastrin in laboratory animals induces gastric mucosal hyperplasia [3]. On the other hand, decreasing circulating gastrin, by antrum removal, results in atrophy of the gastric and duodenal mucosa [4]. Gastrin has also been shown to stimulate the growth of colon cancer xenotransplants that express gastrin receptors [5–7], and the growth responses *in vitro* and *in vivo* of many colon cancer cells [8–11]. The trophic effect of gastrin is independent of its secretagogue action, but dependent on the binding to specific cell-surface receptors [12,13]. Though an autocrine regulatory mechanism can be ascribed to gastrin in gastric and colorectal cancer [14], its physiological influence in the colon and the molecular mechanisms by which it activates colonic cells are largely unknown.

Phosphoinositide-specific phospholipase C (PLC) is the key enzyme triggering the phosphoinositide signalling cascade. Ligand-induced PLC activation catalyses the hydrolytic cleavage of membrane phosphatidylinositol 4,5-bisphosphate (PIP $_2$), generating two second messengers: inositol 1,4,5-trisphosphate (IP $_3$) and 1,2-diacylglycerol [15]. IP $_3$ and 1,2-diacylglycerol are instrumental in inducing Ca $^{2+}$ mobilization and activation of protein kinase C (PKC) [15,16]. Recent studies have revealed the presence of multiple specific isoenzymes of PLC [17–19]. Their heterogeneity and differential expression in various tissues indicate that they have different biological roles in the signal transduction cascade.

In previous investigations, we have shown that gastrin exerts a trophic effect on colonic epithelial cells *in vitro* [20,21]. We have also shown that gastrin induces the translocation of a Ca $^{2+}$ -, phosphatidylserine- and 1,2-diacylglycerol-dependent PKC activity from the cytosol to the membrane [22]. In this paper, we

define and dissect a signalling mechanism in the action of gastrin. A rapid activation, by tyrosine phosphorylation, of PLC γ_1 triggers the breakdown of PIP $_2$, with subsequent Ca $^{2+}$ mobilization and translocation of PKC isoenzymes. To our knowledge, this rapid and early mechanism in the action of gastrin has not been defined in the colonic cells.

MATERIALS AND METHODS

Materials

Synthetic human gastrin I was purchased from Research Plus (Bayonne, NJ, U.S.A.); 2-mercaptoethanol, Tween-20, ion-exchange resins and SDS/PAGE chemicals were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); murine anti-phosphotyrosine (anti-PT; clone 4G10), anti-PLC γ_1 and anti-PLC β_1 monoclonal antibodies (mAbs) were from Upstate Biotechnology (Boston, MA, U.S.A.); an anti-peptide antibody to ζ -PKC was from Gibco-BRL Laboratories (Grand Island, NY, U.S.A.); mAbs to PKC isoenzymes α , β and γ were from Seikagaku America (Rockville, MD, U.S.A.); affinity-purified polyclonal anti-PLC β_2 and -PLC β_4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); purified rat brain PKC was from Lipidex (Westfield, NJ, U.S.A.); D-*myo*-[3 H]inositol 1,4,5-trisphosphate assay system, nitrocellulose membranes and enhanced chemiluminescence (ECL) immunodetection system were from Amersham (Arlington Heights, IL, U.S.A.); fura-2 AM (acetoxymethyl ester; special packaging) was from Molecular Probes (Eugene, OR); Nonidet P40 from Hoefer Scientific (San Francisco, CA, U.S.A.); Protein A-Sepharose was from Pharmacia (Piscataway, NJ, U.S.A.); and all other chemicals and supplies were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Fisher Scientific (Pittsburgh, PA, U.S.A.).

Abbreviations used: PLC, phospholipase C; PKC, protein kinase C; PIP $_2$, phosphatidylinositol 4,5-bisphosphate; IP $_3$, inositol 1,4,5-trisphosphate; mAb, monoclonal antibody; ECL, enhanced chemiluminescence; [Ca $^{2+}$] $_i$, intracellular Ca $^{2+}$ concentration; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed, at the Division of Gastroenterology, MS 131, Medical College of Pennsylvania and Hahnemann University, Broad and Vine, Philadelphia, PA 19102-1192, U.S.A.

Isolation of colonic epithelial cells

Colonic epithelial cells were isolated from the colons of fasted male Sprague-Dawley rats (150–200 g) as previously described by us [20–22].

Treatment of colonic cells

Colonic epithelial cells were resuspended (approx. 2×10^6 cells/ml) in bicarbonate buffer [20] or in Hepes buffer, depending on the type of the study carried out. Each buffer was chosen after it was assessed for the most reliable results, as compared with other studies. The Hepes buffer consisted of (mM): NaCl 145, KCl 5, CaCl_2 1, MgCl_2 1, Hepes 10, pH 7.2, and glucose 5.6; 10 mM LiCl was included in the phosphoinositide studies. The cell suspension was equilibrated for 10–30 min in an oxygen-enriched 37 °C shaker bath before any experimentation. Stimulation was carried out by adding the appropriate concentration of each ligand, for the specified time intervals. Protein concentration of each sample was measured with the Bio-Rad protein assay system, and samples were normalized for their protein concentration before any assay.

Immunoprecipitation assays and protein tyrosine phosphorylation

Immunoprecipitation assays were carried out by the method of Tate et al. [23], after slight modifications. Treated colonic cells were rapidly mixed with 1 vol. of an ice-cold buffer [100 mM Tris/acetate, pH 8.0, 2 mM EDTA, 2 mM EGTA, 100 mM NaF, 2% (w/v) Nonidet P40, 200 mM NaCl, 6 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 2 mM PMSF, 0.4 mM leupeptin and 20 $\mu\text{g}/\text{ml}$ aprotinin]. The samples were kept on ice for 20 min, then were centrifuged at 16000 g for 10 min at 4 °C.

Samples were pre-cleared with Sepharose 4B, and then were incubated with anti-PLC γ_1 (1.5 $\mu\text{g}/\text{mg}$ of cell protein) for 1 h or for 24 h at 4 °C. Then 5 μg of rabbit anti-mouse immunoglobulin/ml was added to the samples for 1 h, followed by incubation for 1 h with Protein A-Sepharose. The samples were centrifuged at 5600 g for 5 min at 4 °C, and the immunoprecipitates were washed consecutively with a series of buffers, starting first with the Nonidet-P40-containing buffer, followed by a buffer consisting of 150 mM NaCl, 10 mM NaH_2PO_4 and 3 mM sodium orthovanadate, pH 7.6. Finally, the immunoprecipitates were washed in a buffer consisting of 100 mM Tris/HCl, pH 7.4, 500 mM LiCl and 3 mM sodium orthovanadate. The immunoprecipitates were boiled in 100 μl of gel sample buffer in accordance with the method of Laemmli [24].

For protein tyrosine phosphorylation, treated colonic cells were rapidly mixed with 1 vol. of an ice-cold buffer (20 mM Tris/HCl, pH 8.0, 13 mM sodium pyrophosphate, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM sodium orthovanadate and 1% Triton X-100) [25]. The lysates were kept on ice for 20 min, and then centrifuged at 16000 g for 10 min at 4 °C. Samples of the supernatant (100 μl) were removed and boiled in gel sample buffer.

Measurement of PIP_2 mass

The mass of PIP_2 was measured by the method of Chilvers et al. [26], which was based on the conversion of PIP_2 into IP_3 through alkaline hydrolysis of crude lipid extracts. Briefly, treated colonic cells were rapidly mixed with 1 vol. of ice-cold 1 M trichloroacetic acid. The cells were kept on ice for 20 min, and then centrifuged at 3000 g for 20 min at 4 °C. The supernatants were removed and the pellets were washed with 2 ml of 1 M trichloroacetic acid/

1 mM EDTA, followed by 2 ml of water. The lipids were extracted with methanol/chloroform/12 M HCl (80:40:1, by vol.) as previously described [26,27]. Samples of the lower phase (lipid extract) were evaporated to dryness under nitrogen, and the dried samples were reconstituted with 250 μl of 1 M KOH, and then incubated at 100 °C for 15 min. After cooling on ice, excess alkali was removed by ion-exchange chromatography on 0.5 ml columns (AG 50W-X8, H^+ from, 200–400 mesh) as previously described [26–28]. A 1 ml portion of the lipid-containing phase was taken and freeze-dried, and then reconstituted in water and assayed for IP_3 content as described below. The results were expressed as pmol/mg of protein.

Measurement of IP_3 formation

Treated colonic cells were rapidly mixed with ice-cold HClO_4 (20%, v/v) and kept on ice for 20 min. The preparations were centrifuged at 2000 g for 15 min at 4 °C, and the supernatants were removed and neutralized with KOH (10 M). The preparations were re-centrifuged at 2000 g for 20 min at 4 °C, and the supernatants were collected and utilized for IP_3 assay as previously described [29]. IP_3 was measured by the competitive binding assay system, which has a high isomeric specificity (Amersham). Assays were assessed for their linearity with respect to various incubation conditions, and the results were expressed as pmol/mg of protein.

Measurement of the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$

Isolated colonic cells were incubated with 10 μM fura-2 AM for 7 min in a 37 °C shaker bath. The cell suspension was then diluted 10-fold with fresh 37 °C buffer and re-incubated for a further 30 min. This loading protocol was found to optimize the uptake of fura-2 AM and its intracellular conversion into fura-2. The cells were collected by centrifugation at 500 g for 5 min and resuspended in fresh Hepes either containing 1 mM CaCl_2 , or Ca^{2+} -free and with or without 1 mM EGTA. Immediately before use, 0.5 ml portions were Microfuged and resuspended at a cell concentration of 2.5×10^6 cells/ml. Fluorescence measurements were carried out with a Spex dual-wavelength fluorimeter with excitation at 340 nm and 380 nm, emission at 505 nm. The cuvette containing the cells was constantly kept at 37 °C, and the ligand was injected directly into the cuvette. $[\text{Ca}^{2+}]_i$ was calculated by the ratio method of Grynkiewicz et al. [30].

Extraction of PKC from the colonic cells

Treated colonic cells were rapidly mixed with 1 vol. of an ice-cold homogenizing buffer (20 mM Tris/HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 0.5 mM benzamidine, 5 mM 2-mercaptoethanol and 10 mg/l leupeptin). The preparations were kept on ice for 5 min and then centrifuged at 200 g for 5 min at 4 °C. The supernatants were discarded, and the pellets were resuspended in 500 μl of chilled homogenizing buffer and then sonicated on ice for 5–10 s. The sonicated preparations were ultracentrifuged at 100000 g for 60 min at 4 °C, and the resulting supernatants (cytosolic fractions) were removed and kept on ice. The pellets were resuspended in 300–500 μl of chilled homogenizing buffer containing 0.1% Nonidet P40, and were shaken for 1–2 h at 4 °C. The suspensions were centrifuged at 100000 g for 60 min at 4 °C, and the resulting supernatants represented the membrane fractions. Cytosolic or membrane fractions were used directly or were further purified on DEAE-cellulose columns (0.8 cm $\times$ 4 cm; Polyprep columns, Bio-Rad Laboratories) as previously described [22].

Figure 1 Phosphorylation on tyrosine of a 145 kDa protein (P145) in colonic cells stimulated with gastrin

The epithelial cells were treated with 10 nM gastrin for 0 min (lane 1), 0.5 min (lane 2), 1 min (lane 3) or 5 min (lane 4), and the stimulation was stopped with chilled lysis buffer. (a) PLC γ_1 was immunoprecipitated from the cell lysates, eluted, electrophoresed, transferred on to nitrocellulose filters and then probed with mAb to PT, followed by ECL. Each lane received similar amounts of proteins, as ascertained by total protein assay and by staining with mAb to PLC γ_1 . Molecular-mass marker is indicated on the left side. The blot is representative of experiments. (b) Time course of tyrosine phosphorylation of PLC γ_1 . The autoradiographs were quantified by laser scanning, and the optical density of the control condition (0 min gastrin) was taken arbitrarily as 100%. Data are means $\pm$ S.E.M. of three independent experiments: * P < 0.05. (c) After stripping the first mAb, immunoprecipitates of PLC γ_1 were probed with anti-PLC γ_1 antibody, followed by ECL. The blot is representative of experiments.

Gel electrophoresis and immunoblotting

Immunoprecipitated proteins, cell lysate preparations, or cytosolic and membrane fractions, equalized for their protein contents, were resolved on SDS/polyacrylamide (7.5% or 10%, w/v) gels under reducing conditions, and then transferred to nitrocellulose blotting papers. The blots were blocked overnight at 4°C with 5% (w/v) dry milk in 20 mM Tris-buffered saline/0.1% Tween 20, pH 7.6 (TBS-T). When blotting with anti-PT, the milk was replaced with 5% (w/v) BSA. After the blocking, the blots were rinsed five times in fresh changes of TBS-T, then were incubated with the designated antibodies, under the following conditions: 2 h at room temperature for anti-PT (1 μ g/ml), and 1 h for anti-PLC γ_1 (0.2 μ g/ml) and anti-PLC β isoenzymes 1,2 and 4 (1 μ g/ml). For detection of PKC isoenzymes, the mAbs were used at concentrations of 0.1–0.2 μ g/ml. The specificity and reactivity of the Abs were assessed by solid-phase immunoassay. For the anti-PT, no staining was obtained in the presence of excess (20 mM) phosphotyrosine, indicating the specific immunoreactivity of the antibodies (results not shown). After incubation with the primary antibody, the blots were washed extensively with TBS-T and then treated with peroxidase-labelled secondary antibody for 1 h at room temperature. The blots were then washed as above, and the immunoreactive bands were revealed by staining with the ECL system. When required, the blots were stripped of the first antibody by incubating in a stripping buffer (62.5 mM Tris/HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) for 30 min at 50°C. The blots were washed three times with TBS-T, then blocked and made to

Table 1 Concentration-dependence of gastrin's effect on tyrosine phosphorylation of PLC γ_1 and IP $_3$ formation in the colonic cells

Colonic epithelial cells were treated with the indicated concentrations of gastrin for 0.5 min. Tyrosine phosphorylation of PLC γ_1 and IP $_3$ concentration were quantified as described in the Materials and methods section. Data are means $\pm$ S.E.M. of the numbers of experiments reported in parentheses: * P < 0.05.

Conditions	Tyrosine phosphorylation (% of control)	IP $_3$ (pmol/mg of protein)
Control	100 (3)	2.11 $\pm$ 0.3 (4)
Gastrin (100 nM)	120 $\pm$ 2 (3)*	3.19 $\pm$ 0.1 (3)*
Gastrin (10 nM)	227 $\pm$ 22 (5)*	4.29 $\pm$ 0.3 (4)*
Gastrin (0.1 nM)	111 $\pm$ 11 (3)	2.25 $\pm$ 0.3 (5)

Table 2 Tyrosine phosphorylation of PLC γ_1 in response to various treatments

Colonic epithelial cells were treated with the indicated agents or their vehicle for 10–15 min, after which gastrin was added for 0.5 min. PLC γ_1 was probed and quantified as described in the Materials and methods section. Data are means $\pm$ S.E.M. of the numbers of experiments reported in parentheses: * P < 0.05 versus control, ** P < 0.05 versus gastrin.

Conditions	Tyrosine phosphorylation (% of control)
Control	100 (5)
Gastrin (10 nM)	227 $\pm$ 22 (5)*
Gastrin + proglumide (10 mM)	117 $\pm$ 3 (2)**
Gastrin + genistein (0.1 mM)	82 $\pm$ 6 (3)**
Gastrin (without orthovanadate)	64 $\pm$ 11 (2)**

react with another antibody as described above. The intensities of the bands were quantified with an SLR laser scanner (BioMed Instruments, Fullerton, CA, U.S.A.).

RESULTS

Gastrin induces phosphorylation of PLC γ_1 on tyrosine

To determine whether gastrin induces tyrosine phosphorylation of PLC γ_1 in the colonic cells, PLC γ_1 was immunoprecipitated from control and gastrin-treated cells, blotted, and then probed with anti-PT mAb. As shown in Figure 1(a), gastrin (10 nM) induced a rapid and transient phosphorylation of a 145 kDa protein, which was also demonstrated in whole-cell lysates (results not shown). Maximum phosphorylation (by densitometry) was measured at 30–60 s before declining back to the baseline level within 5 min (Figure 1b). Stripping the blots and re-probing with anti-PLC γ_1 antibody revealed the presence of similar amounts of PLC γ_1 in each sample processed (Figures 1b and 1c). Tyrosine phosphorylation of PLC γ_1 was concentration-dependent, and was maximally induced with 10 nM gastrin (Table 1).

Tyrosine phosphorylation of PLC γ_1 , elicited by gastrin, was abrogated by pretreating the colonic cells with the gastrin-receptor antagonist proglumide (10 mM) and the competitive tyrosine kinase inhibitor genistein (0.1 mM) (Table 2). The concentration of genistein used in these studies did not cause any significant loss of cell viability, as indicated by the Trypan Blue exclusion. Omission of the phosphotyrosine phosphatase inhibitor orthovanadate from the isolation buffer also decreased the phosphorylation of PLC γ_1 to pre-stimulation level (Table 2).

Figure 2 Time course of IP_3 formation in colonic cells stimulated with gastrin

Cells were challenged with 10 nM gastrin for the specified time intervals, and IP_3 was extracted and assayed as described in the Materials and methods section. Data are means $\pm$ S.E.M. of three to eight independent experiments: * $P < 0.05$.

Effect of gastrin on IP_3 formation and PIP_2 mass

Administration of gastrin (10 nM) caused a rapid and transient increase in epithelial IP_3 concentration (Figure 2). The increase was measurable in 10 s, and reached a peak in 30 s (183%), before declining back to the baseline level within 5 min. The increase was also concentration-dependent, with 10 nM gastrin inducing the maximum effect (Table 1). The increase in IP_3 concentration was concomitant with a decrease in the mass of membrane PIP_2 . As shown in Figure 3, the same concentration of gastrin that evoked maximal IP_3 concentration (10 nM) induced a rapid decrease in the mass of PIP_2 . A maximum fall was measured within 30 s of treatment with gastrin and was sustained for up to 5 min.

To investigate whether tyrosine phosphorylation of $\text{PLC}\gamma_1$ is required for the gastrin-induced increase in IP_3 , the concentration of IP_3 was measured in the presence of the tyrosine kinase inhibitor genistein. Colonic epithelial cells were incubated with genistein (0.1 mM) for 15 min, after which gastrin (10 nM) was added for 30 s. As shown in Table 3, genistein abrogated the stimulatory effect of gastrin on IP_3 . To assess the specificity of the genistein effect on IP_3 , we also included bombesin, which has been shown to stimulate Ca^{2+} mobilization, but not proliferation of human colon cancer cells [31]. Table 3 indicates that genistein did not abrogate the effect of bombesin on IP_3 ; the stimulatory effect of gastrin was also prevented by pretreating the cells with the gastrin-receptor antagonist proglumide.

We also screened the colonic cells for $\text{PLC}\beta$ isoenzymes (β_1 , β_2 and β_4) to ascertain that the effect of gastrin on IP_3 was solely due to activation of $\text{PLC}\gamma_1$. Using the screening methods we applied for $\text{PLC}\gamma_1$, we did not detect the presence of these isoenzymes in rat colonic cells (results not shown).

Effect of gastrin on $[\text{Ca}^{2+}]_i$

Figure 4 illustrates the effect of gastrin on the changes in $[\text{Ca}^{2+}]_i$, as measured with the fluorescent dye fura-2. In a buffer containing 1 mM CaCl_2 , gastrin (10 nM) induced a definite, but transient, increase in $[\text{Ca}^{2+}]_i$. This increase was observed after a lag period which correlated with the time frame of IP_3 formation (Figure 2). Concentrations of gastrin higher or lower than 10 nM were less effective in mobilizing Ca^{2+} . For example, when the cells were stimulated with 100 nM gastrin, the increase in $[\text{Ca}^{2+}]_i$ was 62% of that seen with 10 nM, in agreement with our findings for IP_3 .

Figure 3 Time course of the changes in the mass of membrane PIP_2 in response to gastrin

The epithelial cells were treated with 10 nM gastrin for the specified time intervals, and the stimulation was stopped by the rapid addition of chilled 1 M trichloroacetic acid. PIP_2 mass measurements were carried out as described in the Materials and methods section. Data are means $\pm$ S.E.M. of three to six independent experiments: * $P < 0.05$.

Table 3 Effects of different treatments on IP_3 concentration in the colonic cells

Colonic epithelial cells were treated with the indicated agents or their vehicle for 10–15 min, after which gastrin was added for 0.5 min. IP_3 was extracted and assayed as described in the Materials and methods section. Data are means $\pm$ S.E.M. of the numbers of experiments reported in parentheses: * $P < 0.05$.

Conditions	IP_3 (pmol/mg of protein)
Control	2.75 ± 0.1 (3)
Gastrin (10 nM)	5.54 ± 0.3 (4)*
Bombesin (10 nM)	4.03 ± 0.03 (3)*
Gastrin + genistein (0.1 mM)	3.03 ± 0.0 (3)
Bombesin + genistein (0.1 mM)	4.04 ± 0.2 (3)*
Gastrin + proglumide (10 mM)	2.49 ± 0.4 (6)

Figure 4 Effect of gastrin on $[\text{Ca}^{2+}]_i$

The epithelial cells were loaded with fura-2 AM as described in the text, and then washed and resuspended in HEPES buffer containing 1 mM CaCl_2 . Fluorescence measurements were carried out at excitation wavelengths of 340 nm and 380 nm and emission wavelength of 505 nm. $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. [30]. At the time points indicated on the trace, gastrin (10 nM) was injected directly into the cuvette. A representative trace from five experiments is shown.

Figure 5 Redistribution of PKC isoenzymes in response to ligand treatment

The epithelial cells were treated with 10 nM gastrin (lanes 2 and 5), with 1 μ M PMA (lanes 3 and 6) or with vehicle only (lanes 1 and 4) for 1 min, and the stimulation was stopped by the rapid addition of chilled extraction buffer. Cytosol or membrane fractions were extracted, electrophoresed, transferred on to nitrocellulose and then probed for PKC isoenzymes as described in the Materials and methods section. Molecular-mass markers (kDa) are indicated on the left side and the isoenzymes' positions on the right side. The blot is representative of experiments.

formation. The addition of successive doses of gastrin caused desensitization of the Ca²⁺ response. When the studies were carried out in Ca²⁺-free buffers with or without 1 mM EGTA, the baseline Ca²⁺ concentration, as well as the rise induced by gastrin, were greatly diminished (results not shown). Similar observations were reported in other cells [32,33], and were attributed to a Ca²⁺ requirement for gastrin association with its receptor [33].

Effect of gastrin on PKC isoenzymes

We have shown that the major PKC activity in unstimulated colonic cells is associated with the cytosol (72%), and a smaller activity is associated with the membrane (28%) [22]. Gastrin induced a rapid, transient, and concentration-dependent translocation of the cytosolic activity to the membrane [21,22]. To observe whether the translocation of the cytosolic activity is the result of the physical migration of specific isoenzymes, we probe with specific antibodies to α -, β -, γ - and ζ -PKC.

We detected the presence of a small amount of α -PKC (84 kDa), a prominent β -PKC (82 kDa), and ζ -PKC (79 kDa) in unstimulated colonic cells (Figure 5). We were unable to detect any reactivity to anti- γ -PKC antibody (results not shown). Both α - and β -PKC were responsive, but to variable extents, to gastrin (10 nM) treatment, and translocated rapidly (1 min) from the cytosol to the membrane (Figure 5 and Table 4). However, immunoreactivity of α -PKC lost in the cytosol was not totally recovered in the membrane, probably due to the small amount of

α -isoenzyme present in the colonic cells. In agreement with our previous findings for the overall activity of PKC [22], the translocation of immunoreactive α - and β -PKC was transient and declined back to baseline level within 5 min (results not shown). Phorbol 12-myristate 13-acetate (PMA), which was included for the purpose of comparison, also stimulated the translocation of α - and β -PKC (Figure 5 and Table 4).

The specificities of the mAbs used in these assays were as described in the Materials and methods section. Any cross-reactivity could easily be detected, because each isoenzyme migrated to a different location on the blot. The poorly resolved doublets with variable intensities, seen in these blots, have been reported in many other cells. They could be the product of alternative splicing [34], or basically could represent various phosphorylated forms of PKC which tend to migrate to different locations on the SDS gel [35].

DISCUSSION

The stimulatory effect of gastrin on gastrointestinal cell growth has been widely investigated. The mechanisms by which colonic cells respond to gastrin, however, are largely unknown. We reported previously that gastrin exerts a trophic effect on colonic epithelial cells *in vitro* [20,21]. In this study, we have examined the signalling mechanism mediating gastrin's effect in the colon. From the present data it is apparent that gastrin initiates at least one signalling cascade, by rapid activation through tyrosine phosphorylation of PLC γ_1 , leading to inositol lipid turnover, with subsequent redistribution of PKC isoenzymes.

Little information is available on the effect of gastrin on PLC isoenzymes in target cells. Raid et al. [25] have shown that exposure of the small-intestinal crypt-cell line IEC-6 to gastrin increases tyrosine phosphorylation of many proteins with molecular masses of 42–120 kDa; however, the parameters of protein tyrosine phosphorylation were different from what we have seen in the colonic cells. In our system, PLC γ_1 was readily detectable as a 145 kDa band, and the addition of gastrin induced a rapid and transient tyrosine phosphorylation of this protein. A similar rapid and transient tyrosine phosphorylation of PLC isoenzymes has been reported in other cell systems [23,36].

Tyrosine phosphorylation of PLC γ_1 appears to be receptor-mediated, since colonic cells incubated with the gastrin-receptor antagonist proglumide showed a phosphorylation level similar to that of control cells. In addition, the phosphorylation was abrogated by the tyrosine kinase inhibitor genistein and by removal of the phosphotyrosine phosphatase inhibitor orthovanadate from the isolation buffer, an indication of the transient nature of the tyrosine phosphorylation process.

The data presented also indicated that gastrin induced a rapid and transient increase in the cellular concentration of IP₃ and a

Table 4 Redistribution of PKC isoenzymes in response to gastrin and PMA

Colonic epithelial cells were treated with gastrin (10 nM) or with PMA (1 μ M) for 1–2 min, and the stimulation was stopped by the rapid addition of chilled extraction buffer. Cytosol or membrane fractions were prepared and probed for PKC isoenzymes as described in the Materials and methods section. Quantifications were carried out by laser scanning, and the optical density of the control was taken arbitrarily as 100%. Data are means $\pm$ S.E.M. of the numbers of determinations reported in parentheses: * P < 0.05.

Conditions	Cytosol PKC (% of control)			Membrane PKC (% of control)		
	α	β	ζ	α	β	ζ
Control	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)
Gastrin (10 nM)	54 $\pm$ 8 (3)*	34 $\pm$ 4 (4)*	102 $\pm$ 0.7 (3)	122 $\pm$ 9 (3)	157 $\pm$ 13 (6)*	98 $\pm$ 7 (3)
PMA (1 μ M)	19 $\pm$ 10 (3)*	54 $\pm$ 6 (4)*	103 $\pm$ 5 (3)	214 $\pm$ 22 (3)*	174 $\pm$ 24 (4)*	92 $\pm$ 7 (3)

concomitant decrease in the mass of PIP_2 , the metabolite that is the source of IP_3 . The increase in IP_3 reached a peak in 30 s and declined back to control level within 5 min, in accordance with the time course of $\text{PLC}\gamma_1$ activation. The increase in IP_3 was concentration-dependent and was maximally induced with 10 nM gastrin, the optimal concentration for induction of PLC activation and other trophic responses [20–22]. The increase in IP_3 was also abrogated by proglumide and by genistein, indicating that receptor activation of a tyrosine kinase is necessary to initiate PIP_2 breakdown. Recently, Ishizuka et al. [7] have reported that gastrin induced cyclic AMP formation in three colon cancer cell lines (LOVO, COLO-320, HCT-116), and IP_3 formation in HT-29 cancer cell line. However, peak IP_3 (48% maximum) was induced by 100 nM gastrin [7], and the specific pathway through which the concentration of gastrin triggered IP_3 formation in the HT-29 cells was not clear.

We have also shown that gastrin induced a transient rise in the colonocytes' $[\text{Ca}^{2+}]_i$. This rise came after a lag period which coincided with the increase in the epithelial concentration of IP_3 , an indication that IP_3 may be responsible for the Ca^{2+} response. The rise in $[\text{Ca}^{2+}]_i$ was also concentration-specific, and was desensitized by successive doses of gastrin. The mechanisms by which IP_3 triggers a rise in $[\text{Ca}^{2+}]_i$ have been widely investigated, and most studies have indicated that IP_3 induces the release from an internal storage pool [15,37]. However, there are indications that IP_3 may have an effect at the plasma-membrane level, probably by acting on Ca^{2+} channels [38]. When the colonic-cell Ca^{2+} was monitored in a Ca^{2+} -free buffer with or without EGTA, the basal Ca^{2+} , as well as the rise, were largely diminished. Whether the diminished response we obtained in a Ca^{2+} -free buffer is due to a weak ligand–receptor binding [33], or is due to IP_3 action on membrane Ca^{2+} channels, cannot be deduced from these studies. Further work is necessary to examine the relationship between IP_3 and the Ca^{2+} rise in the colonic epithelial cells.

Previously, we have reported that gastrin induced a rapid and transient translocation of PKC activity from the cytosol to the membrane [21,22]. The translocation of the cytosolic activity was concentration-specific, and was abrogated by proglumide and by prolonged treatment with PMA [22]. The PKC isoenzymes involved in regulating colonic cell responses to gastrin as well as other growth factors are largely unknown.

The data presented showed that colonic epithelial cells possess the Ca^{2+} -dependent isoenzymes α - and β -PKC, and the Ca^{2+} -independent isoenzyme ζ -PKC. both α - and β -PKC were responsive to gastrin treatment, and were mobilized rapidly from the cytosol; however, β -PKC was more responsive to gastrin than α -PKC. The time course of the translocation correlated with other signalling events, and was downstream from $\text{PLC}\gamma_1$ activation. The translocation of α - and β -PKC to the membrane is essential to initiate PKC-dependent protein phosphorylation and subsequent propagation of the activating signal. ζ -PKC may be involved in regulating cell responses that are not Ca^{2+} -dependent. The lack of responsiveness of ζ -PKC to PMA, including PMA-binding activity, has also been reported in other cell systems [39,40].

In summary, our studies have shown that gastrin elicits at least one signalling pathway, through a rapid receptor-mediated tyrosine phosphorylation of $\text{PLC}\gamma_1$. The phosphorylation of $\text{PLC}\gamma_1$ triggers the breakdown of PIP_2 leading to a rise in Ca^{2+} concentration and membrane translocation of PKC isoenzymes. The nature of the activating tyrosine kinases need to be defined in these cells.

This work has been supported by National Institutes of Health Grant DK-44190 (R.R.Y.). We express our appreciation to Dr. Helen Korchak, from the University of Pennsylvania, for all her assistance with the Ca^{2+} studies, and to Dr. Jeffrey Fletcher from the Medical College of Pennsylvania for his assistance with the scanning computer programs.

REFERENCES

- Johnson, L. R. (1977) *Gastroenterology* **72**, 788–792
- Johnson, L. R. (1984) *Prog. Cancer Res. Ther.* **29**, 327–336
- Ryberg, B., Axelsson, J., Hakanson, R., Sundler, F. and Mattsson, H. (1990) *Gastroenterology* **98**, 33–38
- Johnson, L. R. and Chandler, A. M. (1973) *Am. J. Physiol.* **224**, 937–940
- Singh, P., Townsend, C. M., Jr., Walker, J. P. and Thompson, J. C. (1984) *Surg. Forum* **35**, 205–206
- Singh, P., Walker, J. P., Townsend, C. M., Jr. and Thompson, J. C. (1986) *Cancer Res.* **46**, 1612–1616
- Ishizuka, J., Townsend, C. M., Jr., Bold, R. J., Martinez, J., Rodriguez, M. and Thompson, J. C. (1994) *Cancer Res.* **54**, 2129–2135
- Smith, J. P. and Solomon, T. E. (1988) *Gastroenterology* **95**, 1541–1548
- Kusyk, C. J., McNeil, N. O. and Johnson, L. R. (1986) *Am. J. Physiol.* **251**, G597–G601
- Watson, S. A., Durrant, L. G., Crosbie, J. D. and Morris, D. L. (1989) *Int. J. Cancer* **43**, 692–696
- Lamote, J. and Willems, G. (1988) *Regul. Pept.* **20**, 1–9
- Beauchamp, R. D., Townsend, C. M., Jr., Singh, P., Glass, E. J. and Thompson, J. C. (1985) *Ann. Surg.* **202**, 303–309
- Townsend, C. M., Jr., Singh, P., Evers, B. M., Gomez, G., Alexander, R. W. and Thompson, J. C. (1990) in *Gastrointestinal Endocrinology* (Thompson, J. C., ed.), pp. 273–284, Academic Press, San Diego
- Morris, D. L., Watson, S. A., Durrant, L. G. and Harrison, J. D. (1989) *Gut* **30**, 425–429
- Berridge, M. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. and Rhee, S. G. (1988) *Cell* **54**, 161–169
- Suh, P.-G., Ryu, S. H., Cho, K. S., Lee, K. Y. and Rhee, S. G. (1988) *J. Biol. Chem.* **263**, 14497–14504
- Cockcroft, S. and Thomas, G. M. H. (1992) *Biochem. J.* **288**, 1–14
- Yassin, R. R., Clearfield, H. R., Katz, S. M. and Murthy, S. N. S. (1991) *Peptides* **12**, 63–69
- Yassin, R. R. and Murthy, S. N. S. (1991) *Peptides* **12**, 925–927
- Yassin, R. R., Clearfield, H. R. and Little, K. M. (1993) *Peptides* **14**, 1119–1124
- Tate, B. F. and Rittenhouse, S. E. (1993) *Biochim. Biophys. Acta* **1178**, 281–285
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Raid, A., Oliver, B., Abdelrahman, A., Sha'afi, R. I. and Hajjar, J.-J. (1993) *Proc. Soc. Exp. Biol. Med.* **202**, 435–439
- Chilvers, E. R., Batty, I. H., Challiss, R. A. J., Barnes, P. J. and Nahorski, S. R. (1991) *Biochem. J.* **275**, 373–379
- Downes, C. P. and Wusteman, M. W. (1983) *Biochem. J.* **216**, 633–640
- Clark, N. G. and Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306
- Palmer, S., Hughes, K. T., Lee, D. Y. and Wakelam, M. J. O. (1989) *Cell. Signalling* **1**, 147–156
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Hirai, M., Ishizuka, J., Hirai, A., Bold, R. J., Townsend, C. M., Jr. and Thompson, J. C. (1993) *Life Sci.* **53**, 1859–1865
- Tsunoda, Y., Takeda, H., Asaka, M., Nakagaki, I. and Sasaki, S. (1988) *FEBS Lett.* **232**, 83–90
- Muallem, S. and Sachs, G. (1984) *Biochim. Biophys. Acta* **805**, 181–185
- Majumdar, S., Rossi, M. W., Fujiki, T., Phillips, W. A., Disa, S., Queen, C. F., Johnston, R. B., Jr., Rosen, O. M., Corkey, B. E. and Korchak, H. M. (1991) *J. Biol. Chem.* **266**, 9285–9294
- Pears, C., Stabel, S., Cazaubon, S. and Parker, P. J. (1992) *Biochem. J.* **283**, 515–518
- Yang, L., Baffy, G., Rhee, S. G., Manning, D., Hansen, C. A. and Williamson, J. R. (1991) *J. Biol. Chem.* **266**, 22451–22458
- Alkon, D. L. and Rasmussen, H. (1988) *Science* **239**, 998–1005
- Kuno, M. and Gordens, P. (1987) *Nature (London)* **326**, 301–304
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3099–3103
- Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F. and Beaven, M. A. (1993) *J. Biol. Chem.* **268**, 1749–1756