Modulation of bombesin-induced phosphatidylinositol hydrolysis in a small-cell lung-cancer cell line

Jane B. TREPEL,*§ James D. MOYER,† Reino HEIKKILA‡|| and Edward A. SAUSVILLE*

*National Cancer Institute–Navy Medical Oncology Branch, †Laboratory of Biological Chemistry, and ‡Laboratory of Pathology, National Cancer Institute, N.I.H., Bethesda, MD 20814, U.S.A.

Bombesin is an amphibian tetradecapeptide whose mammalian homologue, gastrin-releasing peptide (GRP), is produced by many small-cell lung-cancer (SCLC) cells, and which can function in an autocrine growth-promoting manner in SCLC. Studies reported here show that [Tyr⁴]bombesin and its congeners increase inositol 1,4,5-trisphosphate within seconds in NCI-H345, a SCLC cell line that constitutively produces GRP. After 30 min in the presence of 0.01 M-Li⁺ and [Tyr⁴]bombesin, there is marked accumulation of inositol monophosphates and inositol tetrakisphosphate. Pretreatment with phorbol 12-myristate 13-acetate (PMA) for 20 min inhibited the ability of [Tyr⁴]bombesin to induce phosphatidylinositol (PtdIns) turnover and to increase intracellular free Ca²⁺ ([Ca²⁺]_i). Pretreatment with PMA for 48 h attenuated the ability of subsequently added PMA to decrease the response to [Tyr⁴]bombesin. Pretreatment with pertussis toxin (PT; 1 µg/ml for 18–24 h) decreased by < 30 % [Tyr⁴]bombesin-induced increases in [Ca²⁺]_i and PtdIns metabolites. However, interpretation of this result is complicated by the inability of PT to ADP-ribosylate completely its substrates in intact NCI-H345 cells. In contrast, pretreatment with cholera toxin (1 µg/ml for 18–24 h) lowered basal [Ca²⁺]_i and basal inositol phosphate concentrations, attenuated the response of NCI-H345 to subsequently added [Tyr⁴]bombesin, and was not mimicked by treatments that increase cellular cyclic AMP. These data demonstrate the activation of phospholipase C in SCLC by bombesin congeners. In addition, the results suggest a regulatory role for protein kinase C, a cholera-toxin substrate, and perhaps a pertussis-toxin substrate in the response of SCLC to bombesin.

INTRODUCTION

Bombesin is a peptide originally isolated from amphibian skin on the basis of its ability to act as a secretagogue for several mammalian hormones, including gastrin [1]. Gastrin-releasing peptide (GRP) is a bombesin-like peptide found in mammalian central nervous system, gut, fetal and injured lung, and in small-cell lung cancer (SCLC) [2–4]. Bombesin and GRP share nine of ten C-terminal amino acids and bind to the same cell-surface receptor [5]. In addition to clearly defined roles as secretagogues and neuropeptides, these peptides are mitogenic for Swiss 3T3 cells [6] and normal bronchial epithelial cells [7]. Since some SCLC cell lines produce bombesin-like peptides and respond to them by growth in soft agar or nude-mouse heterotransplants [8,9], bombesin-like peptides may have an autocrine role in promoting the growth of SCLC.

Bombesin-activated signal-transduction events have been extensively studied in Swiss 3T3 cells, where it has been shown that bombesin induces phosphatidylinositol (PtdIns) turnover [10], protein kinase C and tyrosine kinase [11,12] activation, Na⁺/H⁺ antiport activity, increased cytoplasmic free Ca²⁺ ([Ca²⁺]_i) [13,14], and

pertussis toxin (PT)-sensitive [15] and phorbol ester-sensitive [16–20] proto-oncogene activation in quiescent cells. Since the relevance of these phenomena to SCLC has not been established, and is of interest in designing therapeutic strategies in this disease, this detailed study of intracellular signals evoked by bombesin congeners has been conducted in NCI-H345, a SCLC cell line that has receptors for bombesin-like peptides [21], expresses the human prepro-GRP gene, contains bombesin-like immunoreactivity [3,4], and responds to exogenous bombesin with increased [Ca²⁺]_i and increased cloning efficiency in soft agar [9,22].

EXPERIMENTAL

Reagents

[Tyr⁴]bombesin, GRP, GRP-(20–27)-peptide and GRP-(1–16)-peptide were from Peninsula (Belmont, CA, U.S.A.). Quin2, phorbol 12-myristate 13-acetate (PMA), 4β-phorbol, dioctanoylglycerol and reagents for ADP-ribosylation were from Sigma. Cholera toxin (CT) was from Sigma or List Biologicals (Campbell, CA, U.S.A.), with no difference noted in effect. PT was exclusively

Abbreviations used: GRP, gastrin-releasing peptide; SCLC, small-cell lung carcinoma; [Ca²⁺]_i, intracellular free calcium concentration; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; Ins1P, inositol 1-monophosphate; Ins4P, inositol 4-monophosphate; Ins(1,4)P₂, inositol 1,4-bisphosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate; PMA, phorbol 12-myristate 13-acetate; CT, cholera toxin; PT, pertussis toxin; R-SIT-H, RPMI-1640 medium without inositol plus 30 nM-sodium selenite, 5 µg of insulin/ml, 10 µg of transferrin/ml, 0.02 M-Hepes; R-H, RPMI-1640 medium with 0.02 M-Hepes; G-protein, guanine-nucleotide-binding protein.

§ To whom correspondence and reprint requests should be addressed: NCI–Navy Medical Oncology Branch, Bldg. 8, Room 5101, Naval Hospital, Bethesda, MD 20814, U.S.A.

|| Present address: Immunology Laboratory, Radium Hospitalet, Montebello, Norway.

from List. RPMI 1640 medium without inositol was formulated by the NIH media unit according to the components listed by Grand Island Biological Co. Radiolabelled inositol 1-phosphate (Ins1P), inositol 1,4-bisphosphate [Ins(1,4)P₂], inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] were purchased from Amersham Corp. Radiolabelled inositol 4-phosphate (Ins4P), glycerophosphoinositol and inositol 1,3,4-trisphosphate [Ins(1,3,4)P₃] were prepared and characterized as described in ref. [23].

Assay of inositol phosphates

The SCLC cell line NCI-H345 was maintained in culture as described previously [22]. Cells were labelled for 48–96 h in RPMI 1640 without inositol plus 30 nM-sodium selenite, 5 µg of insulin/ml, 10 µg of transferrin/ml and 0.02 M-Hepes (R-SIT-H) with 4–5 µCi of *myo*-[³H]inositol (16.3 Ci/mmol; Amersham)/ml. For determination of inositol phosphates after incubation with hormone for 30 min, cells were washed in RPMI 1640 without inositol plus 0.02 M-Hepes (R-H) and incubated for 60 min at 37 °C in R-H or R-SIT-H as indicated in the Figure legends. Cell suspensions (~1 mg of protein) in 1 ml of the same medium were incubated with 0.01 M-LiCl for 15 min before addition of bombesin congeners to amplify accumulation of inositol phosphates as described by Berridge *et al.* [24]. The cells were collected by centrifugation at 10000 *g* for 12 s and lysed in 5% trichloroacetic acid, which was removed by ether extraction before chromatography.

For assay of inositol phosphates after short periods of incubation (< 1 min), cells were resuspended in Hepes-buffered saline (145 mM-NaCl, 5 mM-KCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 5 mM-dextrose, 20 mM-Hepes, pH 7.4) also containing 0.01 M-LiCl. Bombesin congeners were added to 0.4 ml of this cell suspension (~0.7 mg of protein), and the cells were incubated at 37 °C, followed by addition of 0.04 ml of 100% trichloroacetic acid directly to the suspension.

H.p.l.c. utilized a modification of systems described previously [23]. A Whatman SAX column (10 cm × 0.45 cm) was eluted with a gradient of ammonium phosphate, pH 3.8, at a flow rate of 1 ml/min. The gradient used was from 0.01 M- (buffer A) to 1.4 M- (buffer B) ammonium phosphate, as follows: 2 min 100% buffer A, a 13 min linear gradient to 10% B, a 10 min gradient from 10% to 45% B, a 10 min gradient from 45% to 60% B, and a 6 min gradient to 100% B, followed by 9 min of elution at 100% B. Radioactivity was measured by use of a Raytest radiodetector with a 2 ml flow cell and a ratio of 4:1 (v/v) Tru-count High Salt scintillant:eluate at an efficiency of 17%. The retention times of radiolabelled standards are shown by arrows in the 'control' chromatogram of Fig. 1.

For the experiments in Fig. 4, the separation was performed with an ammonium phosphate gradient from 0.5 M (buffer A') to 1.9 M (buffer B'), pH 3.8. Chromatography was performed with a 3 min linear gradient to 20% B', 2 min to 55% B', 5 min to 100% B', and 9 min of further elution at 1 ml/min with 100% B'. In this system, Ins(1,3,4)P₃ is eluted at 7.5 min, Ins(1,4,5)P₃ at 9.5 min, and Ins(1,3,4,5)P₄ at 17 min, well separated from inositol pentakisphosphate (eluted at 19.5 min). Fractions (0.5 ml) were collected and radioactivity was

determined after mixing in 6.5 ml of ACS (Amersham) scintillant containing 5% water.

Assay of intracellular Ca²⁺

[Ca²⁺]_i was assayed by the quin2 technique of Tsien *et al.* [25] as described previously [22].

ADP-ribosylation by bacterial toxins

Cells were treated with cholera or pertussis toxin for up to 24 h, washed once with 0.15 M-NaCl/0.015 M-sodium phosphate, and resuspended in 0.01 M-Tris/0.1 mM-EDTA, pH 7.4. After swelling for 10 min at 4 °C, the cells were homogenized with 40–60 strokes of a Teflon/glass homogenizer. Unbroken cells were removed by centrifugation for 10 min at 500 *g*, and the supernatant sedimented at 100000 *g* at 4 °C for 30 min. The sedimented membranes were washed once in 5 mM-Hepes, and 50 µg of membrane protein was treated with dithiothreitol-activated CT (40 µg/ml) or PT (10 µg/ml) and [³²P]NAD⁺ (New England Nuclear), essentially as described by Martin *et al.* [26], except that MgCl₂ was omitted and reactions were for 30 min at 32 °C, followed by SDS/polyacrylamide-gel electrophoresis and autoradiography of the acid-fixed dried gel.

RESULTS

Bombesin-stimulated PtdIns turnover in SCLC

Fig. 1 shows the profile of inositol phosphates found in NCI-H345 cells, and the changes resulting from the addition of [Tyr⁴]bombesin. There is marked accumulation of Ins1P, Ins4P and InsP₄ after 30 min of incubation in the presence of Li⁺ and [Tyr⁴]bombesin. This InsP₄ component may be a mixture of isomers

Fig. 1. Separation of inositol phosphates from NCI-H345 SCLC cells

NCI-H345 cells were labelled with *myo*-[³H]inositol and incubated in the presence (lower panel) or absence (upper panel) of 100 nM-[Tyr⁴]bombesin in R-SIT-H and chromatographed as described in the Experimental section. The elution positions of standards are indicated.

Fig. 2. Structure-activity analysis and dose-response relation of *InsP* production after [Tyr⁴]bombesin treatment

Cells were incubated for 30 min in the presence of 10 mM-LiCl and 100 nM of the indicated peptide (upper panel) or the indicated concentration of [Tyr⁴]bombesin in R-H (lower panel: ●, *Ins1P*; ○, *Ins4P*). Values shown are means ± s.d. of triplicate determinations. C, control; B, [Tyr⁴]bombesin; (20-27), GRP-(20-27)-peptide, amidated at C-terminus; (1-16), GRP-(1-16)-peptide.

including *Ins*(1,3,4,5)*P*₄, with which it co-chromatographs. In this experiment, representative of 15 independent experiments performed with triplicate incubations, [Tyr⁴]bombesin increased *Ins1P* by 360%, *Ins4P* by 690% and *InsP*₄ by 82%. Amounts of glycerophosphoinositol were not affected by [Tyr⁴]bombesin treatment of NCI-H345 cells (Fig. 1). A comparison of [Tyr⁴]bombesin, GRP and fragments of GRP shows that [Tyr⁴]bombesin and GRP have equivalent activity in stimulating inositol phosphate production (Fig. 2, upper panels). GRP-(20-27)-peptide, which shares seven of eight C-terminal amino acid residues with [Tyr⁴]bombesin, was active in inducing *PtdIns* turnover, whereas GRP-(1-16)-peptide, with no structural homo-

logy with bombesin, was inactive. The increase in the two inositol monophosphate isomers in response to [Tyr⁴]bombesin has a similar dependence on ligand concentration (Fig. 2, lower panel). A response was first seen at 1 nM-[Tyr⁴]bombesin (0.1 nM was ineffective; results not shown), and was half-maximal at approx. 10 nM. The concentration-dependence of inositol monophosphate production corresponds to that of the bombesin-induced *Ca*²⁺ transient in NCI-H345 cells [22]. Analogous experiments performed with NCI-H82, a SCLC cell line which does not respond to bombesin with increased [*Ca*²⁺]_i [22], shows no evidence of [Tyr⁴]bombesin-induced *PtdIns* turnover, despite comparable proportions of the inositol phosphate precursors phosphatidylinositol 4-phosphate (*PtdIns*4*P*) and phosphatidylinositol 4,5-bisphosphate [*PtdIns*(4,5)*P*₂]; results not shown].

Since bombesin-induced increases in [*Ca*²⁺]_i occur almost immediately after addition of ligand and derive in part from internal *Ca*²⁺ stores [22], inositol phosphates were measured at short times after stimulation. [Tyr⁴]bombesin induced a very rapid increase in *Ins*(1,4,5)*P*₃ (a 3-fold elevation at 8 s), which declined by 30 s (Fig. 3). *Ins*(1,3,4)*P*₃, formed from *Ins*(1,4,5)*P*₃ by sequential action of a 3-kinase, to yield *Ins*(1,3,4,5)*P*₄, and a 5'-phosphomonoesterase [27], was increased by 30 s. *InsP*₄ was not increased at 30 s after [Tyr⁴]bombesin addition, but increased by 50–100% after 30 min in the presence of Li⁺ (Fig. 1, lower panel; also see below).

Phorbol ester-induced inhibition of *PtdIns* turnover

To characterize further bombesin-stimulated events in SCLC, we examined the effect of phorbols on [Tyr⁴]bombesin-stimulated [*Ca*²⁺]_i and inositol phosphate formation. PMA (final concn. 50 nM) had no acute effect on the [*Ca*²⁺]_i of NCI-H345 cells, but attenuated the response to subsequently added [Tyr⁴]bombesin (Fig. 4a). The compound 4β-phorbol, which does not activate protein kinase C, had no effect on the agonist-stimulated [*Ca*²⁺]_i increase. In accord with these data, PMA inhibited in a 30 min incubation the accumulation of *Ins4P* induced by [Tyr⁴]bombesin, and the increase in *Ins*(1,4,5)*P*₃ observed at 8 s (Fig. 4c). Phorbol 12,13-dibutyrate and dioctanoylglycerol, both activators of protein kinase C, also inhibited [Tyr⁴]bombesin-induced *Ins1P* and *Ins4P*, whereas 4β-phorbol had no effect on [Tyr⁴]bombesin-stimulated inositol phosphate accumulation (results not shown) after 30 min in the presence of Li⁺.

If antagonism of bombesin-induced responses by phorbol esters is due to activation of protein kinase C, then this effect should be diminished in cells depleted of protein kinase C. Fig. 5(a) shows that PMA treatment (20 min) attenuates the [Tyr⁴]bombesin-stimulated increase in *Ins4P*, but, in a parallel culture treated with PMA for 48 h (a treatment known to deplete protein kinase C in several cell types [16,28]), re-addition of PMA 20 min before bombesin treatment did not decrease the response to [Tyr⁴]bombesin. This effect is also observed with *InsP*₄ (Fig. 5b).

Effects of pertussis or cholera toxin

Pretreatment of NCI-H345 cells for 20 h with 1 μg of PT/ml inhibited by 10–20% the increase in [*Ca*²⁺]_i evoked by [Tyr⁴]bombesin (Fig. 6a). However, Fig. 7 shows that this pretreatment does not completely protect the predominant ~39 kDa PT substrate in these cells from subsequent ADP-ribosylation by pre-activated PT.

Fig. 3. Effect of [Tyr⁴]bombesin on InsP₃ and InsP₄ production in NCI-H345 cells

□, Water added (control); ▨, 100 nM-[Tyr⁴]bombesin added. At the indicated times, 0.1 vol. of 100% trichloroacetic acid was added to the incubations. The radioactivity chromatographing with the indicated inositol phosphate isomer is indicated. Data are means ± S.D. of triplicate determinations: * $P < 0.001$, ** $P < 0.05$ by Student's t test.

Fig. 4. Phorbol ester inhibition of the [Tyr⁴]bombesin-stimulated signals in NCI-H345 cells

(a) The $[Ca^{2+}]_i$ (nM) was measured by the quin2 technique. Upper trace: $[Ca^{2+}]_i$ after addition of 100 nM-[Tyr⁴]bombesin (B). Middle trace: effect of pretreatment with 50 nM-PMA. Lower trace: effect of pretreatment with 50 nM-4β-phorbol. (b) Effect of PMA pretreatment on [Tyr⁴]bombesin-stimulated accumulation of Ins4P. Cells were pretreated with 50 nM-PMA for 15 min before stimulation with 100 nM-[Tyr⁴]bombesin for 30 min in R-H. (c) Effect of PMA pretreatment on [Tyr⁴]bombesin stimulation of Ins(1,4,5)P₃ production. Cells were pretreated with 50 nM-PMA for 15 min before 8 s stimulation with 100 nM-[Tyr⁴]bombesin. Data represent means ± S.D. of triplicate measurements.

This implies that such pretreatment is not adequate to ADP-ribosylate completely all substrate(s) of PT in NCI-H345 cells. In contrast, pretreatment with 1 μg of CT/ml causes a marked (~50%) decrease in basal $[Ca^{2+}]_i$ and a marked attenuation of the increase in $[Ca^{2+}]_i$ caused by [Tyr⁴]bombesin (Fig. 6a). As shown in Fig. 7, this is accompanied by virtually complete protection of the 45 and 52 kDa CT substrates from ADP-ribosylation *in vitro*.

The correlation of these effects with PtdIns metabolism

is shown in Figs. 6(b) and 6(c). Pretreatment for 20 h with CT at 1 μg/ml significantly decreases Ins(1,4,5)P₃ and attenuates the [Tyr⁴]bombesin-induced increase in Ins(1,4,5)P₃ observed at 8 s after agonist addition, providing a parallel to the observed effect of CT on $[Ca^{2+}]_i$. Pretreatment with PT at 1 μg/ml for the same period of pretreatment had no effect on the basal concentrations of Ins(1,4,5)P₃, and there was a 20–30% attenuation of the [Tyr⁴]bombesin-evoked increase in Ins(1,4,5)P₃. These findings are supported by independent

Fig. 5. Effect of long-term PMA pretreatment on response of SCLC to [Tyr⁴]bombesin

Parallel cultures were maintained in the absence or the presence of 100 nM-PMA for 48 h in R-SIT-H. Portions of both cultures were incubated with 0.01 M-LiCl for 15 min before treatment with or without 100 nM-PMA for 20 min, followed by vehicle or 200 nM-[Tyr⁴]bombesin (B). Incubation continued for 30 min before extraction of inositol phosphates. Data represent means $\pm$ S.D. of triplicate determinations.

experiments observing the effect of toxin pretreatment on the accumulation of Ins1P and Ins4P in 30 min incubations in the presence of Li⁺. As shown in Fig. 6(c), pretreatment with CT at 1 μ g/ml markedly attenuates the increase in Ins4P and Ins1P observed after treatment with [Tyr⁴]bombesin, whereas PT at 1 μ g/ml inhibits this effect by 20–30%.

Treatment with CT or PT at 1 μ g/ml did not affect the viability of the cells, as assessed by Trypan Blue exclusion, for up to 48 h of treatment. Dibutyl cyclic AMP (1 mM for 30 min) or 8-chloro-cyclic AMP (10 μ M for 60 min) had no effect on accumulation of Ins1P and Ins4P induced by [Tyr⁴]bombesin (results not shown) after 30 min of treatment in the presence of Li⁺. Likewise, 0.5 mM-isobutylmethylxanthine with or without 1 mM-dibutyl cyclic AMP for 60 min did not inhibit [Tyr⁴]bombesin-induced increases in Ins4P, Ins1P or InsP₄ after 30 min in the presence of Li⁺ (results not shown). CT treatment at 1 μ g/ml for 3 h or less did not inhibit PtdIns turnover in response to [Tyr⁴]bombesin, but treatment with 100 ng of CT/ml for 18–20 h inhibited by 50% the [Tyr⁴]bombesin-induced increase in Ins4P after 30 min in the presence of Li⁺, without effect on basal Ins4P concentrations (results not shown).

DISCUSSION

The autocrine model of tumour growth [29] proposes that factors elaborated by tumour cells may promote the growth of the tumour. A prediction of this model is that

Fig. 6. Effect of CT or PT pretreatments on bombesin-induced signals in NCI-H345 cells

(a) The $[Ca^{2+}]_i$ (nM) was measured in cells without pretreatment (upper trace) or after pretreatment with 1 μ g of CT/ml (middle trace) or 1 μ g of PT/ml (lower trace) for 18 h. To the right of the traces are the basal and stimulated measured $[Ca^{2+}]_i$ values. The data are representative of three independent experiments. Abbreviation: B, bombesin. Values in parentheses are percentage increases. (b) Parallel cultures were treated with 1 μ g of CT/ml, 1 μ g of PT/ml, or medium alone (C) for 16–18 h and then stimulated by 100 nM-[Tyr⁴]bombesin in Hepes-buffered saline plus 0.01 M-LiCl for 8 s before extraction of inositol phosphates. (c) Parallel cultures were treated with 1 μ g of CT/ml, 1 μ g of PT/ml or medium for 18 h before resuspension in R-H and stimulation with 600 nM-[Tyr⁴]bombesin in the presence of 0.01 M-LiCl for 30 min before extraction of inositol phosphates. Values in (c) are in c.p.m. ($\times 10^{-3}$)/200 μ l. Values for inositol phosphates are means $\pm$ S.D. for three independent determinations in (b) and (c).

Fig. 7. ADP-ribosylation of CT and PT substrates in NCI-H345-cell membranes

Cells were treated with medium alone, 1 μ g of CT/ml or 1 μ g of PT/ml for the indicated periods, followed by preparation of a membrane fraction and re-addition of no toxin or pre-activated CT or PT (indicated as Post Rx) with [32 P]NAD $^{+}$. The autoradiogram of an acid-fixed dried SDS/C polyacrylamide gel is shown.

biochemical effects are elicited by such growth factors in their putative responsive cells. Bombesin-like peptides, including GRP and neuromedins, are mitogens for Swiss 3T3 cells, where their binding to cell-surface receptors and induction of secondary biochemical events have been characterized [2,5,6,10–16]. Experiments presented here show that in some respects SCLC, which in a substantial proportion of cases produce and respond to GRP [3,4,9,22], are similar to Swiss 3T3 cells in the modulation of bombesin-induced responses, but in other respects are distinctive.

Stimulation of PtdIns P_2 hydrolysis occurs with the expected structure–activity relationship, and is half-maximal at a concentration generally consistent with the binding properties of [Tyr 4]bombesin to these cells (Figs. 1–3; [21]). Whereas Swiss 3T3 cells show maximal response of Ins(1,4,5) P_3 at 30 s [10], NCI-H345 cells have a maximal increase in Ins(1,4,5) P_3 which is completed by 8–12 s, and Ins(1,4,5) P_3 is decreasing by 30 s and is not significantly different from control values after 30 min in the presence of Li $^{+}$. Although basal Ins P_4 is more prominently labelled than Ins(1,4,5) P_3 in NCI-H345 cells (in contrast with Swiss 3T3 cells [10]), rapid changes in Ins P_4 are not observed at 30 s or less after stimulation, despite flux of label through that pool as evidenced by increased Ins(1,3,4) P_3 at 30 s (Fig. 3). This is in contrast with the rapid increase in Ins P_4 observed in Swiss 3T3 cells (elevated by 30 s after agonist addition, with decline to less than basal values by 5 min [10]). SCLC, in contrast, show sustained increases in Ins P_4 at 30 min after treatment in the presence of Li $^{+}$ (Figs. 1 and 5b). Recent studies have documented the existence of novel Ins P_4 isomers which either do not change or show only delayed change with agonist stimulation [30,31]. Prominent basal concentrations of these isomers in SCLC could mask increases in Ins(1,3,4,5) P_4 at early times after agonist stimulation by using the methods employed here. Ins(1,3,4,5) P_4 has been proposed as a determinant of plasma-membrane Ca $^{2+}$ permeability [32], although potential second-messenger roles for other Ins P_4 isomers are of importance to define. Increased concentrations of Ins P_4 after bombesin stimulation may mediate a tonic action of the hormone.

The response of the bombesin-stimulated second-messenger systems in SCLC to short-term pretreatment

with active phorbol esters is consistent with studies in Swiss 3T3 cells examining 45 Ca $^{2+}$ flux [17,18] and PtdIns turnover [17] in showing markedly decreased responsiveness to bombesin after short-term phorbol ester pretreatment. Since this effect is eliminated by treatments designed to decrease protein kinase C, responsiveness of SCLC to bombesin peptides may be regulated by the degree to which protein kinase C is activated. In this regard, it is noteworthy that prolonged phorbol ester treatment results in higher basal concentrations of Ins P_4 (Fig. 5b). This could result from decreased inositol-5'-phosphomonoesterase activity in protein kinase C-depleted cells as compared with cells not pretreated for long periods with the phorbol ester [33].

Much interest has been stimulated by reports which link guanine nucleotide-binding (G-)proteins [34] to hormonal activation of phospholipase C by agonists [35,36]. Evidence to support this link is the sensitivity of the signal transduction processes in question to PT or CT. These bacterial toxins specifically modify G-proteins, with concomitant alteration of function. Indeed, bombesin-mediated stimulation of *c-myc* transcription [15] and DNA synthesis [15,37] in 3T3 cells is inhibited by short periods of pretreatment with 10–100 ng of PT/ml. PT does not affect Ca $^{2+}$ mobilization by bombesin in Swiss 3T3 cells [37,38] and does not affect at all [37] or inhibits by less than 30% [39] PtdIns turnover induced by the hormone. Concurrent treatment with insulin eliminates the effect of PT on bombesin-stimulated DNA synthesis [37].

In NCI-H345 cells, CT ADP-ribosylates membrane proteins with approximate molecular masses of 52 and 45 kDa, suggesting the CT-catalysed ADP-ribosylation of both types of α subunits of G $_s$ described by Robishaw *et al.* [40], which may be derived from at least four species of G $_s$ mRNA [41]. After 24 h of pretreatment of intact cells with CT, there has been virtually complete titration of CT-reactive sites (Fig. 7). Analogous PT pretreatment of NCI-H345 cells only partially protects an approx. 39 kDa species from subsequent modification *in vitro* by the toxin, in contrast with 3T3 cells [38], where protection is complete. This entity is expected to represent a mixture of G $_o$ [34], any of the three known isoforms of G $_i$ [42,43], or as yet uncharacterized G-proteins in SCLC.

Since in NCI-H345 cells there is only partial inhibition

of bombesin-induced signal transduction (Fig. 6), at concentrations of PT and durations of treatment approx. 10-fold greater than that employed by others in 3T3 cells [15,34], the participation of a PT-sensitive G-protein in bombesin-induced signalling in SCLC is not clear. Certainly, this is distinct from the inhibition of Ca^{2+} flux by PT observed in chemoattractant-stimulated polymorphonuclear leucocytes [44], or the substantial effect of PT on the inhibition by somatostatin of vasoactive-intestinal-peptide-stimulated secretion in pituitary cells [45].

In contrast, there is clear evidence of altered bombesin-induced signal transduction after CT treatment of NCI-H345 cells in a way not previously described for 3T3 cells (Fig. 6). CT is a mitogen for Swiss 3T3 cells [46], and does not inhibit bombesin-induced *c-myc* or *c-fos* induction in these cells [19]. Interruption of agonist-induced signal-transduction events by CT has been described recently in anti-antigen-receptor-complex-treated T-cells [47] and endothelial-cell-growth-factor-treated aorta-derived cells [48]. Three potential mechanisms can explain the effect of CT to interrupt bombesin-related signals in SCLC.

Since CT is known to stimulate adenylate cyclase [34] after ADP-ribosylation of G_s , elevation of cyclic AMP concentration could modulate the response to bombesin. However, treatment of NCI-H345 cells with a variety of cyclic AMP analogues has not reproduced the CT effect on bombesin-induced signal transduction. CT may affect membrane organization or lipid precursor contents. If this decreased production of $\text{Ins}(1,4,5)P_3$ by decreasing $\text{PtdIns}P_2$ availability, lowered basal $[\text{Ca}^{2+}]_i$ might be expected, in accord with the idea that basal $\text{Ins}(1,4,5)P_3$ contents control $[\text{Ca}^{2+}]_i$, as discussed by Berridge [49]. Indeed, effects of CT pretreatment on precursor lipid synthesis have been described in other systems [50,51], although this effect is cell-type and potentially agonist-specific [52]. Finally, modification by CT of a G-protein which is a transducer of hormone-stimulated phospholipase C [34–36] or which regulates an agonist-sensitive ion channel could modulate responsiveness of NCI-H345 cells to exogenous bombesin agonists or endogenous GRP. The last possibility is of particular interest, in view of the recent demonstration that G_s can modulate the activity of a Ca^{2+} channel in heart in a CT-sensitive manner [53].

The results in the present paper indicate that, in a cell line which produces GRP as a putative autocrine growth factor, the hormone also induces rapid activation of phospholipase C. This activity results, in the presence of Li^+ , in sustained $\text{Ins}4P$, $\text{Ins}1P$ and $\text{Ins}P_4$ accumulation in addition to initial increases in $\text{Ins}(1,4,5)P_3$ associated with Ca^{2+} mobilization. These processes are regulated in part by protein kinase C, and prominently by a CT-sensitive process. PT inhibits this process to a considerably less marked extent. Because NCI-H345 and other SCLC cells can both produce and, as shown in the experiments presented here, respond to bombesin with well-defined biochemical effects, they provide a unique system in which to study the modulation of responsiveness to an autocrine growth factor.

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