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Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125^{FAK} and paxillin induced by bombesin and endothelin

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Abstract In this study we examined the role of *rho* p21 in neuropeptide-stimulated tyrosine phosphorylation. Intact Swiss 3T3 cells were treated with the *Clostridium botulinum* C3 exoenzyme which specifically ADP ribosylates and inactivates *rho* p21. C3 exoenzyme treatment of cells caused a marked decrease in both bombesin- and endothelin-stimulated tyrosine phosphorylation of multiple proteins, including p125 focal adhesion kinase (FAK) and paxillin. Our results suggest that *rho* p21 is a component of the signal transduction pathway linking seven transmembrane domain receptors with tyrosine phosphorylation and cytoskeletal events.

Key words: Neuropeptide; *rho* p21; Tyrosine phosphorylation

1. Introduction

Tyrosine phosphorylation has recently been implicated in the intracellular signaling of neuropeptides that act as potent cellular growth factors through seven transmembrane domain receptors [1,2]. Bombesin and other neuropeptide growth factors stimulate tyrosine phosphorylation of multiple proteins in Swiss 3T3 cells, including broad bands of M_r 110,000–130,000 and 70,000–80,000 [3–6]. Recently the focal adhesion associated proteins p125 focal adhesion kinase (p125^{FAK}), a novel tyrosine kinase [7,8], and paxillin [9,10] have been identified as prominent tyrosine-phosphorylated proteins in Swiss 3T3 cells stimulated by bombesin and other neuropeptides [11,12]. The rapidity of neuropeptide-stimulated tyrosine phosphorylation is consistent with p125^{FAK} and paxillin functioning in a neuropeptide-activated tyrosine kinase pathway. However, the components of this pathway have not been fully identified.

The increases in p125^{FAK} and paxillin tyrosine phosphorylation are accompanied by a profound reorganisation of the actin cytoskeleton and by the assembly of focal adhesion plaques, the distinct sites on the plasma membrane where both p125^{FAK} and paxillin are localised [13,14]. Studies utilising cytochalasin D, which selectively disrupts the network of actin filaments, demonstrated that bombesin-stimulated tyrosine phosphorylation of p125^{FAK} and paxillin is dependent on the integrity of the actin cytoskeleton [12,13]. The rho gene products (*rho* p21s), which are members of the ras superfamily of small GTP-binding proteins, have been implicated in mitogen-stimulated formation of focal adhesions and actin stress fibers [15]. The function of *rho* p21 is specifically impaired by treatment with the *Clostridium botulinum* C3 exoenzyme, which ADP ribosylates the Asn^{41} of *rho* p21 and thereby prevents its interaction with downstream targets [16–18].

Here we report that bombesin- and endothelin-stimulated tyrosine phosphorylation of multiple proteins, including p125^{FAK} and paxillin is markedly inhibited by treatment with botulinum C3 exoenzyme. The results imply that *rho* p21 lies upstream of the tyrosine phosphorylation events in a neuropeptide-stimulated signal transduction pathway.

2. Materials and methods

2.1. Materials

Bombesin, tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin, fluorescein isothiocyanate (FITC)-linked anti-mouse IgG agarose-linked anti-mouse IgG were obtained from Sigma. Anti-Tyr(P) monoclonal antibody (mAb), clone Py72, was obtained from the hybridoma development unit, Imperial Cancer Research Fund, London, UK. Py20 anti-Tyr(P) mAb and the mAb directed against paxillin (mAb165) were from ICN, High Wycombe, UK. 4G10 anti-Tyr(P) mAb, mAb 2A7 directed against p125^{FAK} were from TCS Biologicals Ltd., Buckingham, UK. Anti-p125^{FAK} mAb for Western blotting was obtained from AFFINITI Research Products Ltd., Nottingham, UK. ^{125}I-Labelled sheep anti-mouse IgG (50 μCi/mg) and carrier-free $^{32}P_i$ were from Amersham, UK. All other reagents used were of the purest grade available.

2.2. Preparation of recombinant C3 exoenzyme

The C3 exoenzyme gene [17] was modified by PCR-mediated site-directed mutagenesis to produce a recombinant C3 exoenzyme that lacks the signal peptide and has the dipeptide Met-Ala attached to Ser of the mature exoenzyme. PCR was performed with the cloned gene as the template and with the synthetic oligonucleotides (5'-ACTGTTCA-TATGGCTAGCTATGCAGATACTTTC-ACA-3' and 5'-TTATTG-GATCCTATTATTTAAATATCATTGCTGTAA-3') as primers. The amplified fragment was cleaved with *Nde*I and *Bam*HI and ligated with a pET-3a vector [19]. After confirming the DNA sequence, the recombinant plasmid, pET-3$_R$/C3 was introduced into *Escherichia coli* BL21 (DE3)pLysE and expressed [17,19,20].

2.3. Cell culture and C3 exoenzyme pretreatment

Swiss 3T3 cells were seeded at a density of 1×10^5 per 30 mm dish in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. At 72 h, recombinant C3 exoenzyme at a final concentration of 7.5 μg/ml or diluent was added to the medium. After being cultured for a further 48 h the cells were washed twice with DMEM and then incubated in DMEM/Waymouths (1:1, v/v) in the presence or absence of C3 exoenzyme at 15 μg/ml for 24 h.

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Abbreviations: FAK, focal adhesion kinase; TRITC, tetramethylrhodamine B isothiocyanate; FITC, fluorescein isothiocyanate; Tyr(P), tyrosine phosphate; DMEM, Dulbecco's modification of Eagle's essential medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; MARCKS, myristylated alanine-rich protein kinase C substrate; GRP, gastrin releasing peptide; PKC, protein kinase C; LPA, lysophosphatidic acid.

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This protocol has previously been shown to ADP ribosylate *rho* p21 in intact Swiss 3T3 cells, as shown by the fact that cell homogenates from C3 exoenzyme-treated cells contain markedly reduced levels of native *rho* p21 available for [^{32}P]ADP ribosylation by externally added C3 exoenzyme (data not shown) [21,22].

2.4. Immunoprecipitations

Control and C3 exoenzyme pretreated cultures of Swiss 3T3 cells ($1–2 \times 10^6$) were challenged with bombesin or endothelin for the times indicated and lysed at 4°C in 1 ml of a solution containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM Na_3VO_4 and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 15,000 × *g* for 10 min and precleared by incubation with albumin-agarose for 1 h at 4°C. After removal of albumin-agarose by brief (10 s) centrifugation, the supernatants were transferred to fresh tubes for immunoprecipitation. Proteins were immunoprecipitated at 4°C for 4 h with anti-mouse IgG agarose-linked mAbs directed against either phosphotyrosine (Py 72), p125^{FAK} (mAb 2A7) or paxillin (mAb 165) as indicated. Immunoprecipitates were washed three times with lysis buffer and extracted for 10 min at 95°C in 2 × SDS-PAGE sample buffer (200 mM Tris-HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and analyzed by SDS-PAGE.

2.5. Western blotting

Treatment of quiescent cultures of cells with factors, cell lysis and immunoprecipitations were performed as described above. After SDS-PAGE, immunoprecipitated proteins were transferred to Immobilon transfer membranes. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with either the p125^{FAK} mAb or anti-Tyr(P) mAbs (Py20 and 4G10, 1 μg/ml of each antibody), as indicated, in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualized using ^{125}I-labeled sheep anti-mouse IgG followed by autoradiography.

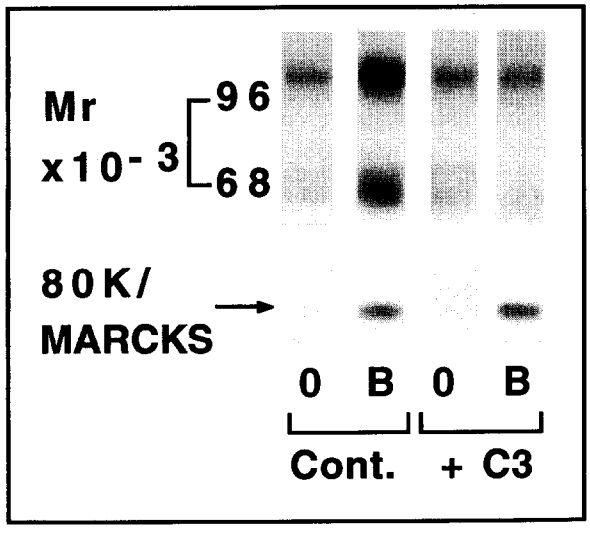

Fig. 1. Effect of C3 exoenzyme pretreatment on bombesin-stimulated tyrosine phosphorylation of proteins and 80K/MARCKS phosphorylation in Swiss 3T3 cells. (Upper panel) Cells pretreated in the presence (+C3) or absence (Cont.) of C3 exoenzyme were incubated with (B) or without (0) bombesin (1 nM) for 20 min at 37°C, lysed and the lysates immunoprecipitated with the anti-Tyr(P) mAb Py 72. Immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) mAbs (Py 20 and 4G10). The positions of molecular weight markers $\times 10^{-3}$ are shown on the left. Similar results were obtained in three independent experiments. (Lower panel) Cells pretreated with or without C3 exoenzyme were further incubated for 12 h at 37°C in phosphate-free DMEM with 50 μCi/ml [^{32}P] P_i. Cells were subsequently stimulated either with (B) or without (0) bombesin (1 nM) for 20 min at 37°C, lysed and the lysates immunoprecipitated with 80K/MARCKS antisera and further analyzed by SDS-PAGE.

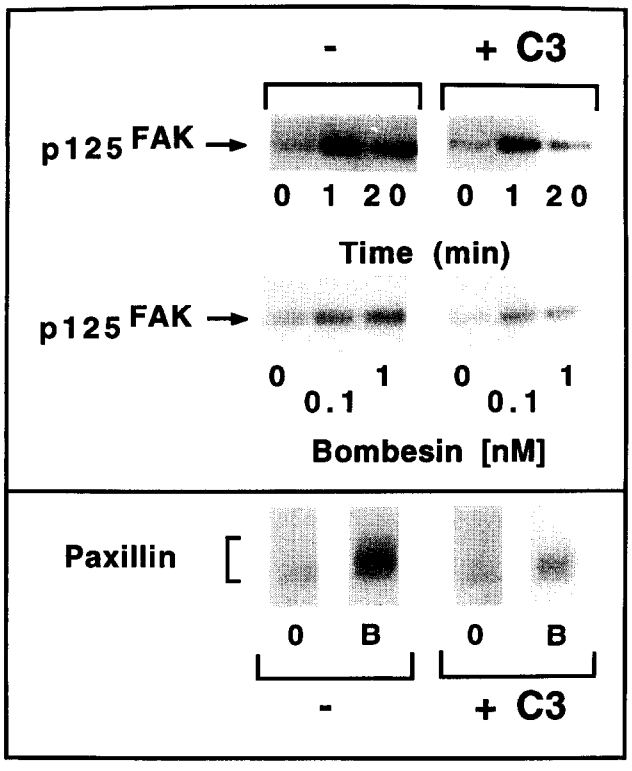

Fig. 2. Effect of C3 exoenzyme pretreatment on bombesin-stimulated tyrosine phosphorylation of p125^{FAK} and paxillin in Swiss 3T3 cells. (Upper panel) Cells pretreated in the presence (+C3) or absence (−) of C3 exoenzyme were incubated with bombesin (1 nM) for 0, 1 or 20 min at 37°C, or with 0, 0.1 or 1 nM bombesin for 20 min at 37°C, lysed and the lysates immunoprecipitated with the anti-p125^{FAK} mAb 2A7. Immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) mAbs. (Lower panel) Cells pretreated in the presence (+C3) or absence (−) of C3 exoenzyme were incubated with (B) or without (0) bombesin (1 nM) for 20 min at 37°C, lysed and the lysates immunoprecipitated with the anti-paxillin mAb 165. Immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) mAbs.

2.6. ^{32}P labeling of cells and analysis of 80K/MARCKS phosphorylation

Quiescent and confluent cultures of Swiss 3T3 cells, previously incubated for 48 h in the presence or absence of the C3 exoenzyme (7.5 μg/ml) were washed twice in phosphate-free DMEM and incubated at 37°C with this medium containing 50 μCi/ml of carrier-free $^{32}P_i$ in the presence or absence of C3 exoenzyme (15 μg/ml). After 24 h the cells were stimulated with bombesin for 20 min. The cells were subsequently lysed as described above and the lysates were immunoprecipitated with an antiserum directed against 80K/MARCKS [23]. These immunoprecipitates were further analysed by SDS-PAGE.

2.7 Immunostaining of cells

Following stimulation, cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and permeabilized with PBS containing 0.2% Triton X-100 for 8 min at room temperature. The cells were then incubated with anti-Tyr(P) mAb (0.3 μg/ml) for 30 min at room temperature, subsequently washed three times in PBS and finally incubated with FITC-labeled anti-mouse IgG as a second antibody at a dilution of 1:100 for another 30 min at room temperature. Cells were visualised utilising a confocal microscope.

2.8. Confocal microscopy

Confocal imaging was performed using a Bio-Rad MRC-600 laser scanning head fitted onto a Nikon Optiphot microscope. A 60× NA 1.4 planapochromat oil immersion lens (Nikon) was used for all imaging. Fluorescein was elicited at 488 nm using a Krypton/Argon mixed gas laser (Bio-Rad). A K1 filter was used that is a double dichromic filter enabling excitation at 488 and 568 nm, and images were collected

using the Kalman filter. Data are presented as projections of sequential optical sections. For Z-series, optical sections were recorded at 0.3 μm. Final images were photographed directly from the VDU screen.

2.9. Other methods

^{125}I-Labelled gastrin releasing peptide (GRP) binding [24], actin staining [14] and ADP ribosylation [22] were performed as described previously.

3. Results and discussions

To assess the role of *rho* p21 in the neuropeptide- stimulated tyrosine phosphorylation of specific proteins, cultures of Swiss 3T3 cells were treated with the *Clostridium botulinum* C3 exoenzyme using a protocol that resulted in ADP ribosylation of *rho* p21 in intact cells (see section 2). This treatment of intact cells with recombinant C3 exoenzyme also caused a marked reduction in actin stress fiber formation in response to bombesin (data not shown).

The effect of C3 exoenzyme pretreatment on the tyrosine phosphorylation of proteins in Swiss 3T3 cells stimulated with 1 nM bombesin for 20 min was examined. As shown in Fig. 1 (upper panel) stimulation of these cells with bombesin induced tyrosine phosphorylation of two clusters of proteins migrating with an apparent M_r of 110,000–130,000 and 70,000–80,000. Treatment of cells with the C3 exoenzyme caused a marked decrease in bombesin-mediated tyrosine phosphorylation of both bands. This result suggests that bombesin-stimulated tyrosine phosphorylation of proteins depends on the activity of *rho* p21.

It was important to establish that the inhibitory effect of the C3 exoenzyme on bombesin-stimulated tyrosine phosphorylation was specific. Bombesin and the structurally related mammalian peptide GRP bind to a single class of high affinity receptors in Swiss 3T3 cells [2,24]. We verified that C3 exoenzyme pretreatment had no effect on the specific binding of [^{125}I]GRP to Swiss 3T3 cells (data not shown). Furthermore, we examined the effect of the C3 exoenzyme pretreatment on bombesin- stimulated activation of protein kinase C (PKC) as measured by the phosphorylation of the specific PKC substrate 80K/MARCKS [23,25]. The results shown in Fig. 1 (lower panel) indicate that C3 exoenzyme pretreatment of cells had no effect on bombesin-stimulated phosphorylation of 80K/MARCKS. Thus the inhibitory effects of the C3 exoenzyme are selective for the tyrosine phosphorylation pathway.

The novel cytosolic tyrosine kinase p125^{FAK} has been identi-

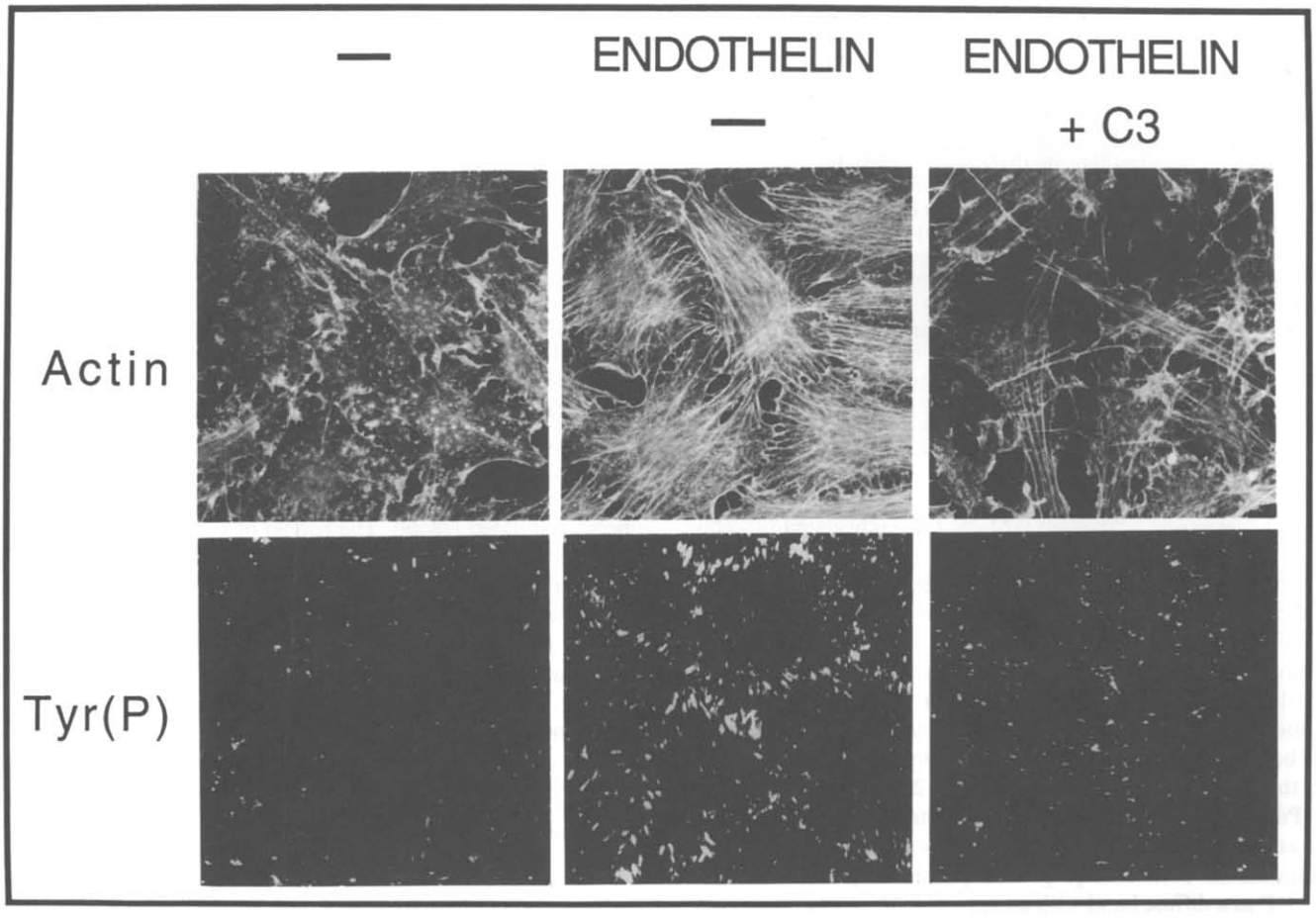

Fig. 3. Effect of C3 exoenzyme pretreatment on endothelin-stimulated changes in the actin cytoskeleton and anti-phosphotyrosine staining. Cells pretreated in the presence (+C3) or absence (Control) of C3 exoenzyme were incubated with (E) or without (0) endothelin (5 nM) for 20 min at 37°C. Cells were then washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Actin was stained by incubation in TRITC-conjugated phalloidin (0.25 μg/ml) in PBS for 10 min at room temperature. Immunostaining for phosphotyrosine was achieved using the specific anti-Tyr(P) mAb 4G10, followed by FITC-labeled anti-mouse IgG. Confocal imaging was performed as described in section 2.

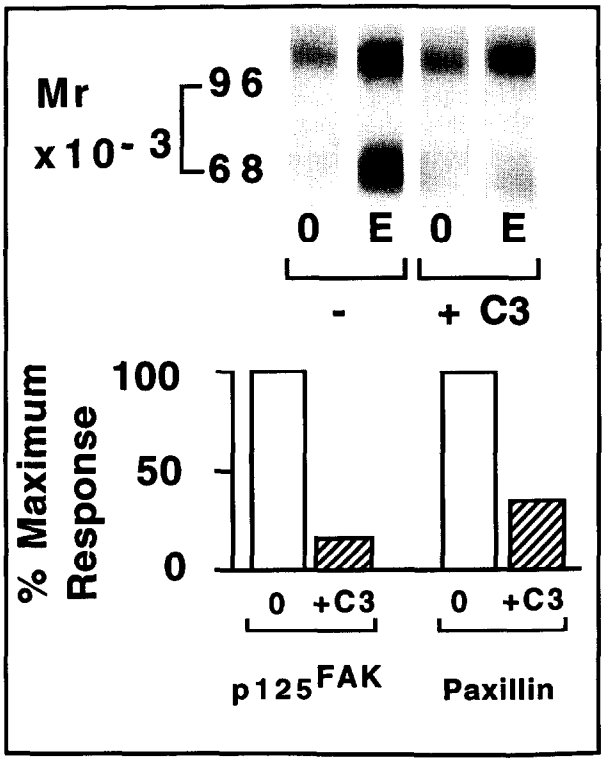

Fig. 4. Effect of C3 exoenzyme pretreatment on endothelin-stimulated tyrosine phosphorylation of total proteins, p125^{FAK} and paxillin in Swiss 3T3 cells. Cells pretreated in the presence (+C3) or absence (−) of C3 exoenzyme were incubated with (E) or without (0) endothelin (5 nM) for 20 min at 37°C, lysed and the lysates immunoprecipitated with either the anti-Tyr(P) mAb, Py 72 (upper panel), the anti-p125^{FAK} mAb 2A7 or the anti-paxillin mAb 165 (lower panel). Immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) mAbs (Py 20 and 4G10). The autoradiogram of the anti-Tyr(P) immunoprecipitates is shown with the positions of molecular weight markers $\times 10^{-3}$ on the left. Autoradiograms of the anti-Tyr(P) immunoblots of p125^{FAK} and paxillin immunoprecipitations were scanned with an LKB Ultrascan XL densitometer to quantify phosphoproteins in terms of peak area. Values correspond to the phosphorylation of either p125^{FAK} or paxillin (as indicated) expressed as a percentage of the maximal response after subtraction of the control.

fied as one of the prominent tyrosine phosphorylated proteins in bombesin-treated Swiss 3T3 cells [11]. As shown in Fig. 2, treatment of Swiss 3T3 cells with C3 exoenzyme reduced the increase in tyrosine phosphorylation of p125^{FAK} as early as 1 min after the addition of bombesin (55% inhibition). The decrease in p125^{FAK} tyrosine phosphorylation was comparable to that in cells treated with bombesin for 20 min. Tyrosine phosphorylation of p125^{FAK} induced by various concentrations of bombesin was similarly inhibited by preincubation of cells in the presence of C3 exoenzyme (Fig. 2).

Paxillin, a cytoskeletal-associated protein, has been identified as another major protein that is phosphorylated on tyrosine in response to bombesin [12]. This protein migrates on SDS-PAGE as a diffuse band with an apparent M_r of 70,000–75,000. As is evident from Fig. 1 there is a striking reduction in the bombesin-stimulated tyrosine phosphorylation of this band in cells pretreated with C3 exoenzyme. Further, direct assessment of the tyrosine phosphorylation of paxillin using a specific anti-paxillin mAb (mAb 165) demonstrated that pretreatment of cells with the C3 exoenzyme attenuated the increase in paxillin tyrosine phosphorylation stimulated by bombesin.

Endothelin, a neuropeptide that acts through a different G-protein-coupled receptor to bombesin, has previously been demonstrated to stimulate tyrosine phosphorylation of proteins, including p125^{FAK} and paxillin [3,11,12]. It is not known, however, whether endothelin, like bombesin [15], stimulates reorganisation of the actin cytoskeleton and the assembly of focal adhesion plaques in Swiss 3T3 cells. Examination of the actin cytoskeleton using TRITC-phalloidin revealed that endothelin stimulated striking actin stress fiber formation (Fig. 3). When cells were immunostained with anti-Tyr(P) mAbs it was evident that the proteins phosphorylated on tyrosine in response to endothelin were localized to small discrete patches at the periphery of the ventral membrane, consistent with focal adhesions (Fig. 3). Vinculin exhibited a very similar distribution in endothelin-stimulated cells (results not shown). Pretreatment of cells with C3 exoenzyme markedly reduced endothelin-stimulated actin stress fiber formation, redistribution of vinculin into focal contacts and tyrosine phosphorylation of proteins (Fig. 3 and data not shown). Western blot analysis was performed to determine the effect of C3 exoenzyme treatment on endothelin-stimulated tyrosine phosphorylation of specific proteins. As shown in Fig. 4 endothelin-stimulated tyrosine phosphorylation of M_r 110,000–130,000 and 70,000–80,000 proteins was inhibited in the C3 exoenzyme-treated cells. We also verified that C3 exoenzyme treatment attenuated the tyrosine phosphorylation of p125^{FAK} and paxillin induced by endothelin (Fig. 4).

The results presented here demonstrate that C3 exoenzyme pretreatment, and consequent selective inactivation of *rho* p21, markedly inhibited neuropeptide-stimulated tyrosine phosphorylation of multiple proteins, including p125^{FAK} and paxillin, in Swiss 3T3 cells. These findings suggest that *rho* p21 is a component of the signal transduction pathway linking neuropeptide receptors with tyrosine phosphorylation. The bioactive lipid, LPA, stimulates actin stress fiber formation and the tyrosine phosphorylation of a similar set of proteins as bombesin in Swiss 3T3 cells [26,27]. It has recently been shown that *rho* p21 is critical for both these responses to LPA [15,22]. However, the putative cell surface receptor for LPA has not as yet been characterized and an intracellular mechanism of action has been postulated [28,29]. The results of this study suggest that there is a common signal transduction pathway activated by seven transmembrane domain receptors, known to mediate the effects of bombesin and endothelin, in which *rho* p21 is upstream of both cytoskeletal responses and the tyrosine phosphorylation of specific proteins.

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