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QUARTERLY

Gastrin activates tyrosine kinase and phospholipase C in isolated rat colonocytes

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Postreceptor regulation of the trophic action of gastrin is not fully elucidated. Tyrosine kinase (Tyr-kinase) has been associated with receptors of a number of growth factors and plays an important role in regulation of cellular growth within the gastrointestinal tract. The aim of this study was to determine, whether Tyr-kinase plays a role in mediating the growth promoting action of gastrin and whether phospholipase C (PLC) is involved in the signal transduction pathway.

Colonocytes isolated from Fischer 344 rats were incubated for 2 min with gastrin (10^{-8} M) and assayed for Tyr-kinase and PLC activities. Incubations with gastrin resulted in 60%–70% rise in Tyr-kinase and 150%–200% rise in PLC activities over the corresponding basal levels. When processed separately, in proximal colon Tyr-kinase activation by gastrin was 15%–20%, while in distal colon 70%–80% as compared to the buffer control. Gastrin activation of both Tyr-kinase and PLC was abolished by Tyr-kinase inhibitor, tyrphostin-25 (3.2 μM) and was not affected by staurosporine (20 ng/ml). We conclude that Tyr-kinase is involved in the mechanism of trophic action of gastrin, and PLC activation appears to be the next step in the signal transduction pathway.

In recent years a variety of evidence has appeared to show that many gastrointestinal (GI) hormones, including gastrin, regulate growth of the GI tract [1, 2].

In animal studies a single or multiple injection of gastrin stimulates GI mucosal cell proliferation [2, 3].

Although gastrin has long been known to stimulate mucosal growth in a large part of the GI tract, the regulatory mechanisms of the growth promoting action of gastrin has not been fully elucidated.

Several *in vitro* experiments have shown that colon is sensitive to gastrin [4, 5]. We have also found high affinity binding of gastrin in colonic mucosal membranes, suggesting that this tissue, responsive to the trophic action of gastrin, possesses specific receptors for this hormone [6].

More recent evidence has also implicated gastrin in colonic neoplasia, since it has been found to induce growth of colonic tumours and to stimulate proliferation of several cancer cell lines [7–9]. Synergistic stimulation of three co-

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Abbreviations: EGF, epidermal growth factor; GI, gastrointestinal; IGF-I, insulin-like growth factor-I; PLC, phospholipase C; TGF, tumour growth factor; Tyr-kinase, tyrosine kinase.

lorectal tumour cell lines proliferation by gastrin and either TGF-α or IGF-I was observed [10]. Gastrin and cholecystokinin antagonists inhibit cell growth *in vitro*, pointing to an autocrine role of gastrin in colon carcinoma [11]. It is not known, however, whether the receptor for the trophic action of gastrin is the same as that which has been identified for its acid-secretory property.

Phosphorylation-dephosphorylation reactions are thought to be of importance for regulatory actions of many hormones [12, 13]. Recent evidence shows that tyrosine kinases, which catalyze tyrosine phosphorylation in proteins, play an important role in regulation of cell proliferation and differentiation [14, 15].

We have shown that Tyr-kinase activation and phosphorylation of some membrane proteins may be the post-receptor regulatory event for the trophic action of gastrin in colonic mucosa [16]. The present investigation, an extension of this observation, examines further the downstream regulatory events in the colonic mucosa in response to gastrin. It was undertaken to evaluate the involvement of Tyr-kinase and tyrosine phosphorylation of proteins in the regulation of gastrin action on colonic mucosal proliferative activity and to further estimate the responsiveness of different regions of the colon to gastrin.

METHODS

Animals and collection of tissues. All experiments were performed utilizing freshly isolated colonocytes from overnight fasted 4–5 month old Fischer-344 rats according to Roediger & Truelove with slight modifications [17]. Briefly, the entire colon was removed, washed thouroughly in cold saline, and immediately everted over a metal rod and washed with 1 mM dithiothreitol to remove adherent mucus. In some experiments the colon was divided into two halves, proximal and distal; colonocytes from both parts were processed separately. The colon was then filled with 10–12 ml of Ca^{2+}/Mg^{2+}-free Hepes-Ringer buffer containing 0.1% bovine serum albumin and ligated at both ends and shaken in the same buffer containing 5 mM EDTA for 45 min at 24°C to facilitate cell removal. The medium containing colonocytes was strained through a wire mesh (150 μm) and cells recovered by low speed centrifugation. The cells were washed several times with complete Hepes-Ringer buffer and finally suspended in the same buffer containing 10 mM sodium butyrate and 0.1% bovine serum albumin. Viability of the cells was assessed by the trypan blue exclusion test. Preparations with viability <90% were not used.

Aliquots (1 ml) of freshly isolated colonocytes were pre-equilibrated at 37°C for 30 min and subsequently incubated at the same temperature for 2 min in the absence (basal) or presence of 1×10^{-8} M gastrin. Those conditions were chosen on the basis of a dose response curve constructed with the use of different concentrations of gastrin and different incubation times. In some series of the experiments colonocytes were additionally incubated with staurosporine (20 ng/ml) or tyrphostin (3.2 μM) alone or together with gastrin. At the end of the incubation period, the colonocytes were recovered by brief centrifugation and used for further assessments.

Tyr-kinase activity. Colonocytes were lysed in RIPA buffer (20 mM Na-phosphate, pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate (DOC), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM 1,10-phenantroline) with constant stirring at 4°C for 2 h.

Tyr-kinase activity was measured according to Dangott *et al.* [18] using as substrate poly-L-Glu-L-Tyr, 4:1 (Sigma Chemical Co., St Louis, MO, U.S.A.), which is known to be highly specific for Tyr-kinase [19]. The reaction mixture in a final volume of 50 μl contained: 2.5 μmol of Tris/HCl; 0.5 nmol of orthovanadate; 0.02% Triton X-100; 3 μmol of ATP; 0.4 μCi [γ-^{32}P]ATP (11.7 Ci/mmol; New England Nuclear, Boston, MA, U.S.A) and 50 μg of Glu-Tyr polymer. The reaction was initiated by adding preparations of lysed cells (10–20 μg protein). Orthovanadate was added to inhibit degradation of ATP and dephosphorylation of the phosphopeptide. The reaction was terminated by applying 20 μl of the reaction mixture onto 3 cm^2 Whatman No. 2 MM filter paper. The filters were washed three times with 10 ml of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, rinsed with ethanol, dried, and radioactivity quantitated in 5 ml of scintillation

coctail. The results were expressed as pmol ^{32}P-incorporated per milligram protein.

Phospholipase C activity (PLC). Colonocytes were homogenized in a buffer containing 60 mM Tris/acetate buffer, 2 mM EDTA, pH 6.5, and centrifuged at 10500 × *g* for 1 h at 4°C. Then the membrane pellet was lysed for 2 h at 4°C in solubilizing buffer (60 mM Tris/acetate, 2 mM EDTA, pH 6.5, 0.5% NP-40, 0.5% Triton X-100).

PLC activity was measured using [^{3}H]phosphatidylinositol (Amersham, 0.02 μCi) as a substrate according to Gupta [20]. The reaction mixture in a final volume of 75 μl contained: 200 μM [^{3}H]phosphatidylinositol, 5 mM $CaCl_2$, 0.6 mg DOC and 60 mM Tris/HCl buffer, pH 6.5. The reaction was initiated by adding membrane preparations (optimal: 25 μg protein). After 20 min the reaction was terminated by adding 300 μl of 1 M HCl. Next, 0.75 ml of chloroform/n-butanol/concentrated HCl (10:10:0.06, by vol.) mixture was added. The mixtures were vortexed and spun at room temperature at 2000 r.p.m. for 10 min and 125 μl of the upper aqueous layer were placed in scintillation vials with 5 ml of scintillation solution for counting. The activity of PLC was estimated on the ground of the radioactivity released in the aqueous layer and expressed as pmole of radioactive phosphatidylinositol hydrolyzed/mg protein.

Phosphorylation of endogenous substrates and identification of phosphorylated proteins. This was carried out with membrane (30000 × *g* pellet) fractions prepared from colonocytes homogenized in HB buffer acccording to Dangott *et al.* [18], as described previously [21]. Briefly, the reaction mixture contained in a final volume of 50 μl: 2.5 μmol Hepes, pH 7.8, 2.5 μmol $MgCl_2$, 0.5 nmol orthovanadate, 0.5 nmol [γ-^{32}P]ATP and 0.02% Triton X-100. The reaction at 0–2°C for 30 min was initiated by adding 20 μl of membrane preparation (50 μg protein) from colonocytes and terminated by adding an equal volume of lysis buffer (20 mM sodium phosphate, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 0.5% DOC, 150 mM NaCl, 5 mM EDTA, 5 mM PMSF, 1 mM sodium vanadate, 10 μg/ml leupeptin and 5 mM sodium pyrophosphate) and 3 μg of antiphosphotyrosine antibody (monoclonal antibody, Boehringer Mannheim). The samples were incubated at 4°C for 4 h and the immune complex was precipitated with pansorbin (25 μg/100 μl sample) by incubating the mixture for 20 h at 4°C, and the immunoprecipitates were suspended in stopping buffer/lysis buffer (1:1), heated at 100°C for 7 min and centrifuged again. The supernatant was then subjected to SDS/polyacrylamide gel electrophoresis. After electrophoresis, the gel was fixed in fixing buffer [21] for 30 min, equilibrated for 2 h in 10% acetic acid/10% isopropanol (v/v), dried and finally exposed to Kodak X-Omat AR film for 3–4 days at –70°C. The relative molecular mass of the labelled bands was calculated from that of marker proteins run concurrently.

In all experiments protein content was determined by the method of Bradford [22].

Statistical analysis: where applicable, the results were statistically evaluated with Student's *t*-test for unpaired values, taking $P < 0.05$ as the level of confidence.

RESULTS

In the first series of experiments Tyr-kinase activity in colonocytes was determined in the absence (basal) and presence of increasing concentrations of human synthetic gastrin G-17. Colonic mucosal Tyr-kinase activity (Fig. 1) increased slightly on increasing gastrin concentration up to 10^{-8} M. Then the activity was found maximal, and exceeded the basal value by 56% ($P < 0.05$). In concentrations of gastrin exceeding 10^{-8} M the stimulatory effect decreased, and Tyr-kinase activity reached the initial value at 10^{-7} M gastrin.

Next, the changes in total Tyr-kinase activity were estimated separately in the colonocytes isolated from proximal and distal colon (Fig. 2). Whereas gastrin produced no demonstrated change in Tyr-kinase activity in proximal colon, it caused a 67% increase in the distal colon. All subsequent experiments were performed using colonocytes from the distal colon.

Taking into account that Tyr-kinase undergoes autophosphorylation and is itself a substrate [23] efforts were made to determine whether the gastrin-induced stimulation of Tyr-kinase activity in colonocytes is associated with tyrosine phosphorylation of some membrane proteins. For this purpose colonocyte membrane preparations were subjected to autophosphorylation with [γ-^{32}P]ATP [24] and the labelled proteins were immunoprecipitated

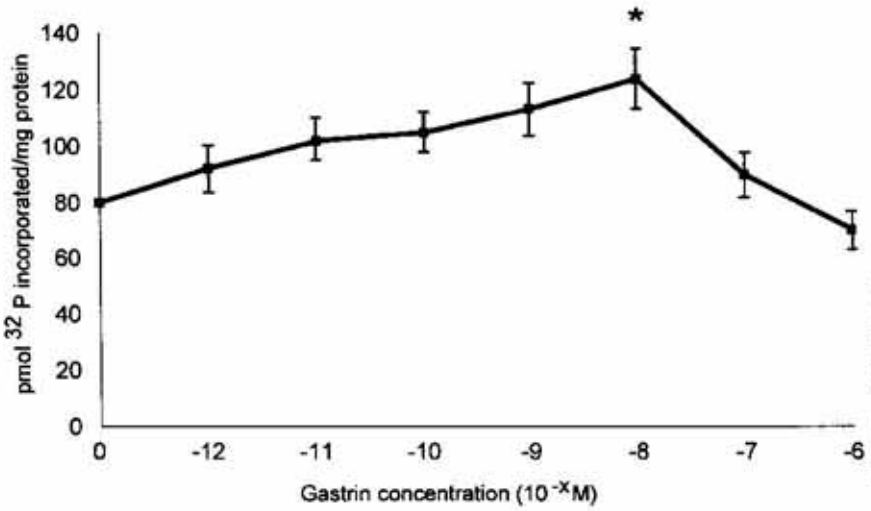

Fig. 1. Gastrin induced Tyr-kinase activity dose response in isolated colonocytes.
Values represent mean ±S.E.M. of at least 6 observations from 3 different preparations. *$P < 0.05$ when compared to basal level.

with antiphosphotyrosine antibody. The immunoprecipitates were then electrophoresed on SDS/polyacrylamide gel. Autoradiographic analysis of the SDS/polyacrylamide gel revealed several distinct bands in both basal and gastrin treated samples. Gastrin-induced stimulation of Tyr-kinase activity in colonocytes (distal) was associated with a marked increase in phosphorylation of proteins with relative molecular mass of 42000, 55000, 60000, 70000, 74000, 94000, 125000, 145000 and 170000 (Fig. 3), as demonstrated by densitometric analysis of the autoradiograph.

To further examine the mechanism of gastrin action tyrphostin, a specific, irreversible inhibitor of Tyr-kinase [25] and staurosporine (protein kinase C inhibitor) were used in the absence and presence of gastrin at 10^{-8} M concentration. In the absence of tyrphostin gastrin caused 61.8% and 150% stimulation of Tyr-kinase and PLC activities, respectively, over the corresponding controls. On the other hand, preincubation of colonocytes with tyrphostin (3.2 μM) completely abolished the gastrin-induced stimulation of Tyr-kinase activity, whereas staurosporine (20 nM) and tyrphostin themselves had no effect on this activity (Fig. 4). Similarly, phospholipase C stimulation by gastrin could be totally abolished by tyrphostin, while neither staurosporine nor tyrphostin alone produced any change in PLC activity (Fig. 5).

Protein kinase C activity in all samples assessed at the same time did not reveal any significant differences (not shown).

DISCUSSION

Inspired by the well known acid secretion stimulatory property of gastrin, numerous investigators have studied the binding charac-

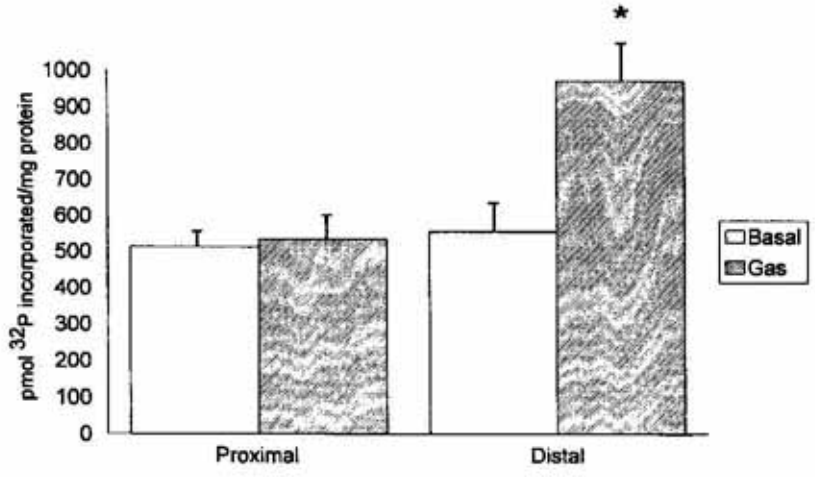

Fig. 2. Total Tyr-kinase activation in colonocytes from proximal and distal colon.
Values represent mean ±S.E.M. of at least 6 observations from 3 different preparations. *$P < 0.01$ when compared to corresponding basal level.

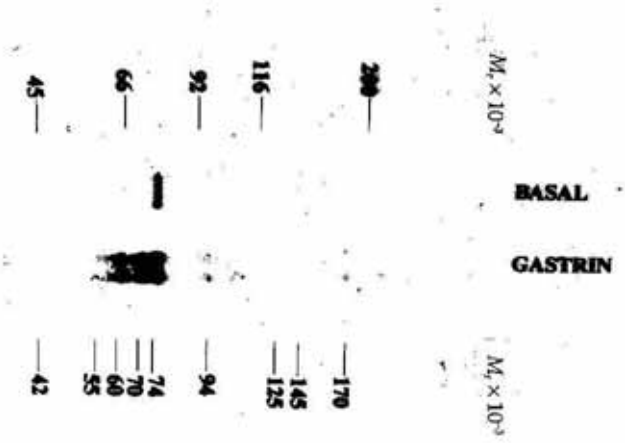

Fig. 3. Tyrosine-specific phosphorylation of membrane proteins from colonocytes incubated either in the absence (basal) or presence of gastrin (10^{-8}M).

teristics of gastrin receptors in gastric mucosa [26, 27].

At present this stimulatory effect of gastrin is completely explained by the stimulation of histamine release in a Ca^{2+}-dependent pathway [27].

Gastrin is now also considered to be a trophic hormone for the mucosa of various regions of the GI tract, including colon [1, 2, 5]. It is not known at present whether the receptor for the trophic action of gastrin is the same as that identified for its acid-secretory property. Moreover the intracellular events that regulate the gastrin-mediated stimulation of cell proliferation have not been fully elucidated.

A number of intracellular events are responsible for regulation of cell proliferation, differentiation and transformation. Recent evidence suggests that tyrosine phosphorylation catalyzed by Tyr-kinase plays an important regulatory role in these events [28]. The observation that several oncogene products [29, 30], as well as receptors of many growth factors [31, 32] show Tyr-kinase activity suggests that induction of cellular growth and transformation are associated with alteration in phosphotyrosine content.

In the GI tract we have observed increased Tyr-kinase activity in gastric mucosa during aging [21, 24], shortly after injury [33], after administration of EGF in young rats [24, 34] and in colonic mucosa after treatment with azoxymethane or azoxymethanol [34, 35]. Each of these conditions was also associated with increased mucosal proliferative activity. To determine, therefore, whether tyrosine kinase plays a role in mediating the growth-promoting action of gastrin in the gastrointestinal mucosa, we studied the effect of gastrin on Tyr--kinase activity and tyrosine-specific phosphorylation of membrane proteins on colonic mu-

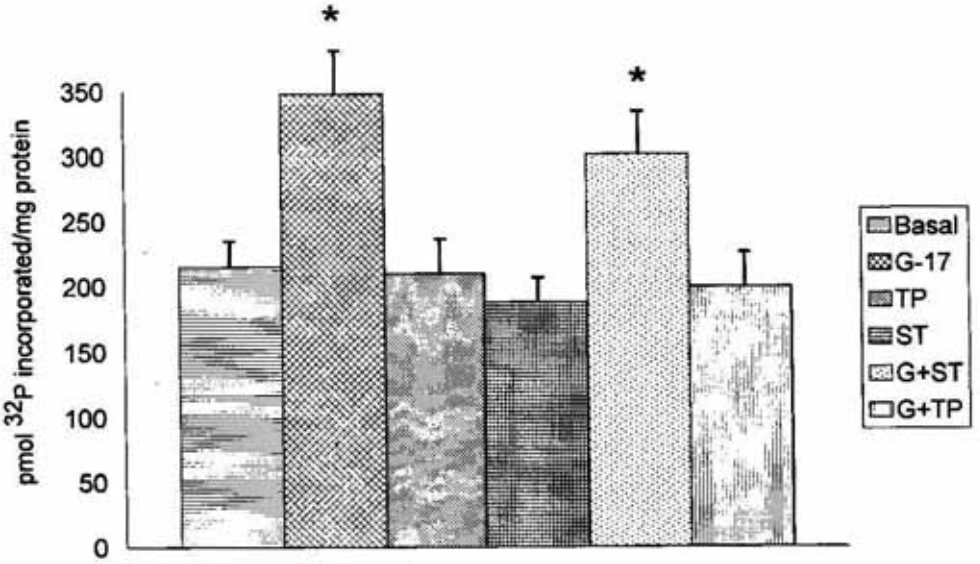

Fig. 4. Gastrin activation of Tyr-kinase in colonocytes.
Values represent mean ± S.E.M. of at least 6 observations from different preparations. *$P < 0.01$ when compared to basal level.

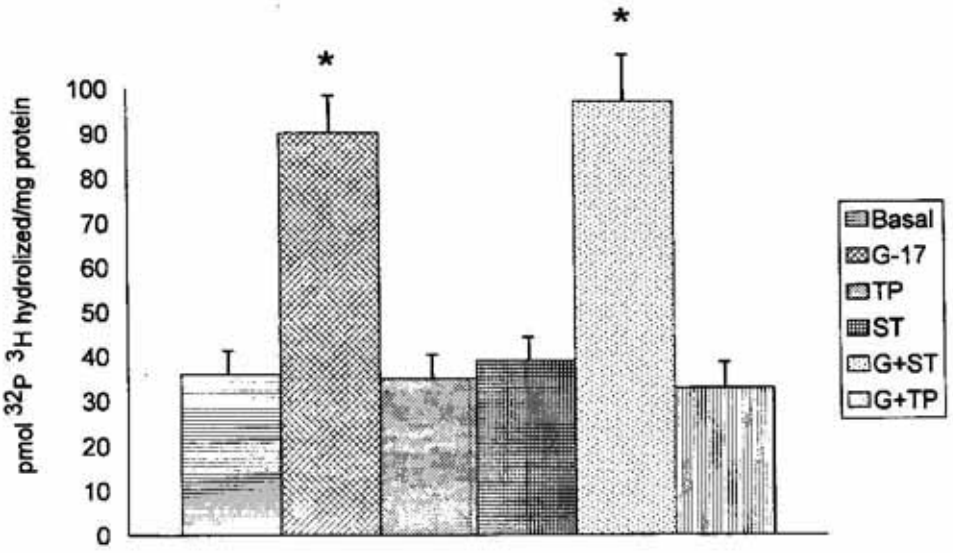

Fig. 5. Phospholipase C activation in colonocytes.
Values represent mean ±S.E.M. of at least 6 observations from different preparations. *$P < 0.001$ when compared to basal level.

cosa in an organ culture system [16]. We have shown that, in colonic mucosal explants, the maximal stimulation (ranging between 70% and 150%) in Tyr-kinase activity occurred within 10–15 min of exposure to gastrin, whereas ODC (a measure of proliferative potential) was stimulated several hours later. Genistein, a specific inhibitor of Tyr-kinase caused a total suppression of the gastrin induced stimulation of both Tyr-kinase and ODC. Furthermore, gastrin also stimulated tyrosine specific phosphorylation of a M_r 55000 membrane protein.

In presented experiments we have observed in our preparations an increase in both Tyr-kinase and PLC activities in response to gastrin. This may suggest, that induction of Tyr-kinase may play a determinant role for the rise in PLC activity by gastrin. If this was the case, then inhibition of Tyr-kinase should supress the gastrin induction of PLC in colonocytes. Indeed, we have observed, that in the presence of tyrphostin (an irreversible inhibitor of Tyr-kinase) the gastrin-mediated stimulation of PLC in our preparations was totally abolished, while a protein kinase C (PKC) inhibitor, staurosporine did not exert any effect on PLC activity. These data correspond with the findings that PLC may be directly tyrosine phosphorylated in response to growth factors and form a physical association with their receptors [36, 37].

We also measured tyrosine-specific phosphorylation of the colonocyte membrane proteins after exposure to gastrin. It appeared that gastrin significantly stimulated tyrosine phosphorylation of a number of proteins. The molecular mass of some of them corresponds to the molecular mass of PLC, EGF receptor and a 55 kDa protein which, according to our previous observations, may be critically involved in colonic mucosa cell proliferation [35].

The significance of increased Tyr-kinase activity with a resultant rise in tyrosine phosphorylation of some membrane proteins is not fully understood. However, it should be noted that, cellular transformation and neoplasia have been found to be associated with increased phosphotyrosine content [23, 28, 29].

The role of gastrin in colorectal carcinogenesis is not fully elucidated. Some investigators point to the correlation between colon cancer and elevated serum gastrin levels [38, 39] whereas others do not see any clear relationship between this cancer and gastrin [40, 41]. The Zollinger Ellisone Syndrome has been found to be associated with colorectal neoplasia [42]. Gastrin receptors with varying degrees of affinity have been found on gastrin-responsive tumour cell lines [43, 44].

The disparate results on the role of gastrin in colorectal carcinoma may be explained by complexity of the interrelationship between growth factors, GI hormones and normal and neoplastic cells, different response to gastrin of individual tumours and the possible impact of differences in etiology of hypergastrinemia [9].

In our study the Tyr-kinase activation by gastrin was observed only in colonocytes isolated

from the distal colon and not from the proximal one. It is also known, that in humans the frequency of colorectal tumours is significantly higher in the distal than in the proximal part of the colon [45]. We may speculate, that Tyr-kinase could be involved in gastrin induced carcinogenesis, neverthless more data would be necessary to confirm this hypothesis.

In conclusion, we have shown that gastrin stimulates Tyr-kinase activity in colonocytes from the distal but not from the proximal colon, which is associated with phosphorylation of several membrane proteins. Gastrin also stimulates PLC activity in the distal colon and Tyr-kinase may be required for regulation of this process. To the best of our knowledge activation of tyrosine kinases in gastric and colonic mucosa by gastrin is one of the earliest post-receptor events in the trophic action of gastrin.

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