Neuromedin B Receptor Activation Causes Tyrosine Phosphorylation of p125^{FAK} by a Phospholipase C Independent Mechanism Which Requires p21^{rho} and Integrity of the Actin Cytoskeleton

Takaharu Tsuda, Takashi Kusui, and Robert T. Jensen*

Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-1804

Received June 17, 1997; Revised Manuscript Received October 22, 1997[⊗]

ABSTRACT: Recent studies show that tyrosine phosphorylation by a number of neuropeptides may be an important intracellular pathway in mediating changes in cell function, particularly related to growth. Neuromedin B (NMB), a mammalian bombesin related peptide, functions through a distinct receptor, the neuromedin B receptor (NMB-R), of which little is known about its cellular basis of action. In the present study we explored the ability of NMB-R activation to cause tyrosine phosphorylation of focal adhesion kinase (p125^{FAK}), an important substrate for tyrosine phosphorylation by other neuropeptides. NMB caused rapid increases in p125^{FAK} phosphorylation which reached maximum at 2 min in both rat C6 glioblastoma cells which possess native NMB-Rs and rat neuromedin B receptor (rNMR-R) transfected BALB 3T3 cells. NMB had a half-maximal effect was at 0.4 nM and was 30-fold more potent than gastrin-releasing peptide (GRP). The stoichiometric relationships between increased p125^{FAK} tyrosine phosphorylation and other cellular processes was similar in both C6 cells and rNMB-R transfected cells. TPA (1 μM) caused 45% and the calcium ionophore, A23187, 11% of maximal tyrosine phosphorylation of p125^{FAK} seen with NMB. A23187 potentiated the effect of TPA. Pretreatment with the selective PKC inhibitor, GF109203X, inhibited TPA-induced p125^{FAK} tyrosine phosphorylation, but it had no effect on the NMB stimulation. Pretreatment with thapsigargin completely inhibited NMB-stimulated increases in $[Ca^{2+}]_i$, but had no effect on NMB-stimulation of p125^{FAK} phosphorylation either alone or with GF109203X. The tyrosine kinase inhibitor, tyrphostin A25, inhibited NMB-induced phosphorylation of p125^{FAK} by 52%. However, tyrphostin A25 did not inhibit NMB-stimulated increases in [^{3}H]inositol phosphates. Cytochalasin D, an agent which disrupts actin microfilaments, inhibited BN- and TPA-induced tyrosine phosphorylation of p125^{FAK} completely. In contrast, colchicine, an agent which disrupts microtubules, had no effect. Pretreatment with Clostridium botulinum C3 exoenzyme which inactivates the small GTP-binding protein *rho* p21, also inhibited tyrosine phosphorylation of p125^{FAK} by 55%. These results demonstrate that activation of NMB-R can cause rapid tyrosine phosphorylation of p125^{FAK}. NMB-induced tyrosine phosphorylation of p125^{FAK} is independent of NMB-induced changes in $[Ca^{2+}]_i$ or PKC. The integrity of the actin cytoskeleton but not of microtubules is necessary for NMB-stimulated phosphorylation of p125^{FAK}. The ras-related small GTP-binding protein *rho* p21 is at least partially involved in mediating NMB-induced tyrosine phosphorylation of p125^{FAK}. These results suggest that similar to some other neuropeptides, activation of this pathway may be an important mechanism in mediating cellular changes by this receptor such as growth.

The mammalian bombesin (Bn)-related peptides gastrin-releasing peptide (GRP) and neuromedin B (NMB) have effects in many diverse cell systems (*1*): in the central nervous system (thermoregulation, thyrotropin release, regulation of circadian rhythm, regulation of appetite) (*2*−*5*); in the immune system (stimulation of macrophage migration, regulation of lymphocyte activity) (*6, 7*); in development (pulmonary budding, stimulation of chondrocytes) (*8, 9*); in the gastrointestinal tract (smooth muscle contraction, stimulation of pancreatic secretion) (*1, 10, 11*); stimulation of the release of numerous hormones (*12*−*14*). Bombesin-related peptides also have potent growth effects in normal and tumorous tissues (*1, 15*−*22*). Gastrin-releasing peptide has been shown to stimulate the growth of both normal tissues (Swiss 3T3 cells, bronchial endothelial cells, pulmonary neuroendocrine cells, uterine stromal cells) (*15*−*18*) and various tumor tissues such as small cell lung cancer, prostatic cancer cells, hepatocellular cancer, and breast cancer (*18, 23*−*25*). NMB has been shown to stimulate growth of small cell lung cancer cells C6 glioma cells, BALB 3T3 cells transfected with the rat NMB receptor, and adrenal cortical cells (*19*−*22*).

The actions of the mammalian Bn-related peptides, GRP and NMB, are now known to be mediated by two different subtypes of Bn receptors, a gastrin-releasing peptide receptor (GRP receptor) and a recently described neuromedin B receptor (NMB receptor), which are members of the seven transmembrane G protein linked receptor superfamily (*26*−*29*). These receptors are coupled in all tissues examined to phospholipase C, through heterotrimetric G proteins and activation results in increase in inositol phosphates and consequently mobilization of intracellular Ca^{2+} and activation of PKC (*15, 21, 22, 30*−*33*). The phospholipase C cascade has been extensively studied particularly in the case of the

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.

S0006-2960(97)01448-7

GRP receptor; however, recent studies suggest similar to other neuropeptides, tyrosine phosphorylation of various proteins may be an important intracellular cascade (*34*−*36*).

Recent studies show that a number of neuropeptides including angiotensin, bombesin, vasopressin, endothelin, and bradykinin can cause tyrosine phosphorylation of multiple proteins, including broad bands of M_r 110000−130000 and 70000−80000 (*37*−*41*). A number of studies show that a novel cytosolic tyrosine kinase, focal adhesion kinase (p125^{FAK}), is a major component of these tyrosine phosphorylated proteins and that it is localized to the cellular focal adhesion plaque (*39*, *41*−*43*). In addition to neuropeptides, integrin activation by cell attachment to fibronectin causes an increase of tyrosine phosphorylation of multiple proteins including p125^{FAK} (*44*−*48*), and *src* transformation also results in an increase in p125^{FAK} tyrosine phosphorylation (*45*, *49*). These results suggest that tyrosine phosphorylation of p125^{FAK} is a point of convergence in the action of integrins, oncogenes, and some neuropeptides (*36*). p125^{FAK} seems to play an important role in different kinds of cell regulation such as adhesion, shape, motility, and in the case of the neuropeptides, it may be particularly important in mediating growth effects. Recent studies show with some stimuli of tyrosine phosphorylation of these two proteins that the integrity of the actin cyloskeleton and the participation of the ras-related small GRP-binding protein p21^{rho} (*50*, *51*) may be important for this stimulation.

The ability of GRP receptor activation to stimulate tyrosine phosphorylation particularly of p125^{FAK} and paxillin and its relationship to other intracellular signaling cascades caused by this activation has been extensively studied (*34*−*39*, *51*, *52*). In contrast very little is known about the ability of the NMB receptor to activate tyrosine phosphorylation. A recent study reports NMB receptors transfected into rat-1 cells can cause p125^{FAK} tyrosine phosphorylation (*22*). It is unknown whether native NMB receptors can cause tyrosine phosphorylation or phosphorylation of p125^{FAK}. It is unclear if NMB receptor activation causes p125^{FAK} tyrosine phosphorylation in nontransfected cells, whether NMB-induced PKC activation or changes in cytosolic calcium are involved in this stimulation. It is also unclear whether cytoskeletal components or small GTP-binding proteins are important in the stimulation of the p125^{FAK} tyrosine phosphorylation mediated by NMB receptor activation, as they are with a number of other stimuli (*50*−*57*). The present study was aimed at addressing these issues.

MATERIALS

Neuromedin B (NMB), porcine gastrin-releasing peptide (GRP), and [Tyr^4]bombesin were obtained from Peninsula Laboratories, Belmont, CA; antifocal adhesion kinase (p125^{FAK}) antibody from Transduction Laboratories, Lexington, KY; agarose conjugated anti-phosphotyrosine Ab clone 4G10, Upstate, Lake Placid, NY; 12-*o*-tetradecanoylphorbol 13-acetate(TPA), cytochalasin D, deoxycholic acid, colchicine, *Clostridium botulinum* C3 exoenzyme, and tyrphostin A25 from Calbiochem, La Jolla, CA; thapsigargin and GF109203X from Biomol, Plymouth Meeting, PA; phosphate buffer saline (PBS), pH 7.4, and Waymouth's MB 752/1 medium from Biofluids, Rockville, MD; Dulbecco's modified essential media (DMEM), fetal bovine serum, and the aminoglycoside G-418 were from Gibco, Waltham, MA; Tris and glycine from Schwarz/Mann Biotech, Cleveland, OH; ethylenediaminetetraacetic acid (EDTA), bacitracin, soybean trypsin inhibitor (SBTI), dimethyl sulfoxide (DMSO), triton X-100 from Sigma, St. Louis, MO; phenylmethanesulfonyl fluoride (PMSF) from Fluka, Ronkonkoma, NY; bovine serum albumin (BSA) fraction V from Miles Inc., Kankakee, IL; aprotinin, leupeptin and 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (Hepes) from Boehringer Mannheim Biochemicals, Indianapolis, IN; goat anti-mouse IgG-peroxidase conjugated and IODO-GEN from Pierce, Rockford, IL; sodium dodecyl sulfate (SDS), 2-mercaptoethanol and protein assay dye reagent from BIO-RAD, Richmond, CA; Hyperfilm ECL and enhanced chemiluminescence detection reagents and Na^{125}I from Amersham, Arlington Heights, IL. *myo*-[2-^{3}H]inositol (22.9 Ci/mmol) from Dupont NEN, Boston, MA; BALB/3T3 fibroblasts and rat glioblastoma C-6 cells were obtained from the American Type Culture Collection, Rockville, MD; nitrocellulose membrane from Schleicher & Schuell, Keene, NH; and fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes, Eugene, OR. Standard buffer consisted of 98 mM NaCl, 6 mM KCl, 25 mM HEPES, 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate and 0.1% soybean trypsin inhibitor.

METHODS

Growth of the Rat Glioblastoma Tumor C-6 Cell Line and Transfected BALB 3T3 Cells. As described previously (*30*), BALB 3T3 cells expressing a stably transfected rat NMB receptor were obtained using calcium phosphate precipitation of a full length NMB-preferring bombesin receptor clone generated from rat esophagus and subcloned into modified version of the pCD_2 plasmid (*58*). Cells were maintained in DMEM containing 10% fetal bovine serum plus 300 μg/mL G-418, and passaged every 3−4 days at confluence, using 0.1% trypsin in 1 mM EDTA. Rat glioblastoma C-6 tumor cells were maintained in DMEM containing 10% fetal bovine serum and were passaged weekly at confluence. All cell lines were cultured at 37 °C in a 5% CO_2 atmosphere.

Immunoprecipitation. Quiescent and confluent cultures of cells in 100-mm dishes were preincubated twice with DMEM for 1 h, treated with NMB and TPA at the concentrations indicated, and lysed at 4 °C in 1 mL of a solution containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 2.5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 1 mM PMSF, and 0.2 mM Na_3VO_4. Lysates were centrifuged at 15000*g* for 15 min. The supernatants were incubated with 15 μL of agarose conjugated anti-phosphotyrosine mAb overnight at 4°C. The immunoprecipitates were washed three times with PBS and further analyzed by SDS-PAGE and Western blotting.

Western Blotting. Immunoprecipitates were fractionated by SDS-PAGE, and the proteins were then transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C using blotto (5% nonfat dried milk in a solution containing 50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3) and incubated for 2−3 h at 22 °C with 1 μg/mL anti-p125^{FAK} antibody. The membranes were washed twice for 10 min with blotto and incubated for 40 min at 22 °C with anti-mouse IgG-horseradish peroxidase conjugate. The mem-

brane were finally washed 4 times for 10 min with washing solution (50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3), incubated with enhanced chemiluminescence detection reagents (ECL) for 60 s and exposed to Hyperfilm ECL for up to 10 min. The density of bands on the film were measured using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Binding of ^{125}I-[DTyr^0]NMB to rNMB-R Transfected and C-6 Cells. ^{125}I-[DTyr^0]NMB (2200 Ci/mmol) was prepared as described previously (*26*, *30*, *32*). Binding studies using rat glioblastoma C-6 cells or NMB-R-transfected cells were performed as described previously (*30*) by suspending disaggregated cells in binding buffer (standard buffer additionally containing 1 mM $MgCl_2$, 2.2 mM $KHPO_4$, 2 mM glutamine, 11 mM glucose, 0.1% (w/v) bacitracin, and 0.2% (w/v) bovine serum albumin (pH 7.4)). NMB-R-transfected cells were suspended at a concentration of 1×10^6 cells/mL, whereas C-6 cells were suspended at a concentration of 15×10^6 cells/mL. Incubations contained 50 pM ^{125}I-[DTyr^0]NMB for 30 min at 22°C. Nonsaturable binding of ^{125}I-[DTyr^0]NMB was the amount of radioactivity associated with C-6 cells or NMB-R-transfected cells when the incubation mixture contained 1 μM NMB. Nonsaturable binding was <15% of total binding in all experiments, and all values in this paper are reported as saturable binding (i.e., total minus nonsaturable binding).

Measurement of Inositol Phosphates. Cells were split 1:4 into 24-well plates and grown to confluence in regular medium and then loaded with 3 μCi/mL *myo*-[2-^{3}H]inositol in Dulbecco's modified essential medium supplemented with 2% fetal bovine serum at 37 °C for 24 h. Cells were washed and incubated for 15 min with phosphoinositide buffer (standard buffer additionally containing 10 mM LiCl, 2 mM $CaCl_2$, 2% bovine serum albumin, and 1.2 mM $MgSO_4$) and were then incubated with varying concentrations of NMB for 60 min at 37 °C. Reactions were halted by using 1% HCl in methanol. Total [^{3}H]inositol phosphates were isolated by anion exchange chromatography, as described previously (*59*, *60*).

Measurement of $[Ca^{2+}]_i$. Cells were mechanically disaggregated, resuspended in binding buffer at a concentration of 2×10^6 cells/mL containing 2 μM fura-2/AM, and incubated at 22 °C for 45 min. After loading with fura-2, cells were washed three times in binding buffer. For measurement of $[Ca^{2+}]_i$, 2 mL samples were placed in quartz cuvettes in a Delta PTI Scan-1 spectrometer (PTI Instruments, Gaithersburg, MD). Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340})and 380 nm (F_{380}). Autofluorescence of unloaded cells was subtracted from all measurements. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (*61*), using the formula $[Ca^{2+}]_i = K_d \times (R - R_{min}) (R_{max} - R_{min}) \times Sf/Sb$, where K_d is the affinity of fura-2 for Ca^{2+}, determined to be 225 nM in our experiments, $R = F_{340}/F_{380}$ and is the ratio of the fluorescence with the two excitation wavelengths, R_{max} is F_{340}/F_{380} in saturated Ca^{2+} environment after the addition of 0.1% Triton X-100 and 10 mM calcium, R_{min} is the fluorescence ratio at virtually zero calcium after addition of 25 mM EGTA, *Sf* is the F_{380} at zero calcium and *Sb* is the F_{380} at saturated $[Ca^{2+}]_i$.

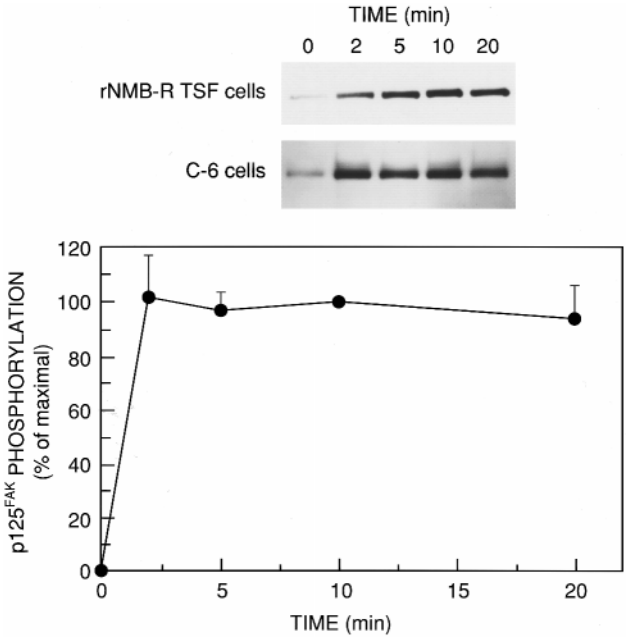

FIGURE 1: Time course of NMB stimulation of p125^{FAK} tyrosine phosphorylation in rNMB-R transfected BALB 3T3 cells and C6 rat glioblastoma cells. rNMB-R transfected BALB 3T3 cells (rNMB-R TSF cells) and C6 rat glioblastoma cells (C6 cells) were treated for various times as indicated with 0.1 μM NMB and lysed. Tyrosine phosphorylation of p125^{FAK} was analyzed by immunoprecipitation using phosphotyrosine mAb. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to nitrocellulose membrane. Phosphorylated p125^{FAK} was detected using anti-p125^{FAK} mAb and chemiluminescent detection as described in Methods. In the top panel are shown results from a representative experiment with each cell type. These results are representative of at least three others. In the bottom panel is shown the quantitation from five experiments in rNMB-R transfected BALB 3T3 cells. Quantitation was performed using scanning densitometry, and values are the means ±SE ($n = 5$) expressed as the percentage of the maximal increase above the control unstimulated values at 10 min. The maximal increase in p125^{FAK} tyrosine phosphorylation in rNMB-R transfected cells was 5.4 ± 0.8 fold over the pretreatment level and 3.9 ± 0.8-fold in C6 cells ($n = 5$).

RESULTS

NMB (0.1 μM) stimulated a rapid increase in p125^{FAK} tyrosine phosphorylation of 5.4 ± 0.8-fold in rNMB-R transfected 3T3 cells and 3.9 ± 0.8-fold in C6 cells (Figure 1) with maximal stimulation occurred at 2 min. Stimulation was maintained for at least 20 min in both rNMB-R transfected cells and C6 cells (Figure 1). In preliminary studies with longer incubation periods (>30 min) NMB-stimulated p125^{FAK} phosphorylation decreased (data not shown). NMB caused a detectible increase at 10 pM, half maximal effect at 0.43 ± 0.31 nM and maximal effect at 100 nM (Figure 2). GRP was 26-fold less potent than NMB causing a half maximal effect at 11 ± 20 nM (Figure 2). Similar results were obtained in C-6 glioblastoma cells (not shown). We compared the ability of various concentration of NMB to alter p125^{FAK} tyrosine phosphorylation, stimulate increases in $[Ca^{2+}]_i$, and cause the generation of [^{3}H]inositol phosphates in rNMB-R transfected cells and C6 cells with its ability to occupy the NMB receptor (Figure 3). In rNMB-R transfected BALB 3T3 cells, NMB caused detectible receptor occupation at 0.1 nM, half-maximal receptor occupation at 3.0 nM, and maximal occupation at 1.0 μM (Figure 3, top). The NMB dose−response curve for generation of inositol phosphates was identical to that for receptor

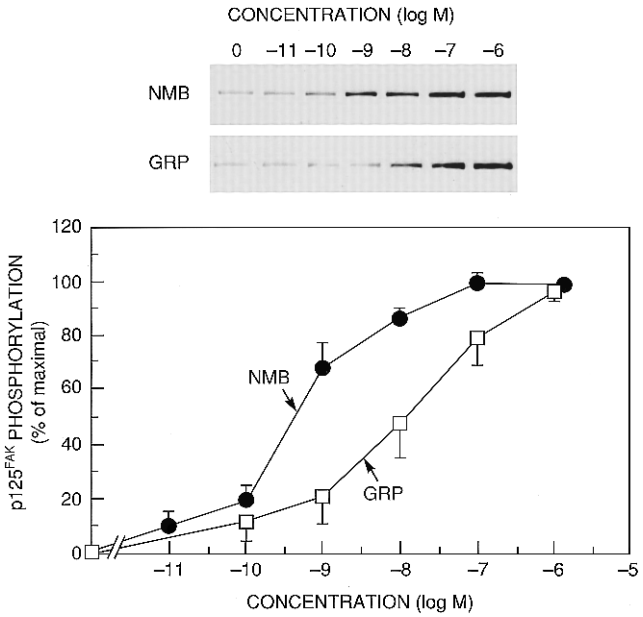

FIGURE 2: Ability of NMB and GRP to stimulate p125FAK tyrosine phosphorylation in rNMB-R transfected cells. Quiescent rNMB-R transfected BALB 3T3 cells grown to confluence in 100 mm dishes were treated with various concentration of NMB and GRP for 10 min and p125FAK tyrosine phosphorylation was determined by immunoprecipitation and Western blotting as described in Figure 1 legend. The top two panels show the results with no additions or various concentrations of GRP and NMB from one experiment representative of four others. In the bottom panel is shown the quantitation of p125FAK tyrosine phosphorylation performed using scanning densitometry. Values are the means ±SE ($n = 4$) expressed as the percentage of maximal increase caused by 1 μM NMB above the control unstimulated values. The maximal increase (1 μM NMB) was 5.4 ± 0.1 fold in the rNMB-R transfected cells.

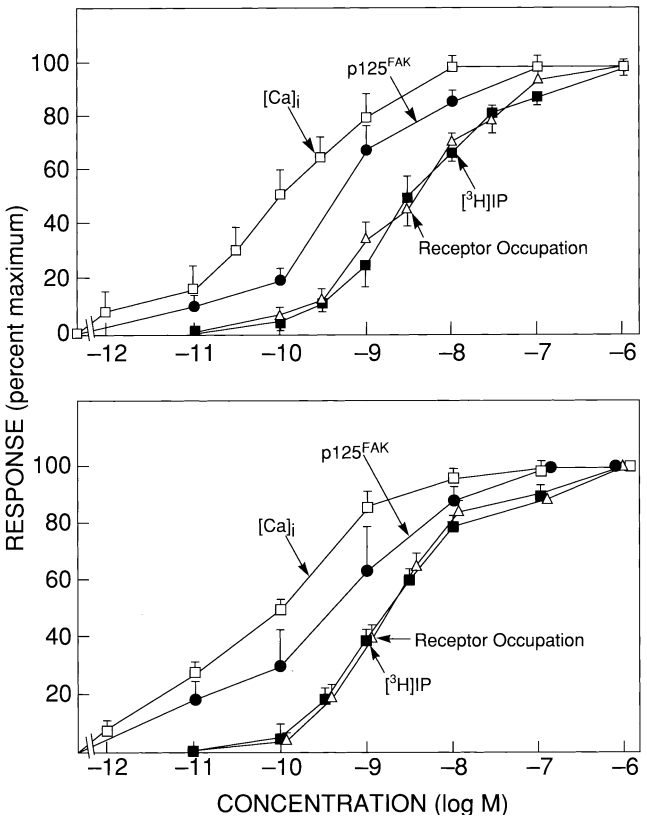

FIGURE 3: Relationship between the ability of NMB to occupy NMB receptors and stimulate p125FAK tyrosine phosphorylation and other intracellular responses in rNMB-R transfected cells (top) and C6 cells (bottom). rNMB-R transfected BALB 3T3 cells and C6 rat glioblastoma cells were treated with various concentrations of NMB for 10 min at 37 °C and p125FAK tyrosine phosphorylation determined as described in Figure 1 legend. Results are expressed as the percentage of the maximal increase caused by 1 μM NMB. The maximal increase in p125FAK tyrosine phosphorylation was 5.4 ± 0.1-fold in rNMB-R transfected cells and 3.9 ± 0.8-fold in C6 cells. The ability of NMB to inhibit binding of 75 pM ^{125}I-[DTyr^0]-NMB to rNMB-R transfected cells and C6 cells was determined at 37 °C after a 30 min incubation. Results are expressed as the percentage of receptors occupied (triangles) with a given NMB concentration after analyzing the dose−inhibition curves using a least-square curve-fitting program, LIGAND (*92*). Parallel cultures were preloaded with 2 μM fura-2/AM and $[Ca^{2+}]_i$ (open squares) determined as described in Methods. The peak increase in $[Ca^{2+}]_i$ at each NMB concentration was determined, and results were expressed as the percentage of the maximal increase caused by 1 μM NMB. Control and 1 μM NMB stimulated $[Ca^{2+}]_i$ were 100 ± 10 nM and 360 ± 40 nM, respectively, for rNMB-R transfected cells ($n = 6$) and 100 ± 10 nM and 367 ± 36 nM for C6 cells, respectively ($n = 5$). Parallel cultures were incubated with *myo*-[2-^{3}H]inositol for 24 h, washed, and incubated either alone or with various concentration of NMB for 60 min at 37°C. [^{3}H]Inositol phosphates were determined (closed squares) as described in methods. Results are expressed as the percentage of the maximal increase caused by 1 μM NMB. Control and 1 μM NMB-stimulated [^{3}H]Inositol phosphates were 9500 ± 500 dpm and 123000 ± 2100 dpm, respectively, for rNMB-R transfected cells ($n = 4$) and 8200 ± 700 dpm and 68000 ± 1400 dpm, respectively for C6 cells ($n = 4$).

occupation (Figure 3, top). The dose−response curve of tyrosine phosphorylation of p125FAK was to the left of that for binding and generation of inositol phosphates with a half-maximal effect at 0.4 nM. The dose−response curve for NMB-stimulated increases in $[Ca^{2+}]_i$ was further to the left with NMB having a half-maximal effect at 0.1 nM. Identical results were obtained with C-6 cells (Figure 3, bottom).

NMB receptor activation of phospholipase C results in increases in both protein kinase C (PKC) and changes in a cytosolic calcium (*21*, *22*, *30*, *32*, *62*, *63*). To see whether activation of one or both pathways could alter p125FAK tyrosine phosphorylation we compared the ability of the phorbol ester TPA and the calcium ionophore A23187 to cause p125FAK phosphorylation in rNMB-R transfected cells (Figure 4). TPA (0.1 μM) caused 33 ± 2.4% of the maximal stimulation of p125FAK tyrosine phosphorylation caused by NMB (0.1 μM). However, calcium ionophore A23187 (0.1 μM) caused only 11 ± 2.6% of the maximal stimulation caused by NMB (0.1 μM) (Figure 4). A 10-fold higher concentration of A23187 (1 μM) caused no greater stimulation. Stimulation with both TPA and A23187 increased p125FAK tyrosine phosphorylation to 63 ± 3.8% of the stimulation caused by NMB (0.1 μM), which was significantly greater than the sum of the values obtained with each alone (63 ± 3.8 vs 45 ± 3.8, $p < 0.05$) (Figure 4).

To determine whether A23187 was only potentiating the efficacy of TPA or whether it was also increasing the potency of TPA, we examined the effect of A23187 on the dose−response curve of TPA-induced tyrosine phosphorylation of p125FAK. TPA alone caused tyrosine phosphorylation of p125FAK with a half-maximal effect at 24 ± 5.6 nM and maximal effect at 1 μM (Figure 5). When A23187 (0.1 μM) was present, TPA caused maximal tyrosine phosphorylation of p125FAK at 1 μM, which was 66% higher than TPA alone. In the presence of A23187 (0.1 μM), TPA had a half-maximal effect at 4.6 ± 0.5 nM which indicated TPA was significantly more potent in the presence than absence of

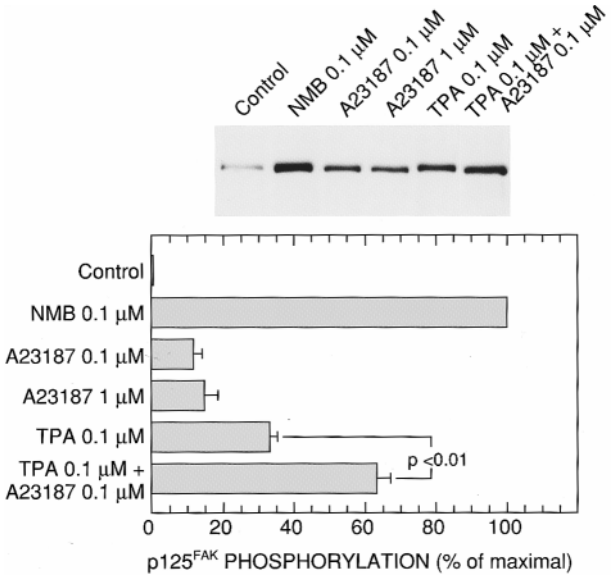

FIGURE 4: Effect of the calcium ionophore, A23187, and the phorbol ester, TPA, on stimulation of $p125^{FAK}$ tyrosine phosphorylation in rNMB transfected BALB 3T3 cells. rNMB-R transfected BALB 3T3 cells were treated with the indicated agents for 10 min at 37 °C and $p125^{FAK}$ phosphorylation determined as described in Figure 1 legend. Results shown at the top are from one experiment representative of four others. In the bottom panel is shown the quantitation of $p125^{FAK}$ tyrosine phosphorylation determined by scanning densitometry. The values are the means ±SE ($n = 5$) expressed as percentage of the maximal increase in $p125^{FAK}$ tyrosine phosphorylation caused by 0.1 μM NMB above control which was a 5.4 ± 0.8-fold increase over the untreated control.

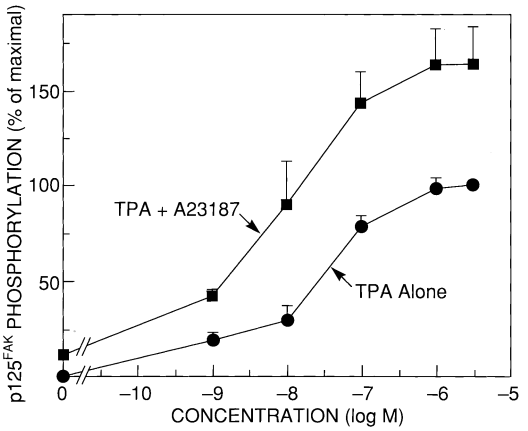

FIGURE 5: Effect of the calcium ionophore, A23187, on the dose−response curve of TPA-induced tyrosine phosphorylation of $p125^{FAK}$. rNMB-R transfected BALB 3T3 cells were treated with the indicated concentration of TPA with or without 0.1 μM A23187 for 10 min at 37 °C and $p125^{FAK}$ phosphorylation determined as described in Figure 1 legend. Values are the means ±SE ($n = 4$) expressed as the percentage of maximal increase caused by 1 μM TPA alone above the control unstimulated values with no A23187 present which was a 2.2 ± 0.3-fold increase.

A23187 (i.e., EC_{50} 4.6 ± 0.5 vs 24 ± 5.6 nM, $p < 0.05$).

To determine whether PKC activation might be involved in mediating the NMB-stimulated changes in $p125^{FAK}$ tyrosine phosphorylation, we examined the effect of GF109203X, a staurosporine-related compound which is a selective inhibitor of PKC (*64*) (Figure 6). In C6 cells pretreatment with 5 μM GF109203X caused almost complete inhibition (90 ± 7%) of TPA-stimulated tyrosine phosphorylation of $p125^{FAK}$ (Figure 6, bottom). In rNMB-R transfected cells pretreatment with 5 μM GF109203X for 1 h causes a 76 ± 8% decrease in the phosphorylation of $p125^{FAK}$

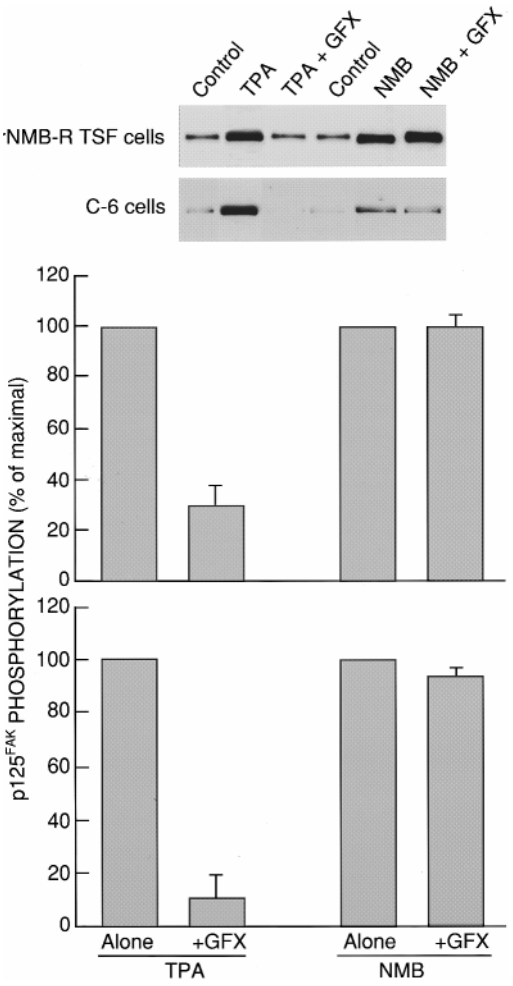

FIGURE 6: Effect of the selective PKC inhibitor, GF109203X, on $p125^{FAK}$ tyrosine phosphorylation. rNMB-R transfected BALB 3T3 cells (top) and C6 cells (bottom) were pretreated for 1 h either in the absence or presence of 5 μM GF109203X. Control cells received an equivalent volume of solvent. Cells were then incubated for a further 10 min with no additions (control), with 0.1 μM NMB or with 0.1 μM TPA. $p125^{FAK}$ phosphorylation was determined as described in Figure 1 legend. The top panel shown a single experiment representative of two others. In the lower panel is shown the quantitation of $p125^{FAK}$ tyrosine phosphorylation determined by scanning densitometry. The values are mean ±SE from three experiments and are expressed as percentage of the maximal phosphorylation caused by TPA or NMB above the control level. NMB (0.1 μM) caused a 5.4 ± 0.8−fold increase over untreated cells in rNMB-R transfected cells and 3.9 ± 0.8-fold in C6 cells. TPA (0.1 μM) caused a 2.4 ± 0.4-fold increase over untreated cells in rNMB-R transfected cells and 1.8 ± 0.2-fold in C-6 cells ($n = 3$).

by 0.1 μM TPA stimulation (Figure 6 top). However, GF109203X did not alter NMB stimulation of tyrosine phosphorylation of $p125^{FAK}$ in either rNMB-R transfected cells or C6 cells (Figure 6).

To determine whether NMB-induced changes in $[Ca^{2+}]_i$ were important in mediating the ability of NMB to stimulate tyrosine phosphorylation of $p125^{FAK}$, we examined the effect of thapsigargin, which inhibits microsomal Ca^{2+}-ATPase and depletes intracellular calcium stores (*65*) (Figure 7). The addition of thapsigargin for 30 min prior to NMB stimulation completely inhibited the ability of NMB to stimulate changes in $[Ca^{2+}]_i$ (Figure 7, insert). However, thapsigargin had no effect on the NMB-stimulated increase in $p125^{FAK}$ tyrosine phosphorylation (Figure 7). Furthermore, thapsigargin had no effect on TPA-induced $p125^{FAK}$ tyrosine phosphorylation.

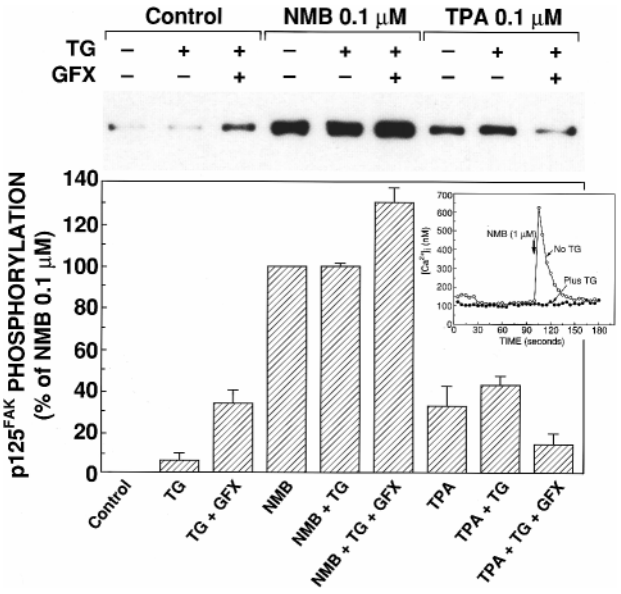

FIGURE 7: Effect of the thapsigargin alone or with GF109203X on p125FAK tyrosine phosphorylation. rNMB-R transfected BALB 3T3 cells were pretreated for 1 h at 37 °C either in the absence or presence of 5 μM GF109203X. During the final 30 min of pretreatment, 0.1 μM thapsigargin was added to one-half the cells. Control cells received an equivalent volume of DMSO. Cells were then incubated for a further 10 min with no additions (control), with 0.1 μM NMB or with 0.1 μM TPA. p125FAK phosphorylation was determined as described in Figure 1 legend. The top panel shows a single experiment representative of two others. In the lower panel is shown the quantitation of p125FAK tyrosine phosphorylation determined by scanning densitometry. The values are mean ±SE from three experiments and are expressed as percentage of the phosphorylation caused by 0.1 μM NMB above the control level. NMB (0.1 μM) caused a 4.7 ± 0.6-fold increase over the untreated control (n = 3). Inset. Effect of thapsigargin on NMB-induced changes in $[Ca^{2+}]_i$ in rNMB transfected cells. Fura-2 loaded cells were incubated in the absence of presence of 0.1 μM thapsigargin for 30 min, and then 1 μM NMB was added. $[Ca^{2+}]_i$ was continuously measured in a spectrofluorimeter as described in Methods. This result is representative of three others.

Pretreatment with 5 μM GF109203X for 1 h and addition of 0.1 μM thapsigargin had no effect on NMB-induced tyrosine phosphorylation of p125FAK, however it inhibited TPA-induced p125FAK tyrosine phosphorylation by 57% (Figure 7).

Tyrphostins are reported to be specific inhibitors of tyrosine kinases (*66*−*68*), and thus we examined the effect of tyrphostin A25 on phosphorylation of p125FAK by NMB and TPA in rNMB-R transfected cells (Figure 8). Pretreatment with 20 μM tyrphostin A25 inhibited by 100% TPA-stimulated-increases and by 52 ± 2% NMB-stimulated increases in p125FAK tyrosine phosphorylation (Figure 8). Preincubation with 20 μM tyrphostin A25 under identical conditions to those that inhibited p125FAK tyrosine phosphorylation had no effect on the generation of inositol phosphates stimulated by NMB. Tyrphostin A25 preincubation neither changed the efficacy nor the potency of NMB for stimulating changes in [^{3}H]inositol phosphates (data not shown).

Previous studies shows that the integrity of the cytoskeleton, and particularly the actin filament network is important for the stimulation of p125FAK tyrosine phosphorylation by a number of agents (*52*−*55*). We examined the effect of cytochalasin D, an agent that disrupts actin filaments (*69*) and colchicine, an agent that disrupts microtubules (*70*, *71*) on NMB- and TPA-stimulated changes in p125FAK tyrosine

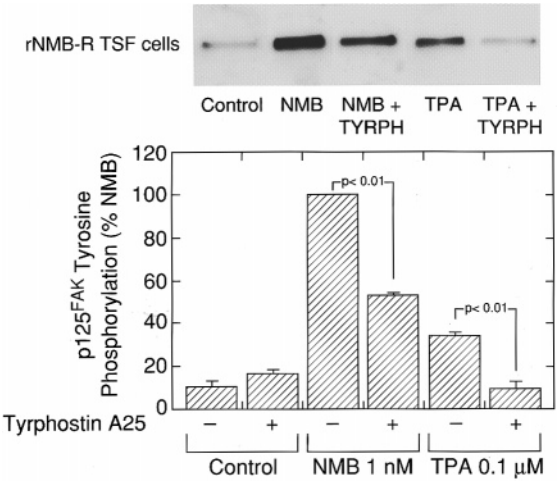

FIGURE 8: Effect of Tyrphostin A25 on NMB- and TPA-stimulation of p125FAK tyrosine phosphorylation in rNMB-R transfected BALB 3T3 cells. rNMB-R transfected BALB 3T3 cells were incubated for 18 h in DMEM/Waymouths medium in the absence or presence of 20 μM tyrphostin A25. Cells were then incubated for a further 10 min either in the presence of 1 nM NMB or 0.1 μM TPA. p125FAK tyrosine phosphorylation was determined as described in Figure 1 legend. In the top panel is shown the results from one experiment representative of two others. In the lower panel is shown the quantitation of p125FAK tyrosine phosphorylation determined by scanning densitometry. The values are mean ±SE from three experiments. Results are expressed as percentage of the maximal phosphorylation which was induced by 1 nM NMB which was a 3.2 ± 0.5-fold increase over untreated cells.

phosphorylation. Cytochalasin D inhibited both the NMB and TPA stimulation of p125FAK tyrosine phosphorylation completely (Figure 9, top). In contrast to the effect of cytochalasin D, pretreatment with 0.3 μM colchicine had no effect on either NMB- or TPA-stimulation of p125FAK tyrosine phosphorylation (Figure 9, bottom).

The small GTP-binding protein, p21rho is required for growth factor-induced formation of stress fibers and focal adhesions (*51*, *72*, *73*). To determine whether this small GTP binding protein could be involved in the ability of NMB to stimulate tyrosine phosphorylation of p125FAK, we examined the effect of *Clostridium botulinum* C3 exoenzyme on NMB-stimulated changes in p125FAK tyrosine phosphorylation (Figure 10). *Clostridium botulinum* C3 exoenzyme has been shown to cause ADP ribosylation of p21rho which inactivates p21rho (*74*−*76*). Pretreatment with the C3 exoenzyme inhibited by 55 ± 11% the NMB-stimulated increase in tyrosine phosphorylation of p125FAK in rNMB-R transfected cells (Figure 10).

DISCUSSION

A number of results in the present study demonstrate that NMB receptor activation can stimulate tyrosine phosphorylation of the focal adhesion kinase p125FAK. In both the rNMB-R transfected cells and C6 rat glioblastoma cells which contain native NMB receptors (*20*, *32*), NMB stimulated a rapid increase in tyrosine phosphorylation of p125FAK. Phosphorylation of p125FAK by NMB in each cell type occurred over a similar concentration range to that caused by NMB in other well-studied changes in cell function such as increases in $[Ca^{2+}]_i$ (*22*, *30*, *32*, *63*, *77*). GRP also caused tyrosine phosphorylation of p125FAK in rNMB-R transfected cells, but NMB was 30-fold more potent than GRP for stimulation of p125FAK tyrosine phosphorylation.

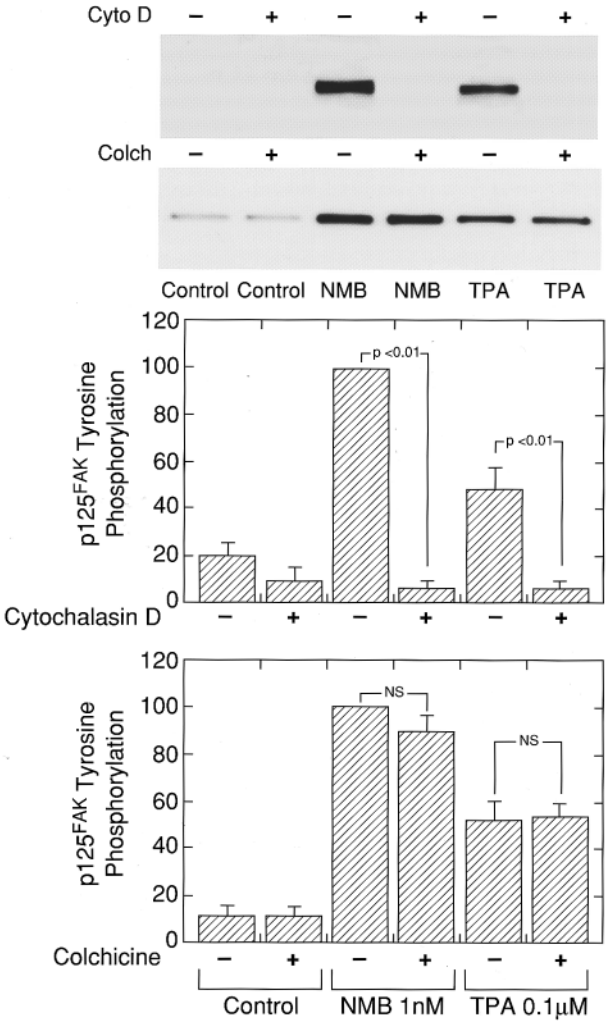

FIGURE 9: Effect of cytochalasin D or colchicine on NMB- and TPA-stimulation of p125^{FAK} tyrosine phosphorylation. rNMB-R transfected BALB 3T3 cells were treated for 2 h either in the absence or presence of 1.25 μM cytochalasin D or 0.3 μM colchicine. Cells were then incubated for a further 10 min either in the presence of 0.1 μM NMB or 0.1 μM TPA. p125^{FAK} phosphorylation was determined as described in Figure 1 legend. The top panel shown a result from a single experiment representative of two others. In the lower panel is shown the quantitation of p125^{FAK} tyrosine phosphorylation determined by scanning densitometry. The values are mean ±SE from three experiments. Results are expressed as the percentage of the maximal phosphorylation caused by 0.1 μM NMB in untreated cells which was a 5.4 ± 0.2-fold increase.

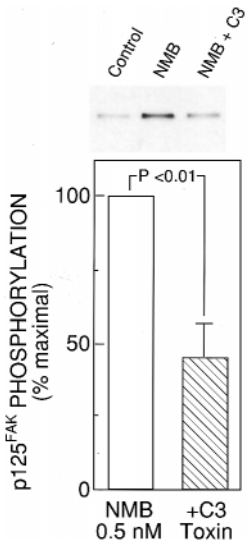

FIGURE 10: Effect of Botulinum clostridium C3 exoenzyme on NMB stimulation of p125^{FAK} tyrosine phosphorylation in rNMB-R transfected BALB 3T3 cells. rNMB-R transfected cells (1.5×10^5 per 35 mm dish) were cultured in 2 mL of DMEM supplemented with 10% fetal bovine serum for 48 h, and then C3 exoenzyme (at 7.5 μg/mL) was added. After a additional 48 h of culture 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 more. Cells were then incubated for a further 10 min in the presence or absence NMB (0.5 nM). p125^{FAK} tyrosine phosphorylation was determined as described in Figure 1 legend. In the top panel is shown the results from one experiment representative of two others. In the lower panel is shown the quantitation of p125^{FAK} tyrosine phosphorylation determined by scanning densitometry. The values are mean ±SE from three experiments. Results are expressed as percentage of the maximal phosphorylation which was induced by 0.5 nM NMB in cells not treated with C3 exoenzyme which was a 2.7 ± 0.4-fold increase.

This difference in the ability of GRP and NMB to increase phosphorylation of p125^{FAK} is similar to the differences in affinities of these two peptides for the NMB receptor demonstrated by binding studies in each cell type (*30*, *63*) and therefore reflects the relative abilities of these two peptides to occupy the NMB receptor.

The ability of NMB to cause tyrosine phosphorylation of p125^{FAK} in relation to its ability to occupy the NMB receptor and cause other changes in cellular function have both similarities and differences from that previously reported for the closely related GRP receptor (*1*, *14*, *15*, *27*, *28*). In both rNMB-R containing cells in this study the NMB dose−response curve for generation of inositol phosphates was identical to that for receptor occupation. The NMB dose−response curve for stimulation of p125^{FAK} tyrosine phosphorylation was to the left of that for binding and generation of inositol phosphates by approximately 1 log unit and the dose−response curve for stimulation of increases in $[Ca^{2+}]_i$ was further to the left by approximately 1/2 of a log unit. These results demonstrate with both cell types there were spare NMB receptors for tyrosine phosphorylation of p125^{FAK} and calcium mobilization with the result that submaximal receptor occupation results in maximal responses for these two cellular processes. The intracellular coupling of the NMB receptor is similar to that reported for the GRP receptor (*52*) in that with both receptors the dose−response curves for agonist activation of tyrosine phosphorylation of p125^{FAK} and increases in $[Ca^{2+}]_i$ are to the left of those for binding and changes in inositol phosphates. However, the two receptors differ in the coupling relationship of receptor occupation to increases in $[Ca^{2+}]_i$ and tyrosine phosphorylation of p125^{FAK}. With the NMB receptor, greater receptor spareness exists for changes in $[Ca^{2+}]_i$ than for changes in stimulation of tyrosine phosphorylation of p125^{FAK}. In contrast with agonist activation of the GRP receptor (*52*) the relationship is reversed with greater receptor spareness for stimulation of tyrosine phosphorylation of p125^{FAK} than for increases in $[Ca^{2+}]_i$.

In recent studies the mouse GRP receptor mGRP receptor stably transfected into either BALB 3T3 cells (*31*) or into frog melanophores (*78*) is reported to couple in a different manner than to the native mGRP receptor in murine Swiss 3T3 cells. In contrast, our study demonstrates the rNMB receptor stably transfected into BALB 3T3 cells appears to behave in an identical fashion to the wild type rNMB receptor in rat C6 glioblastoma cells. This conclusion is supported by the similar stoichiometric relationships between receptor

occupation and agonist stimulation of changes in $[Ca^{2+}]_i$, generation of phosphoinositides, and phosphorylation of p125^{FAK} in the transfected and mild type NMB receptor cell lines. Furthermore, the kinetics of tyrosine phosphorylation of p125^{FAK} and the effects of cytochalasin D and the PKC inhibitor, GF109203X, on both rNMB-R containing cells were similar. These results demonstrate that not only are these rNMB-R transfected cells useful in studying NMB receptor modulation (internalization, desensitization, down-regulation) as recently reported (*30*, *79*), they will also be a useful system to explore the receptor structural requirements for tyrosine phosphorylation of p125^{FAK} because these cells behave in an identical fashion to cells possessing the wild type rNMB receptor.

Activation of the NMB receptor by NMB-related peptides causes stimulation of phospholipase C which results in increases in inositol phosphates and elevation of cellular calcium (*21*, *22*, *30*, *32*, *33*). In numerous other systems, agents which activate phospholipase C also cause generation of diacylglycerol and activation of PKC (*80*). A number of recent studies suggest that with some neuropeptides, activation of phospholipase C may be important for agonist-induced tyrosine phosphorylation of p125^{FAK}. The possibility that changes in cytosolic calcium could be an important mediator is supported by studies which show that angiotensin II increases tyrosine phosphorylation in liver cells including a 125 kDa protein through a Ca^{2+}-dependent pathway (*81*) and epinephrine stimulation of p125^{FAK} phosphorylation in platelets is blocked when increases in cytosolic calcium are inhibited (*57*). However, in the present study we demonstrated that the calcium ionophore A23187 caused minimal tyrosine phosphorylation of p125^{FAK} in rNMB-R transfected cells, suggesting that activation of this pathway at least alone was not likely involved in mediating NMB-stimulated increases in phosphorylation of p125^{FAK}. This conclusion was strongly supported by the results with thapsigarin pretreatment which by inhibiting the Ca^{2+}-ATPase (*65*) and depleting intracellular calcium stores completely inhibited NMB receptor increases in cytolic calcium. Thapsigargin pretreatment, however, had no effect on NMB-receptor stimulated increases in p125^{FAK} tyrosine phosphorylation directly demonstrating changes in $[Ca^{2+}]_i$ are not necessary for this stimulation to occur with NMB. This conclusion is similar to results with GRP receptor activation in Swiss 3T3 cells (*52*) and CCK_A receptor stimulation of p125^{FAK} tyrosine phosphorylation in pancreatic acini (*82*). Furthermore, in streptolysin O-permeabilized Swiss 3T3 cells increasing Ca^{2+} concentration did not induce any significant tyrosine phosphorylation (*50*), whereas bombesin and vasopressin stimulated p125^{FAK} tyrosine phosphorylation in these cells (*37*). These results suggest that NMB receptor activation differs from angiotensin II receptor activation in liver cells or epinephrine's action in platelets, but resembles CCK_A and GRP receptor activation in that increases in $[Ca^{2+}]_i$ alone have no effect on their ability to stimulate tyrosine phosphorylation of p125^{FAK}.

The possibility that phospholipase C activation of PKC could be important in agonist-induced p125^{FAK} tyrosine phosphorylation is also supported in studies of some neuropeptide/neurotransmitter receptors. Recent studies demonstrate that epinephine stimulation of p125^{FAK} tyrosine phosphorylation in platelets is inhibited when PKC activation is reduced (*57*). Similarly, endothelin-1 stimulation of p125^{FAK} tyrosine phosphorylation in RAT-1 cells (*83*) and angiotensin II stimulation in aortic smooth muscle cells (*84*) was partially inhibited by PKC inhibitors. Furthermore, p125^{FAK} has a PKC phosphorylation sequence (*42*) and activation of PKC by phorbol 12,13-dibutyrate (PDB) or 1-oleoyl-2-acetylglycerol (OAG) have been shown to cause tyrosine phosphorylation of p125^{FAK} (*52*). However, p125^{FAK} tyrosine phosphorylation due to activation of CCK_A receptors in pancreatic acinar cells (*82*) or GRP receptors in Swiss 3T3 cells (*52*) was not effected by PKC inhibition. In the present study the PKC activator, TPA, caused an increase in tyrosine phosphorylation that was equal in magnitude to 50% of that seen with NMB receptor activation. However, our results show that it is unlikely PKC activation alone is involved in tyrosine phosphorylation of p125^{FAK} caused by NMB. This conclusion is supported by the inability of the PKC inhibitor GF 109203X to inhibit NMB-induced p125^{FAK} tyrosine phosphorylation at a concentration that completely inhibited TPA-simulated p125^{FAK} tyrosine phosphorylation.

Simultaneous activation of the PKC pathway and increases in cytosolic calcium can have synergistic effect on a number of cellular responses such as phosphorylation of various proteins (*85*), stimulation of amylase release from pancreatic acini (*14*, *86*), or pepsinogen release from chief cells (*87*). In our study the simultaneous activation of PKC with TPA and increases in cytosolic calcium caused by the calcium ionophore, A23187, had a synergistic effect on the magnitude of p125^{FAK} tyrosine phosphorylation in both rNMB-R transfected cells and C6 cells. Furthermore, increases in cytosolic calcium caused a leftward shift in the TPA-stimulated p125^{FAK} tyrosine phosphorylation dose−response curve demonstrating it increased the affinity of PKC in stimulating this cascade. Therefore it is possible that changes in $[Ca^{2+}]_i$ simultaneous with activation of PKC could be responsible for some of the stimulation caused by NMB. Our results suggest that with NMB receptor activation, it is unlikely that a synergistic effect of PKC activation and increases in cytosolic calcium are important in mediating the NMB stimulation of p125^{FAK} tyrosine phosphorylation. The combination of thapsigargin at a concentration that completely inhibited NMB-stimulated increases in $[Ca^{2+}]_i$ and GF109203X at a concentration that markedly inhibited TPA-stimulation of p125^{FAK} tyrosine phosphorylation had no effect on NMB-stimulation of p125^{FAK} tyrosine phosphorylation. The results suggest that NMB-stimulates tyrosine phosphorylation of p125^{FAK} by a phospholipase C independent pathway. This result differs from a recent study (*57*) examining p125^{FAK} tyrosine phosphorylation in platelets by thrombin and CCK_A receptor activation in pancreatic acini (*82*). In both of these studies the p125^{FAK} tyrosine phosphorylation caused by either agent was not altered by inhibiting cellular calcium mobilization and only minimally inhibited by a PKC inhibitor; however, inhibition of both pathways simultaneously completely inhibited thrombin-stimulation (*57*), and decreased CCK_A receptor stimulation of p125^{FAK} tyrosine phosphorylation by 50% (*82*).

An additional finding in the present study which demonstrates that NMB-R activation of phospholipase C is distinct and separate from its ability to cause tyrosine phosphorylation of p125^{FAK} is the results with tyrophostin A25. Tyrphostins are series of synthetic tyrosine kinase inhibitors (*68*) which have been shown to selectively inhibit both membrane and cytosolic protein tyrosine kinases (*66*, *67*). In our study

pretreatment with 20 μM tyrphostin A25 caused a 52% inhibition of NMB- and TPA-stimulated tyrosine phosphorylation of p125^{FAK}. However, tyrphostin A25 had no effect on activation of phospholipase C and the generation of inositol phosphates by NMB. These results further support the conclusion that phospholipase C activation is not needed for NMB-stimulated tyrosine phosphorylation of p125^{FAK}.

Recent studies demonstrate that various extracellular matrix proteins (*43*−*45*, *47*, *49*), various transforming oncogenic forms of pp60^{src} (*45*, *49*), various bioactive lipids such as lysophosphatidic acid (LPA) (*55*), various growth factors such as PDGF (*53*), and various neuropeptides and hormones such as endothelin, vasopressin, angiotensin, bradykinin, and bombesin (*52*, *56*, *83*, *84*, *88*) can stimulate tyrosine phosphorylation of p125^{FAK}. With a number of these stimulants, studies suggest that this stimulation requires the integrity of the actin cytoskeleton (*52*−*56*). Our studies provide evidence that the integrity of actin microfilament network but not of microtubular network is necessary for the ability of NMB receptor activation to cause tyrosine phosphorylation of p125^{FAK}. Specifically, pretreatment with cytochalasin D, an agent which disrupts the actin microfilament network (*69*) inhibited tyrosine phosphorylation of p125^{FAK} caused by stimulation with NMB or TPA completely. In contrast, colchicine which disrupts the microtubules (*70*, *71*) had no effect on tyrosine phosphorylation of p125^{FAK} by NMB and TPA. These results support the conclusion that similar to a number of other stimuli, tyrosine phosphorylation of p125^{FAK} by NMB receptor activation depends on the integrity of actin cytoskeleton.

Recent studies show that tyrosine phosphorylation and some of the cytoskeletal changes induced by various growth factors and neuropeptides require functional p21^{rho} (*50*, *51*, *72*, *73*, *89*, *90*), a member of the ras-related small GTP-binding protein (*91*). These findings raised the possibility that p21^{rho} may be involved in mediating NMB-induced tyrosine phosphorylation of p125^{FAK}. Botulinum C3 exoenzyme is known to cause ADP ribosylation of Asn^{21} of p21^{rho} which inactivates p21^{rho} (*74*, *76*). In our study pretreatment with C3 exoenzyme markedly inhibited tyrosine phosphorylation of p125^{FAK} in rNMB-R transfected BALB 3T3 cells. This results is consistent with the conclusion that p21^{rho} is important in mediating NMB-induced changes in tyrosine phosphorylation of p125^{FAK}.

In conclusion, activation of NMB receptor can cause rapid tyrosine phosphorylation of p125^{FAK} in both the C6 rat glioblastoma cells natively possessing NMB receptors and rNMB-R transfected BALB 3T3 cells. Although GF109203X inhibited TPA-induced tyrosine phosphorylation of p125^{FAK}, it had no effect on NMB-induced p125^{FAK} phosphorylation. Furthermore, the calcium ionophore A23187 had a little effect on tyrosine phosphorylation of p125^{FAK} and thapsigargin at concentrations which completely inhibited NMB-stimulated changes in $[Ca^{2+}]_i$, had not effect on NMB-stimulated p125^{FAK} tyrosine phosphorylation. Furthermore, the combination of GF10920X and thapsigargin did not alter NMB-stimulates p125^{FAK} tyrosine phosphorylation. Tyrphostin A25 inhibited tyrosine phosphorylation of p125^{FAK} but not generation of [^{3}H]inositol phosphates. These results demonstrate that stimulation of tyrosine phosphorylation of p125^{FAK} by NMB receptor activation differs from a number of other stimulators of p125^{FAK} tyrosine phosphorylation such as thombin, angiotensin, endothelin, and CCK, in that it is independent of agonist-induced changes in $[Ca^{2+}]_i$ or PKC and that activation of phospholipase C is not necessary for activation of this pathway. Pretreatment with either cytochalasin D or C3 exoenzyme inhibited tyrosine phosphorylation of p125^{FAK}, but not colchine pretreatment. These results indicate that the integrity of the actin cytoskeletal network but not of microtubular network is necessary for NMB-stimulated phosphorylation of p125^{FAK}. The ras-related small GTP-binding protein p21^{rho} is at least partially involved in mediating NMB-induced tyrosine phosphorylation of p125^{FAK}. These results suggest that similar to some other neuropeptides, stimulation of p125^{FAK} tyrosine phosphorylation may be an important pathway in mediating cellular changes caused by activation of the NMB receptor.

REFERENCES

1. Tache, Y., Melchiorri, P., and Negri, L. (1988) *Ann. N. Y. Acad. Sci. 547*, 1−541.
2. Brown, M. R., Carver, K., and Fisher, L. A. (1988) *Ann. N. Y. Acad. Sci. 547*, 174−182.
3. Rettori, V., Pazos-Moura, C. C., Moura, E. G., Polak, J., and McCann, S. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3035−3039.
4. Albers, H. E., Liou, S. Y., Stopa, E. G., and Zoeller, R. T. (1991) *J. Neurosci. 11*, 846−851.
5. McCoy, J. G., and Avery, D. D. (1990) *Peptides 11*, 595−607.
6. Ruff, M., Schiffmann, E., Terranova, V., and Pert, C. B. (1985) *Clin. Immunol. Immunopathol. 37*, 387−396.
7. DelaFuente, M., DelRio, M., and Hernanz, A. (1993) *J. Neuroimmunol. 48*, 143−150.
8. Hill, D. J., and McDonald, T. J. (1992) *Endocrinology 130*, 2811−2819.
9. Sunday, M. E., Hua, J., Reyes, B., Masui, H., and Torday, J. S. (1993) *Anat. Rec. 236*, 25−32.
10. Falconieri Erspamer, G., Severini, C., Erspamer, V., Melchiorri, P., Delle Fave, G., and Nakajima, T. (1988) *Regul. Pept. 21*, 1−11.
11. Jensen, R. T., Coy, D. H., Saeed, Z. A., Heinz-Erian, P., Mantey, S., and Gardner, J. D. (1988) *Ann. N. Y. Acad. Sci. 547*, 138−149.
12. Ghatei, M. A., Jung, R. T., Stevenson, J. C., Hillyard, C. J., Adrian, T. E., Lee, Y. C., Christofides, N. D., Sarson, D. L., Mashiter, K., MacIntyre, I., and Bloom, S. R. (1982) *J. Clin. Endocrinol. Metab. 54*, 980−985.
13. Severi, C., Jensen, R. T., Erspamer, V., D'Arpino, L., Coy, D. H., Torsoli, A., and Delle Fave, G. (1991) *Am. J. Physiol. 260*, G683−G690.
14. Jensen, R. T. (1994) in *Physiology of the Gastrointestinal Tract*, 3rd ed. (Johnson, L. R., Jacobsen, E. D., Christensen, J., Alpers, D. H., and Walsh, J. H., Eds.) pp 1377−1446, Raven Press, New York.
15. Rozengurt, E. (1988) *Ann. N. Y. Acad. Sci. 547*, 277−292.
16. Willey, J. C., Lechner, J. F., and Harris, C. C. (1984) *Exp. Cell Res. 153*, 245−248.
17. Endo, T., Fukue, H., Kanaya, M., Mizunuma, M., Fujii, M., Yamamoto, H., Tanaka, S., and Hashimoto, M. (1991) *J. Endocrinol. 131*, 313−318.
18. Seglen, P. O., Skomedal, H., Saeter, G., Schwartze, P. E., and Nesland, J. M. (1989) *Carcinogenesis 10*, 21−29.
19. Markowska, A., Nussdorfer, G. G., and Malendowicz, L. K. (1993) *Histol. Histopathol. 8*, 359−362.
20. Moody, T. W., Fagarasan, M., and Zia, F. (1995) *Peptides 16*, 1133−1140.
21. Dobrzanski, D., Sharoni, Y., Wada, E., Battey, J., and Sausville, E. (1993) *Regul. Pept. 45*, 341−352.
22. Lach, E. B., Broad, S., and Rozengurt, E. (1995) *Cell Growth Differ. 6*, 1427−1435.
23. Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A., and Minna, J. D. (1985) *Nature 316*, 823−826.

24. Bologna, M., Festuccia, C., Muzi, P., Biordi, L., and Ciomei, M. (1989) *Cancer 63*, 1714−1720.
25. Schrey, M. P., Patel, K. V., and Tezapsidis, N. (1992) *Cancer Res. 52*, 1786−1790.
26. von Schrenck, T., Heinz-Erian, P., Moran, T., Mantey, S. A., Gardner, J. D., and Jensen, R. T. (1989) *Am. J. Physiol. 256*, G747−G758.
27. Battey, J. F., Way, J. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Mann, E., and Feldman, R. I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 395−399.
28. Spindel, E. R., Giladi, E., Brehm, P., Goodman, R. H., and Segerson, T. P. (1990) *Mol. Endocrinol. 4*, 1956−1963.
29. Wada, E., Way, J., Shapira, H., Kusano, K., Lebacq-Verheyden, A. M., Coy, D., Jensen, R., and Battey, J. (1991) *Neuron 6 (3)*, 421−430.
30. Benya, R. V., Wada, E., Battey, J. F., Fahti, Z., Wang, L. H., Mantey, S. A., Coy, D. H., and Jensen, R. T. (1992) *Mol. Pharmacol. 42(6)*, 1058−1068.
31. Benya, R. V., Fathi, Z., Pradhan, T., Battey, J. F., Kusui, T., and Jensen, R. T. (1994) *Mol. Pharmacol. 46*, 235−245.
32. Wang, L. H., Battey, J. F., Wada, E., Lin, J. T., Mantey, S., Coy, D. H., and Jensen, R. T. (1992) *Biochem. J. 286*, 641−648.
33. Moody, T. W., Staley, J., Zia, F., Coy, D. H., and Jensen, R. T. (1992) *J. Pharmacol. Exp. Ther. 263*, 311−317.
34. Rozengurt, E. (1991) *Eur. J. Clin. Invest. 21*, 123−134.
35. Seckl, M., and Rozengurt, E. (1993) *J. Biol. Chem. 268*, 9548−9554.
36. Zachary, I., and Rozengurt, E. (1992) *Cell 71*, 891−894.
37. Zachary, I., Gil, J., Lenhmann, W., Sinnett-Smith, J., and Rozengurt, E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4577−4581.
38. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1991) *J. Biol. Chem. 266*, 24126−24133.
39. Leeb-Lundberg, L. M. F. and Song, X. H. (1991) *J. Biol. Chem. 266*, 7746−7749.
40. Schieffer, B., Bernstein, K. E., and Marrero, M. B. (1996) *J. Mol. Med. 74*, 85−91.
41. Turner, C. E., Pietras, K. M., Taylor, D. S., and Molloy, C. J. (1995) *J. Cell. Sci. 108*, 333−342.
42. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5192−5196.
43. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8487−8491.
44. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J. Biol. Chem. 267*, 23439−23442.
45. Guan, J. L., and Shalloway, D. (1992) *Nature 358*, 690−692.
46. Vuori, K., and Ruoslahti, E. (1993) *J. Biol. Chem. 268*, 21459−21462.
47. Burridge, K., Turner, C. E., and Romer, L. H. (1992) *J. Cell Biol. 119*, 893−903.
48. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) *J. Cell Biol. 119*, 905−912.
49. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3328−3332.
50. Seckl, M. J., Morii, N., Narumiya, S., and Rozengurt, E. (1995) *J. Biol. Chem. 270*, 6984−6990.
51. Rankin, S., Morii, N., Narumiya, S., and Rozengurt, E. (1994) *FEBS Lett. 354*, 315−319.
52. Sinnett-Smith, J., Zachary, I., Valverde, A. M., and Rozengurt, E. (1993) *J. Biol. Chem. 268*, 14261−14268.
53. Rankin, S., and Rozengurt, E. (1994) *J. Biol. Chem. 269*, 704−710.
54. Seufferlein, T., and Rozengurt, E. (1994) *J. Biol. Chem. 269*, 27610−27617.
55. Seufferlein, T., and Rozengurt, E. (1994) *J. Biol. Chem. 269*, 9345−9351.
56. Leeb-Lundberg, L. M. F., Song, X. H., and Mathis, S. A. (1994) *J. Biol. Chem. 269*, 24328−24334.
57. Shattil, S. J., Haimovich, B., Cunningham, M., Lipfert, L., Parsons, J. T., Ginsberg, M. H., and Brugge, J. S. (1994) *J. Biol. Chem. 269*, 14738−14745.
58. Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) in *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., Inc., New York.
59. Qian, J. M., Rowley, W. H., and Jensen, R. T. (1993) *Am. J. Physiol. 264*, G718−G727.
60. Berridge, M. J., Dawson, R. M., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) *Biochem. J. 212*, 473−482.
61. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem. 260*, 3440−3450.
62. Spindel, E. R. (1986) *Trends Neurosci. 9*, 130−133.
63. Wang, L. H., Mantey, S. A., Lin, J. T., Frucht, H., and Jensen, R. T. (1993) *Biochim. Biophys. Acta 1175*, 232−242.
64. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) *J. Biol. Chem. 266*, 15771−15781.
65. Thastrup, O. (1990) *Agents Actions 29*, 8−15.
66. Bilder, G. E., Krawiec, J. A., McVety, K., Gazit, A., Gilon, C., Lyall, R., Zilbertstein, A., Levitzki, A., Perrone, M. H., and Schreiber, A. B. (1991) *Am. J. Physiol. 260*, C721−C730.
67. Lyall, R. M., Zilbestein, A., Gazit, A., Gilon, C., Levitzki, A., and Schlessinger, J. (1989) *J. Biol. Chem. 264*, 14503−14509.
68. Gazit, A., Yaish, P., Gilon, C., and Levitzki, A. (1989) *J. Med. Chem. 32*, 2344−2352.
69. Cooper, J. A. (1987) *J. Cell Biol. 105*, 1473−1478.
70. Leung, M. F., and Sartorelli, A. C. (1992) *Leukemia Res. 16*, 929−935.
71. Salmon, E. D., McKeel, M., and Hays, T. (1984) *J. Cell Biol. 99*, 1066−1075.
72. Ridley, A. J., and Hall, A. (1992) *Cell 70*, 389−399.
73. Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. (1993) *J. Biol. Chem. 268*, 24535−24538.
74. Aktories, K., Weller, U., and Chhatwal, G. S. (1987) *FEBS Lett. 212*, 109−113.
75. Nemoto, Y., Namba, T., Kozaki, S., and Narumiya, S. (1991) *J. Biol. Chem. 266*, 19312−19319.
76. Sekine, A., Fujiwara, M., and Narumiya, S. (1989) *J. Biol. Chem. 264*, 8602−8605.
77. Battey, J., and Wada, E. (1991) *Trends Neurosci. 14*, 524−527.
78. Graminski, G. F., Jayawickreme, C. K., Potenza, M. N., and Lerner, M. R. (1993) *J. Biol. Chem. 268*, 5957−5964.
79. Benya, R. V., Kusui, T., Battey, J. F., and Jensen, R. T. (1994) *J. Biol. Chem. 269*, 11721−11728.
80. Nishizuka, Y. (1992) *Science 258*, 607−614.
81. Huckle, W. R., Dy, R. C., and Earp, H. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8837−8841.
82. Garcia, L. J., Rosado, J. A., Gonzalez, A., and Jensen, R. T. (1997) *Biochem. J. 327*, 461−472.
83. Saville, M. K., Graham, A., Malarkey, K., Paterson, A., Gould, G. W., and Plevin, R. (1994) *Biochem. J. 301*, 407−414.
84. Leduc, I., and Meloche, S. (1995) *J. Biol. Chem. 270*, 4401−4404.
85. Itoh, H., Shimomura, A., Okubo, S., Ichikawa, K., Ito, M., Konishi, T., and Nakano, T. (1993) *Am. J. Physiol. 265*, C1319−C1324.
86. Williams, J. A., and Yule, D. I. (1993) in *PANCREAS: Biology, Pathobiology, and Disease* (Go, V. L. W., DiMagno, E. P., Gardner, J. D., Lebenthal, E., Reber, H. A., and Scheele, G. A., Eds.) pp 167−189, Raven Press, New York.
87. Raufman, J. P., Kasbekar, D. K., Jensen, R. T., and Gardner, J. D. (1983) *Am. J. Physiol. 245*, G525−G530.
88. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992) *J. Biol. Chem. 267*, 19031−19034.
89. Ridley, A. J. (1995) *Curr. Opin. Genet. Dev. 5*, 24−30.
90. Ridley, A. J. (1994) *J. Cell Sci. 18*, 127−131.
91. Hall, A. (1990) *Science 249*, 635−640.
92. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem. 107*, 220−229.

BI971448O