Ca²⁺ and protein kinase C-dependent mechanisms involved in gastrin-induced Shc/Grb2 complex formation and P44-mitogen-activated protein kinase activation

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The proliferative effects of gastrin on normal and neoplastic gastro-intestinal tissues have been shown to be mediated by the gastrin/CCK_B (G/CCK_B) G-protein-coupled receptors. We have recently reported that gastrin stimulates the tyrosine phosphorylation of Shc proteins and their subsequent association with the Grb2/Sos complex, leading to mitogen-activated protein kinase (MAPK) activation, a pathway known to play an important role in cell proliferation. We undertook the present study to characterize the signalling pathways used by this receptor to mediate the activation of the Shc/Grb2 complex. Since G/CCK_B receptor occupancy leads to the activation of the phospholipase C (PLC)/protein kinase C (PKC) pathway, we examined whether PKC stimulation and Ca²⁺ mobilization contribute to the phosphorylation of Shc proteins and their association with Grb2 in response to gastrin. Our results indicate that Shc proteins are tyrosine phosphorylated and associate with Grb2 in response to phorbol esters, suggesting that activation of PKC is a potential

signalling pathway leading to activation of the Shc/Grb2 complex. Inhibition of PKC by GF109203X completely blocked the effect of PMA on Shc tyrosine phosphorylation and its subsequent association with Grb2, but had a partial inhibitory effect on the response to gastrin. Depletion of the intracellular Ca²⁺ pools by treatment with thapsigargin blocked the increase in intracellular free calcium concentration induced by gastrin and diminished the ability of the peptide to stimulate Shc phosphorylation and recruitment of Grb2. In addition, removal of extracellular Ca²⁺ partially inhibited the effect of gastrin on Shc phosphorylation as well as its association with Grb2, indicating that the effects of gastrin are also mediated by Ca²⁺-dependent mechanisms. Furthermore, we show that blockage of the two major early signals generated by activation of PLC, which induced the activation of the Shc/Grb2 complex, also blocked gastrin-induced MAPK activation.

INTRODUCTION

The mammalian gene *shc* encodes three proteins of 46, 52 and 66 kDa [1] which have been shown to play an important role in mitogenesis and cell transformation. Their overexpression in fibroblasts induces malignant transformation and increases cell proliferation in response to growth factor stimulation [1]. In contrast, microinjections of antibodies specific for Shc proteins inhibit growth factor-induced DNA synthesis [2].

Shc proteins have been found to be phosphorylated on tyrosine residues by a large number of oncogene products and growth factor receptors that contain intrinsic tyrosine-kinase activity [1,3,4]. The three isoforms of Shc act as adaptor proteins that link the Ras pathway to tyrosine-kinase receptors. The Shc SH2 domain interacts with specific phosphotyrosine-containing sequences of these tyrosine kinases. Following phosphorylation on tyrosine residues, Shc proteins also associate with the SH2 domains of Grb2, a second intermediate protein [3,5]. Grb2 also possesses SH3 domains which interact with the proline-rich motif of the p21-Ras activator termed Sos [6–11]. P42-mitogen-activated protein kinase (MAPK) and p44-MAPK, also known as ERK-1 and ERK-2 (extracellular-regulated protein kinase-1 and -2), are key downstream effectors of the p21-Ras signalling pathway. They have been shown to be stimulated by growth factor receptors with intrinsic tyrosine-kinase activity or by

growth hormone and cytokine receptors coupled to cytosolic protein tyrosine kinases [12,13].

Recently, several growth factors, whose signalling is transduced by G-protein-coupled receptors, have been reported to activate the Ras-dependent MAPK pathway by inducing Shc phosphorylation and its subsequent association with the Grb2/Sos complex [14–17]. However, the signalling pathways used by this receptor family to mediate the tyrosine phosphorylation of Shc proteins remain to be elucidated.

We have previously reported that gastrin exerts growth promoting effects on a tumour-derived pancreatic acinar cell line (AR4-2J) through the gastrin/CCK_B (G/CCK_B) receptor [18,19]. This receptor, which belongs to the family of G-protein-coupled receptors, is known to be linked to the phospholipase C/protein kinase C (PLC/PKC) pathway. Gastrin-dependent activation of the G/CCK_B receptor has been shown to induce the rapid hydrolysis of phosphatidylinositol biphosphate by PLC to generate diacylglycerol and inositol triphosphate, which activate PKC and mobilize intracellular Ca²⁺ [20,21]. We have recently demonstrated that gastrin induces a rapid and transient increase in tyrosine phosphorylation of several proteins, including two isoforms of the adaptor protein Shc (p46- and p52-kDa Shc) [17]. However, the mechanisms by which gastrin regulates Shc phosphorylation remain unknown. In the present study, we examined whether PKC activation, intracellular Ca²⁺ mobilization and

Abbreviations used: G/CCK_B, gastrin/CCK_B; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium.

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extracellular Ca^{2+} influx contribute to gastrin-induced Shc tyrosine phosphorylation and MAPK activation.

MATERIALS AND METHODS

Materials

Human gastrin₂₋₁₇NS was purchased from Bachem (Switzerland). ^{125}I -labelled NaI (100 mCi/ml) was obtained from Amersham and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) from Isotopchim. Myelin basic protein, Protein A-Sepharose-CL4B, Protein G-Sepharose-CL4B, orthovanadate, aprotinin, leupeptin, PMSF and BSA 7030 were purchased from Sigma. Anti-phosphotyrosine and anti-Shc antibodies were from Upstate Biotechnology. Anti-p44-MAPK and anti-Grb2 antibodies were obtained from Santa Cruz.

Cell culture

AR4-2J cells, originally obtained by Jessop and Hay [22] from a rat exocrine pancreatic tumour (azaserine induced), were given by Dr. C. Logsdon (Ann Arbor, MI, U.S.A.). The cells, plated at 75000 cells/ml, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum. The medium was changed every 2 days.

Immunoprecipitation

AR4-2J cells growing in 100 mm culture dishes were serum starved in DMEM for 18 h before peptide addition. After stimulation, the cells were washed with ice-cold buffer A [50 mM Hepes/150 mM NaCl/10 mM EDTA/10 mM $\text{Na}_4\text{P}_2\text{O}_7$ /100 mM NaF/2 mM orthovanadate (pH 7.5)] and homogenized in 500 μl of lysis buffer [buffer A containing 1% (v/v) Triton X-100, 0.5 mM PMSF, 20 μM leupeptin and 100 units/ml Trasylol] for 15 min at 4 °C. The solubilizes were clarified by centrifugation at 12000 g for 10 min at 4 °C and immunoprecipitated with the indicated antibodies preadsorbed on Protein A-Sepharose or Protein G-Sepharose. Samples for immunoblotting were washed twice with 30 mM Hepes buffer (pH 7.5) containing 30 mM NaCl and 0.1% Triton X-100, resuspended in SDS sample buffer and boiled for 5 min.

Western blotting analysis

Whole cell lysates or immunoprecipitates, prepared as described above, were separated by SDS/PAGE. Proteins were transferred to PVDF membrane (Immobilon PVDF, Millipore). Membranes were blocked with saline buffer [10 mM Tris/140 mM NaCl (pH 7.4)] containing 5% (w/v) BSA or non-fat dried milk and incubated overnight with the indicated antibodies. Membranes were washed 3 times with saline buffer containing 0.5% BSA or non-fat dried milk and 1% Triton X-100 and incubated with ^{125}I -Protein A (500000 c.p.m./ml) for 1 h at room temperature. Membranes were washed and autoradiographed.

Immune complex assays for MAPK activation

Cells growing in 60 mm culture dishes were serum starved in DMEM for 18 h before peptide addition. Cell lysates were immunoprecipitated as described above with anti-p44-MAPK antibodies. Precipitated immune complexes were washed twice in lysis buffer and 3 times in 20 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 200 μM orthovanadate, 0.1% Triton X-100, 10% (v/v) glycerol, 0.5 mM PMSF, 20 μM leupeptin and 100 units/ml Trasylol. The phosphorylation was performed

for 30 min at room temperature with myelin basic protein (at 0.2 mg/ml), 10 mM magnesium acetate and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol). The reaction was stopped by depositing the samples on P81 Whatman chromatography paper immersed in 1% (v/v) orthophosphoric acid. After three washes in 1% orthophosphoric acid, the radioactivity was counted.

Intracellular calcium measurement

The AR4-2J cells were plated at 75000 cells/ml into 35 mm culture wells containing a single glass coverslip (7 mm $\times$ 22 mm). The cells were loaded with 4 mM fura 2/AM for 45 min at 37 °C and transferred into a quartz cuvette into which gastrin was perfused. The fluorescence ratio (340 nm/380 nm) was measured with a dual-wavelength modular fluorimeter (Spex Fluorolog 2, Spex Industries, Edison, NJ, U.S.A.).

RESULTS

Tyrosine phosphorylation of Shc proteins and their association with Grb2 in response to phorbol esters and gastrin

We first investigated whether direct activation of PKC by phorbol esters increased the tyrosine phosphorylation of Shc proteins. AR4-2J cells were incubated with 100 nM PMA for the time indicated (Figure 1). Cell lysates were immunoprecipitated with an anti-Shc antibody and precipitates were analysed by immunoblot using an anti-phosphotyrosine antibody. As previously described for numerous tumour cell lines [23], Shc proteins were tyrosine phosphorylated in unstimulated AR4-2J cells despite their serum starvation (Figure 1). However, the basal constitutive phosphorylation of p46 and p52 Shc could be

Figure 1 Tyrosine phosphorylation of Shc proteins and their association with Grb2 in response to phorbol esters

(A) and (B) AR4-2J cells were treated for the times indicated with 100 nM PMA, 100 nM 4α-PDD or 10 nM gastrin (G). Cell lysates were immunoprecipitated with an anti-Shc antibody and the precipitates were Western blotted with an anti-phosphotyrosine antibody. (C) Cells were treated for 15 min with 100 nM PMA. The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and immunoblotting with an anti-Shc antibody. The migration of Shc proteins is indicated by the arrows.

Figure 2 Effects of PKC down-regulation on PMA and gastrin-stimulated Shc tyrosine phosphorylation

AR4-2J cells were incubated for 9 h in the absence or presence of 100 nM PMA and then treated with either gastrin (G) at 10 nM for 3 min or with 100 nM PMA for 15 min; C = control. Cells were lysed and proteins immunoprecipitated with an anti-Shc antibody. Precipitates were immunoblotted with an anti-phosphotyrosine antibody. The autoradiogram was exposed for 12 h (A) or 24 h (B). The arrows indicate the migration of precipitated Shc proteins.

enhanced by PMA (Figure 1A) or a growth-promoting agent such as gastrin (Figure 1B). The increase in tyrosine phosphorylation of both Shc isoforms by PMA was time dependent, reaching a maximum at 15–20 min (p46-Shc, 2.71 ± 0.33 -fold; p52-Shc, 3.83 ± 0.30 -fold; $n = 5$) before decreasing towards the basal level at 30–45 min. No significant increase in Shc phosphorylation was observed in AR4-2J cells treated with the biologically inactive phorbol ester 4 α -phorbol-12,13 didecanoate (PDD) (Figure 1B) (p46-Shc, 1.09 ± 0.15 -fold; p52-Shc, 1.17 ± 0.22 -fold; $n = 3$), whereas the phosphorylation was maximally enhanced in response to 10 nM gastrin as previously described (p46-Shc, 6.52 ± 1.00 -fold; p52-Shc, 10.62 ± 2.31 -fold; $n = 5$).

We next examined the association of Grb2 with Shc. The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and Western blotting with an anti-Shc antibody. Similarly to Shc phosphorylation, a basal constitutive association between Shc and Grb2 was observed in control cells (Figure 1C). However, treatment of the cells with 100 nM PMA for 15 min (Figure 1C) significantly increased the amount of Shc protein co-precipitated with the Grb2 antibodies (p46-Shc, 2.03 -fold ± 0.19 ; p52-Shc, 1.88 -fold ± 0.23 ; $n = 3$).

Effects of PKC down-regulation on PMA or gastrin-stimulated Shc phosphorylation

Since G/CCK_B receptors have been shown to be linked to the PLC/PKC pathway [20,21], we examined whether tyrosine phosphorylation of Shc proteins induced by gastrin could be mediated by PKC activation. For these experiments, we down-

Figure 3 Effects of PKC inhibition on PMA or gastrin-stimulated Shc/Grb2 complex formation

AR4-2J cells were pretreated for 30 min in the absence or presence of 5 μ M or 10 μ M GF109203X as indicated. (A) and (B) Cells were then incubated with 100 nM PMA for 15 min or with 10 nM gastrin for 3 min as indicated. Cell extracts were immunoprecipitated with an anti-Shc antibody and proteins were revealed by Western blotting using an anti-phosphotyrosine antibody. (C) and (D) The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and immunoblotting with an anti-Shc antibody. The migration of Shc proteins is indicated by the arrows.

regulated PMA-sensitive PKC isoforms by preincubation of AR4-2J cells for 9 h with 100 nM PMA. PKC down-regulated cells or control cells were subsequently stimulated with 10 nM gastrin for 3 min or with 100 nM PMA for 15 min. Immunoblots with anti-phosphotyrosine antibodies were performed on anti-Shc immunoprecipitates. PMA and gastrin-stimulated p52-Shc phosphorylation could be detected following a 12 h exposure of the autoradiogram (Figure 2A), whereas prolonged exposure (24 h) was necessary to detect p46-Shc phosphorylation (Figure 2B).

The increase in Shc phosphorylation by PMA was completely blocked following chronic treatment of the cells with PMA. In contrast, tyrosine phosphorylation of p46-Shc (Figure 2B) and p52-Shc (Figure 2A) in response to gastrin was partially inhibited in PKC down-regulated cells (respectively $43 \pm 6\%$ and $42 \pm 8\%$, $n = 3$).

Together, these results suggest that the induction of Shc tyrosine phosphorylation by gastrin can be mediated by both PKC-dependent and -independent mechanisms.

Effects of PKC inhibition on PMA- or gastrin-stimulated Shc/Grb2 complex formation

To provide additional evidence that only a portion of the gastrin-induced Shc phosphorylation is PKC dependent, we used a potent and specific inhibitor of PKC, GF109203X [24]. Cells were preincubated for 30 min with 5 μ M GF109203X and subsequently stimulated with 10 nM gastrin for 3 min or 100 nM PMA for 15 min (Figures 3A and 3B). The concentration of GF109203X used in these experiments completely abolished PMA-stimulated Shc phosphorylation. In contrast, Shc phosphorylation in response to 10 nM gastrin was only partially inhibited by 5 μ M GF109203X (p46-Shc, $54 \pm 5\%$; p52-Shc, $48 \pm 6\%$; $n = 3$). The basal phosphorylation of Shc proteins was

Table 1 Effect of thapsigargin on gastrin-induced Ca^{2+} mobilization

AR4-2J cells were treated with 10 nM gastrin and/or with the tumour promoter thapsigargin (1 μM , 30 min) as indicated. This agent specifically inhibits the endoplasmic reticulum Ca^{2+} -ATPase and thereby depletes intracellular compartments of Ca^{2+} . Intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) was measured in fura-2-loaded cells as described in the Materials and methods section. Results are means $\pm$ S.E. of four separate experiments.

Treatments	$[\text{Ca}^{2+}]_i$ (nM)
Basal	132 $\pm$ 23
Thapsigargin 1 μM	185 $\pm$ 33
Gastrin 10 nM	557 $\pm$ 23
Gastrin 10 nM + thapsigargin 1 μM	118 $\pm$ 19

not significantly affected by pretreatment of the cells with the inhibitor.

The partial inhibitory effects of 5 μM GF109203X on the Grb2/Shc complex formation induced by gastrin were consistent with those observed for the phosphorylation of Shc proteins (Figures 3C and 3D) (p46-Shc, 49 $\pm$ 8 %; p52-Shc, 64 $\pm$ 11 %; $n = 3$).

Effect of thapsigargin on gastrin-induced Ca^{2+} mobilization and Shc/Grb2 complex activation

We have previously reported that PLC activation by gastrin in AR4-2J cells leads to a rapid increase in inositol trisphosphate, which in turn mediates intracellular Ca^{2+} mobilization [21]. To evaluate the role of Ca^{2+} mobilization in gastrin-induced Shc tyrosine phosphorylation, we used thapsigargin, a selective inhibitor of the endoplasmic reticulum ATPase which is responsible for maintaining intracellular Ca^{2+} stores [25]. We first investigated the effect of thapsigargin on intracellular Ca^{2+} mobilization induced by gastrin (Table 1). As previously described in the literature [26,27], we observed in cells treated with 1 μM thapsigargin alone a weak but significant increase in the basal level of intracellular calcium (basal = 132 $\pm$ 23 nM, thapsigargin = 185 $\pm$ 33 nM). However, despite this weak stimulation, pretreatment of the cells with 1 μM thapsigargin for 30 min blocks completely the intracellular calcium increase induced by a subsequent addition of gastrin (basal = 132 $\pm$ 23 nM, gastrin = 557 $\pm$ 23 nM, gastrin + thapsigargin = 118 $\pm$ 19 nM, $n = 4$).

Tyrosine-phosphorylated Shc proteins were visualized by immunoprecipitation with an anti-Shc antibody and Western blotting with an anti-phosphotyrosine antibody (Figure 4A). We also observed a weak increase in Shc phosphorylation by thapsigargin alone which might reduce the inhibitory effects observed on gastrin signals. However, gastrin-induced Shc phosphorylation was still partially inhibited by pretreatment of the cells with 1 μM thapsigargin (p46-Shc, 49 $\pm$ 4 %; p52-Shc, 70 $\pm$ 3 %; $n = 3$), indicating the involvement of at least two mechanisms, one dependent on calcium release and the other one independent.

The partial inhibitory effects of thapsigargin on the Grb2/Shc complex formation induced by gastrin (Figure 4B) were consistent with those observed for the phosphorylation of Shc proteins (p46-Shc, 40 $\pm$ 3 %; p52-Shc, 58 $\pm$ 2 %; $n = 3$), indicating that the recruitment of Grb2 to phosphorylated Shc stimulated by gastrin can be mediated by mechanisms dependent or independent of Ca^{2+} release from intracellular stores.

Figure 4 Effect of thapsigargin on gastrin-induced Shc/Grb2 complex formation

AR4-2J cells were pretreated for 30 min in the absence or presence of the tumour promoter thapsigargin as indicated. Cells were subsequently incubated in the absence or presence of 10 nM gastrin for 3 min. (A) Cell lysates were immunoprecipitated with an anti-Shc antibody and immunoblotted with an anti-phosphotyrosine antibody. (B) The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and immunoblotting with an anti-Shc antibody. The arrows indicate the migration of Shc proteins.

Involvement of intracellular Ca^{2+} and PKC in gastrin-induced Shc/Grb2 complex activation

We performed additional experiments to examine the activation of the Shc/Grb2 complex in cells treated with both PMA and the calcium ionophore A23187. The Western blot Figure 5(A) indicates that PMA and A23187 synergize to produce 85 $\pm$ 5 % ($n = 3$) of the gastrin response on Shc/Grb2 complex activation. In addition, calcium chelation by thapsigargin (1 μM) and PKC inhibition by GF109203X (5 μM) inhibit by 87 $\pm$ 6 % ($n = 3$) gastrin-induced Shc/Grb2 complex formation (Figure 5B).

Figure 5 Role of PKC and Ca^{2+} in gastrin-induced Shc/Grb2 complex activation

Cells were treated with 100 nM PMA for 15 min, 10 μM A23187 (the calcium ionophore) for 30 min or 10 nM gastrin for 3 min as indicated in (A), or with 10 nM gastrin for 3 min, 5 μM GF109203X for 30 min or 1 μM thapsigargin for 30 min as indicated in (B). The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and immunoblotting with an anti-Shc antibody. The arrows indicate the migration of Shc proteins.

Figure 6 Role of Ca^{2+} influx in gastrin-induced Shc tyrosine phosphorylation and its association with Grb2

AR4-2J cells were incubated as indicated in the absence or presence of 5 mM EGTA and subsequently stimulated with 10 nM gastrin for 3 min. (A) Cell lysates were immunoprecipitated with an anti-Shc antibody and immunoblotted with an anti-phosphotyrosine antibody. (B) The Shc/Grb2 complex formation was visualized by immunoprecipitation with an anti-Grb2 antibody and immunoblotting with an anti-Shc antibody. The arrows indicate the migration of Shc proteins.

Role of Ca^{2+} influx in gastrin-induced Shc tyrosine phosphorylation and its association with Grb2

Previous studies from our laboratory have demonstrated that gastrin induces an initial increase in intracellular Ca^{2+} , which is independent of the presence of extracellular Ca^{2+} , followed by a small sustained elevation which is dependent on extracellular Ca^{2+} influx [21]. We therefore examined whether tyrosine phosphorylation of Shc and subsequent complex formation with Grb2, induced by gastrin, could be regulated by Ca^{2+} influx (Figure 6). AR4-2J cells were incubated in DMEM in the absence or presence of 5 mM EGTA. Elimination of extracellular Ca^{2+} by addition of EGTA to the culture medium partially blocked gastrin-induced tyrosine phosphorylation of Shc proteins (p46-Shc, $79 \pm 8\%$; p52-Shc, $65 \pm 5\%$).

Chelation of extracellular Ca^{2+} also decreased the association of Shc proteins with Grb2 induced by gastrin, indicating that Ca^{2+} influx plays an important role in Shc/Grb2 complex activation.

Role of PKC and Ca^{2+} in gastrin-induced MAPK activation

To examine whether PKC activation is a potential signalling pathway leading to MAPK activation in AR4-2J cells, we investigated the effect of PMA on MAPK activity by performing immune complex assays for MAPK activation (as described in the Materials and methods section). As shown in Figure 7, the time course of PMA-stimulated p44-MAPK activity (max. 2.11 ± 0.03 -fold at 15–20 min) paralleled the tyrosine phosphorylation of Shc proteins and their association with Grb2 induced by this phorbol ester.

In order to determine whether PKC activation contributes to

Figure 7 PKC-dependent activation of MAPK activity

Cells were treated with 100 nM PMA for the times indicated. Cells extracts were then immunoprecipitated by antibodies to p44-MAPK and MAPK activation measured by using myelin basic protein (MBP) as substrate as described in the Materials and methods section. Results are means $\pm$ S.E. of three separate experiments.

Table 2 Role of PKC and Ca^{2+} in gastrin-induced MAPK activation

AR4-2J cells were treated as indicated with 100 nM PMA for 15 min, 10 nM gastrin for 3 min, 10 μM A23187, 5 μM GF109203X (GF), 1 μM thapsigargin (TG) or 5 mM EGTA for 30 min. Cell lysates were immunoprecipitated by antibodies to p44-MAPK and MAPK activation was measured by using myelin basic protein (MBP) as substrate, as described in the Materials and methods section. Results are means $\pm$ S.E. of three separate experiments.

Treatments	Phosphorylation of MBP (c.p.m.)
No treatment	819 $\pm$ 54
PMA	1728 $\pm$ 62
Gastrin	9096 $\pm$ 649
A23187	1703 $\pm$ 136
PMA + A23187	7550 $\pm$ 566
PMA + GF	857 $\pm$ 75
Gastrin + GF	2888 $\pm$ 75
Gastrin + TG	1613 $\pm$ 71
Gastrin + GF + TG	1294 $\pm$ 65
Gastrin + EGTA	5205 $\pm$ 111

gastrin-induced p44-MAPK activity, we performed additional experiments on GF109203X-pretreated AR4-2J cells. As shown in Table 2, pretreatment of the cells for 30 min with 5 μM GF109203X completely blocked the subsequent effect of PMA but had only a partial effect ($70 \pm 3\%$) on gastrin-induced MAPK activity. The basal level of myelin basic protein phosphorylation was not significantly affected by pretreatment of the cells with the inhibitor.

To assess whether intracellular Ca^{2+} mobilization participates in MAPK activation in the AR4-2J cell line, MAPK activity was measured in cells treated for 30 min with the calcium ionophore A23187. p44-MAPK activity was increased 2.08-fold $\pm$ 0.08 in response to 10 μM A23187.

In addition, despite a weak increase in basal MAPK activity observed with thapsigargin alone (1.3-fold $\pm$ 0.2; results not shown), gastrin-induced MAPK activation was partially inhibited ($82 \pm 4\%$) by pretreatment of the cells with 1 μM thapsigargin (Table 2), indicating that elevation of intracellular Ca^{2+} concentrations is involved in MAPK activation by gastrin.

The results in Table 2 also indicate that PMA and A23187

synergize to produce $83 \pm 7\%$ of the gastrin effect, which induces a $11.1\text{-fold} \pm 0.7$ increase in MAPK activity. In addition, calcium chelation by thapsigargin ($1\text{ }\mu\text{M}$) and PKC inhibition by GF109203X ($5\text{ }\mu\text{M}$) inhibit, by $86 \pm 5\%$, gastrin-induced MAPK activation.

Finally, we analysed the possibility that MAPK activation in response to gastrin was dependent on the presence of extracellular Ca^{2+} . As shown in Table 2, the effects of gastrin were partially inhibited ($43 \pm 2\%$) by preincubation of the cells with 5 mM EGTA, indicating a role for Ca^{2+} influx in the MAPK signalling pathway activated by gastrin.

DISCUSSION

The present study demonstrates that the phosphorylation of Shc proteins on tyrosine residues and their association with Grb2 were enhanced in response to the biologically active phorbol ester PMA, indicating that activation of PMA-sensitive PKC isoforms is a potential mechanism involved in the recruitment of the Shc/Grb2 complex.

Gastrin, a regulatory peptide known to activate the PLC/PKC pathway through G-protein-coupled receptors, also activates the complex Shc/Grb2. In AR4-2J cells, the PLC/PKC pathway is activated within 40 s of gastrin addition [20]. This activation precedes the Shc/Grb2 complex formation and MAPK activation which occur $3\text{--}5\text{ min}$ after gastrin stimulation [17]. These results are in agreement with the hypothesis that the PLC/PKC pathway may be involved upstream of gastrin-induced Shc phosphorylation and MAPK activation.

Recent data obtained in our laboratory (F. Clemente, A. Estival, N. Vaysse, personal communication) demonstrate that the major PKC isoform in this cell line is PKC α . However, PKC ϵ and PKC δ are also expressed. All these isoforms are sensitive to diacylglycerol and can be down-regulated by prolonged exposure to PMA [28]. We did not observe, in our experiments, any effect of PMA on Shc phosphorylation after chronic treatment of the cells with this phorbol ester, indicating that all PMA-sensitive PKCs involved in Shc phosphorylation have been down-regulated. Under these experimental conditions, gastrin-induced Shc phosphorylation was partially decreased. A partial inhibition of gastrin-induced Shc/Grb2 complex activation was also observed when cells were pretreated with a bisindolylmaleimide (GF109203X), known to inhibit completely several PKC isoforms including PKC α , PKC ϵ and PKC δ [29,30].

Taken together, these results provide evidence of gastrin stimulating the recruitment of the Shc/Grb2 complex through the activation of at least two different pathways, one involving PMA-sensitive PKC and the other one independent of these enzymes.

In AR4-2J cells, gastrin has been shown to stimulate the release of Ca^{2+} from inositol-triphosphate-sensitive intracellular stores. In this cell line, the level of intracellular Ca^{2+} is also regulated by extracellular Ca^{2+} influx. The results presented in this study demonstrate that Ca^{2+} plays an important role in the formation of the Shc/Grb2 complex. Indeed, depletion of intracellular Ca^{2+} pools by thapsigargin, which completely blocked the mobilization of Ca^{2+} stimulated by gastrin, partially inhibited the gastrin-induced Shc phosphorylation and subsequent association with Grb2. These results demonstrate that gastrin activates the complex Shc/Grb2 through a signal transduction pathway that is partly dependent on intracellular Ca^{2+} . In addition, elimination of extracellular Ca^{2+} by addition of EGTA to the culture medium decreased the tyrosine phosphorylation of Shc proteins and their association with Grb2 induced by

gastrin, indicating that Ca^{2+} influx also plays a role in this response.

We [17] and others [31] have previously shown that occupancy of the G/CCK $_B$ receptors leads to MAPK activation. We show in this study that blockage of the two major early signals generated by activation of PLC, which induces the activation of the Shc/Grb2 complex, also blocked gastrin-induced MAPK activation. These results suggest that tyrosine phosphorylation of Shc proteins and subsequent complex formation with Grb2 may be one mechanism by which the G/CCK $_B$ G-protein-coupled receptor mediates p21-Ras-dependent MAPK activation.

Thrombin and thyrotropin-releasing hormone [14,16], two ligands that bind G-protein-coupled receptors, have also been shown to stimulate Shc phosphorylation on tyrosine residues. However, the effects of these ligands on Shc phosphorylation are mediated by Ca^{2+} and PKC-independent mechanisms. These results and ours suggest that the role of PKC and Ca^{2+} in the phosphorylation of Shc proteins may depend on the cell system and the specific agonist.

Further studies are required to identify the Ca^{2+} and/or PKC-dependent tyrosine kinases stimulated by gastrin that may be responsible for the phosphorylation of Shc proteins.

Different protein tyrosine kinases activated by G-protein-coupled receptors may be involved. In particular, Src-family protein tyrosine kinases are potential candidates for mediating Ca^{2+} -induced tyrosine phosphorylation of Shc proteins. This family of kinases have been shown to be activated by a number of seven-transmembrane-domain receptors including the G/CCK $_B$ receptors [32–34]. In addition, published data have demonstrated that *shc* gene products become highly tyrosine phosphorylated in cells transformed with Src oncoproteins, suggesting that Shc proteins may be biologically relevant substrates of Src tyrosine kinases [35].

Recently, a novel cytoplasmic protein tyrosine kinase, termed PYK2, has been cloned. This non-receptor protein tyrosine kinase has been shown to be activated by extracellular signals that lead to Ca^{2+} influx or Ca^{2+} release from internal stores, including ligands that bind G-protein-linked receptors. Moreover, PYK2 appears to be regulated by both PKC-dependent and -independent mechanisms. In addition, dramatic tyrosine phosphorylation of Shc proteins was observed in cells over-expressing PYK2, suggesting that activation of PYK2 may be one mechanism by which G-protein-coupled receptors mediate Shc phosphorylation [36].

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