Bombesin, Vasopressin, Lysophosphatidic Acid, and Sphingosylphosphorylcholine Induce Focal Adhesion Kinase Activation in Intact Swiss 3T3 Cells*

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Treatment of quiescent Swiss 3T3 cells with bombesin rapidly increased focal adhesion kinase (FAK)-associated tyrosine kinase activity in immune complexes. The effect was rapid (maximum at 2.5 min) and dose dependent (half-maximum response at 0.05 nm). Addition of vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine also elicited a rapid increase in FAK-associated tyrosine kinase activity. Addition of the selective Src inhibitor pyrazolopyrimidine directly to the cell line potently inhibited Src kinase activity induced by bombesin but did not affect the kinase activity of FAK measured by autophosphorylation or by synthetic substrate phosphorylation in parallel assays. In addition, Src activity was not detected in FAK immunoprecipitates using an optimal Src peptide substrate. Thus, agonist-induced tyrosine kinase activity measured in FAK immunoprecipitates is mediated by FAK activation rather than by co-immunoprecipitating Src. Bombesin-induced FAK activation is not dependent either on protein kinase C or Ca²⁺ mobilization but was completely blocked by treatment with cytochalasin D or by placing the cells in suspension. These findings indicate that FAK activation requires an intact actin cytoskeleton. Our results demonstrate that agonists that act via 7-transmembrane domain receptors stimulate FAK kinase activation.

Neuropeptides stimulate DNA synthesis and cell proliferation in cultured cells and are implicated as growth factors in a variety of fundamental processes including development, inflammation, tissue regeneration, and tumorigenesis (1, 2). In particular, bombesin and its mammalian counterpart gastrin-releasing peptide bind to a G-protein-coupled receptor (3, 4) that promotes Gₐ₆-mediated activation of β isomers of phospholipase C (5, 6) to produce second messengers: inositol 1,4,5-trisphosphate, that mobilizes Ca²⁺ from internal stores and diacylglycerol that activates protein kinase C (PKC) (7–9). Subsequently, bombesin induces activation of phosphorylation cascades including p42MAPK/P44MAPK and p70S6K (10–13) leading to increased expression of immediate early response genes, stimulation of cell cycle events, and subsequent cell proliferation (9, 14–17).

The binding of bombesin to its receptor also induces rapid tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells (9, 18, 19). Focal adhesion kinase (FAK) (20, 21) has been identified as a prominent tyrosine-phosphorylated protein in cells stimulated with bombesin (22, 23) and other neuropeptides (22, 24, 25). Tyrosine phosphorylation of FAK is also increased by diverse signaling molecules that mediate cell growth and differentiation, including bioactive lipids such as lysophosphatidic acid (LPA) and sphingosylphosphorylcholine (Sphingosylphosphorylcholine) (SPC) (26–28), polypeptide growth factors (29, 30), bacterial toxins (31, 32), activated variants of pp60src (33, 34), and extracellular matrix proteins (21, 35–37). These results indicate that FAK is a point of convergence in a variety of signal transduction pathways (38, 39). The importance of FAK-mediated signal transduction is underscored by recent experiments showing that this tyrosine kinase is implicated in embryonic development (40) and in the control of cell migration (41–43), proliferation (42, 44), and apoptosis (45–47).

While an increase in the tyrosine phosphorylation of FAK is recognized as an early event in the action of multiple agents, much less is known about the regulation of the tyrosine kinase activity of FAK in response to extracellular stimuli. Integrin engagement with fibronectin and v-Src transformation have been shown to increase FAK-associated tyrosine kinase activity in immunoprecipitates (33, 36). Subsequent studies, however, showed that these conditions also promote the formation of a complex between autophosphorylated FAK at Tyr-397 and the SH2 domain of Src (48–53). Consequently, it is not clear whether the increase in the kinase activity of FAK immune complexes is mediated by FAK or by co-immunoprecipitating Src. Furthermore, the effect of neuropeptide agonists or bioactive lipids on FAK-associated tyrosine kinase activity has not been examined.

In the present study we demonstrate that stimulation of Swiss 3T3 cells with bombesin, vasopressin, LPA, and SPC induces a rapid increase in FAK-associated tyrosine kinase activity. Our results show that this increase in FAK activity can be dissociated from the presence of Src kinase members in the FAK immunoprecipitates. Bombesin induces FAK activation through a PKC- and Ca²⁺-independent pathway that requires the integrity of the actin filament network.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DME), supplemented with 10% fetal bovine serum, in a humidified atmosphere containing 10% CO₂ and 90% air at 37 °C. For experimental purposes, Swiss 3T3
cells were plated in 35-mm Nunc Petri dishes at 105 cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent (14).

**Immunoprecipitation—**Quiescent cultures of Swiss 3T3 cells (1–2 × 107) were diluted twice with DMEM, equilibrated for 1 h at 37 °C for at least 15 min and then treated with peptide factors in 1 ml of DMEM for the times indicated. The stimulation was terminated by aspirating the medium and solubilizing the cells in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.35, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 1% Triton X-100, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM sodium orthovanadate, and 2 mM 2-mercaptoethanol). Lysates were clarified by centrifugation at 14,000 rpm for 10 min and the pellets were discarded. After centrifugation, supernatants were transferred to fresh tubes and proteins were immunoprecipitated at 4 °C for 3 h with either protein A-agarose linked polyclonal anti-FAK (C-20) or polyclonal anti-src family (SRC-2) antibodies or with protein G-agarose linked mAb directed against FAK (mAb 2A7) as described above. When C-20 polyclonal antibody was used, immunoprecipitates were washed three times with lysis buffer, extracted in 2 × SDS-PAGE sample buffer (200 mM Tris-HCl, pH 6.8, 0.1 mM sodium orthovanadate, 1 mM EDTA, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol), by boiling 5 min, fractionated by one-dimensional SDS-PAGE, and further analyzed as described under “Results” and in the figure legends. Immunoprecipitates obtained with C-20, 2A7, or SRC-2 were also used for in vitro kinase reactions.

**In Vitro Kinase Reactions—**FAK immunoprecipitates were washed and pelleted (2,500 rpm for 10 min in the cold) 3 times in lysis buffer. When C-20 polyclonal antibody was used, immunoprecipitates were washed twice with FAK kinase buffer A (20 mM Hepes, pH 7.35, 3 mM MnCl2). Pellets were dissolved in 40 μl of kinase buffer and reactions were started by adding 10 μl of [γ-32P]ATP. The reactions were carried out at 30 °C for 15 min, and stopped on ice by adding 10 mM EDTA. In some experiments poly(Glu-Tyr) (4:1) (40 μg) was added to the C-20 immunocomplex. The incorporation of 32P label into poly(Glu-Tyr) (4:1) was stopped by removing the supernatant from the agarose beads and adding 2 × SDS-PAGE sample buffer. Samples were then analyzed by SDS-PAGE and autoradiography.

When mAb 2A7 was used, immunoprecipitates were washed twice with FAK kinase buffer B (50 mM Hepes, pH 7.5, 0.1 mM EDTA, 0.01% Brij-35, 0.1% β-mercaptoethanol, 0.01% bovine serum albumin, 150 mM NaCl). These immunoprecipitates were then dissolved in 40 μl of kinase buffer and reactions were started by adding 20 μl of ATP mixture composed of 30 mM MgCl2, 30 μM cold ATP, pH 7.0, and 10 μl of [γ-32P]ATP. The reactions were carried out at 30 °C for 40 min and were stopped on ice by adding 10 mM EDTA. After the in vitro kinase reactions the pellet was washed twice in lysis buffer containing 5 mM EDTA, extracted for 5 min at 95 °C in 2 × SDS-PAGE sample buffer and analyzed by SDS-PAGE. After fixing and drying of the gels, autoradiography was performed at ~80 °C. Autoradiographs were scanned using an LKB Ultrascan XL densitometer and labeled bands were quantitated using Ultrascan XL internal integrator.

**Phosphorylation of Src Peptide and Raytide Substrates—**Phosphorylation of the highly specific Src peptide, p-Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Ala-Lys-Lys-Lys-Lys-NH2 (56), and RaytideTM substrate were performed as follows. Immunoprecipitates were washed three times with lysis buffer as above and twice with kinase assay buffer (50 mM Hepes, pH 7.5, 0.1 mM EDTA, 0.01% Brij-35) and resuspended in 20 μl of this buffer. Kinase reactions were initiated by the addition of 100 μM ATP, 10 mM MgCl2, and 2 μCi of [γ-32P]ATP, in the presence of 100 μM Src peptide or RaytideTM substrate, in a total volume of 30 μl. After incubation at 30 °C for 5 min (Src peptide) or 30 min (RaytideTM substrate), peptide phosphorylation was stopped by the addition of 120 μl of 10% phosphoric acid, and the reaction mixture was then applied onto P-81 ion exchange chromatography paper. Papers were washed five times in 0.5% phosphoric acid, once with acetone, dried, and counted in a scintillation counter.

**Western Blotting—**Treatment of quiescent cultures of cells with factors, cell lysis, and immunoprecipitations were performed as described above. After SDS-PAGE, proteins were transferred to Immobilon membranes for 2 h at 0.1 mA/cm2 in transfer buffer (192 mM glycine, 25 mM Tris base, 0.1% SDS, 0.06 mM sodium orthovanadate, and 0.05% Brij-35). After transfer, membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 2 h at 22 °C either with the anti-FAK mAb from Transduction Laboratories, or with the polyclonal anti-FAK C-20 antibody, both diluted 1:500 in phosphate-buffered saline containing 5% nonfat dried milk. After incubating membranes with secondary antibodies (horseradish peroxidase-conjugated goat antibodies to rabbit or mouse immunoglobulin), immunoreactive bands were visualized using ECL reagents.

**Phosphoamino Acid Analysis—**This was carried out according to Ref. 57.

**RESULTS**

**Bombesin Stimulation of FAK-associated Tyrosine Kinase Activity in Swiss 3T3 Cells—**To examine the effect of bombesin on FAK-associated tyrosine kinase activity, quiescent cultures of Swiss 3T3 cells were treated with or without 10 nM bombesin for 2.5 min and lysed. The lysates were incubated with the 2A7 mAb, which recognizes the C-terminal sequence of FAK (58). The resulting immune complexes were incubated with [γ-32P]ATP and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1A (2A7, upper) bombesin induced an increase in the phosphorylation of a 125-kDa band that exactly comigrated with immunoreactive FAK. In order to confirm that the radiolabeled 125-kDa band was FAK, the phosphorylated band was eluted by denaturation and incubated with a different anti-FAK mAb that recognizes an epitope in the kinase domain of FAK (corresponding to amino acids 354–533 of the chicken protein). The re-immunoprecipitation of the 125-kDa radiolabeled band (Fig. 1A, a-FAK) confirmed that it is phosphorylated FAK. Immunoblotting with anti-FAK mAb of anti-FAK immunoprecipitates prepared in parallel with those used for the assays of kinase activity verified that similar amounts of FAK were recovered after bombesin treatment (Fig. 1A, ip:2A7, wb:a-FAK).

An increase in FAK phosphorylation following in vitro kinase assays was also obtained when lysates of Swiss cells stimulated with bombesin were immunoprecipitated with C-20, a polyclonal antibody raised against a peptide corresponding to amino acids 1033–1052 mapping at the carboxyl terminus of human FAK (Fig. 1A, C-20, upper). Densitometric scanning showed that bombesin induced a 2.5 ± 0.5-fold (n = 15) and 2.3 ± 0.5-fold (n = 9) increase in the phosphorylation of FAK in immunoprecipitates obtained with 2A7 mAb and C-20 polyclonal antibody, respectively (Fig. 1A, lower panels). Phosphoamino acid analysis of the phosphorylated FAK band obtained after the in vitro kinase reaction showed that only tyrosine residues were phosphorylated (Fig. 1B).

An increase in the activity of FAK-associated kinase after bombesin treatment of intact Swiss 3T3 cells was also demonstrated when lysates of quiescent and bombesin-stimulated cells were immunoprecipitated with 2A7 mAb and kinase activity was determined by the ability of the immunoprecipitates to phosphorylate exogenously added substrates, including RaytideTM (1:3-fold stimulation, Fig. 1C), or poly(Glu-Tyr) (4:1) (not shown). In all cases, no kinase activity was detected when the lysates were immunoprecipitated with non-immune serum (results not shown). Bombesin, Vasopressin, LPA, and SPC Rapidly Increase FAK-associated Tyrosine Kinase Activity—An increase in FAK-associated tyrosine kinase in anti-FAK immunoprecipitates
was a rapid consequence of the addition of bombesin to intact Swiss 3T3 cells, reaching a maximum between 1 and 2.5 min of incubation (Fig. 2A). Immunoblotting with anti-FAK mAb of anti-FAK immunoprecipitates prepared in parallel with those used for the assays of kinase activity verified that similar amounts of FAK were recovered after different times of bombesin treatment (Fig. 2A). Bombesin induced FAK phosphorylation in a concentration-dependent manner reaching half-maximum and maximum effects at 0.05 and 1 nM, respectively (Fig. 2B).

To examine whether activation of other G-protein coupled receptors also increases FAK-associated tyrosine kinase activity, Swiss 3T3 cells were stimulated with 20 nM bombesin, 2 μM LPA, or 5 μM SPC for various times. As shown in Fig. 3 (A and B), vasopressin and LPA induced a rapid and sustained increase in the phosphorylation of FAK which reached a maximum 2 min after agonist stimulation. SPC also induced a very rapid increase in FAK-associated kinase activity, but it was transient rather than sustained (Fig. 3C). Western blotting with anti-FAK mAb of anti-FAK immunoprecipitates, carried out in parallel with those used for the assays of in vitro kinase activity, confirmed that similar amounts of FAK protein were recovered after vasopressin, LPA, and SPC treatment (Fig. 3).

Dissociation of FAK Kinase Activity from Src Kinases in Swiss Cells—Autophosphorylation of FAK at Tyr-397 either in v-Src transformed cells or in cells replated on fibronectin, creates a binding site for the SH2 domain of members of the Src family that leads to the formation of a signaling complex in which Src kinases are active (48–53). Consequently, the increase in tyrosine kinase activity measured in FAK immunocomplexes under these conditions could be due to co-immunoprecipitating Src rather than to FAK. Similarly, the increase in FAK-associated tyrosine kinase activity induced by bombesin and other agonists shown in the present study could be mediated by co-precipitating Src kinases rather than to FAK activation and autophosphorylation. Here, we provide several lines of evidence indicating that bombesin and other agonists induce FAK activation in Swiss 3T3 cells.

We found that lysis of Swiss 3T3 cells in a buffer solution containing 1% Triton (the composition of the lysis buffer is given under “Experimental Procedures”) rather than in modified RIPA buffer greatly diminished complex formation between FAK and Src family members, as judged by Western blot analysis with anti-FAK C-20 antibody of Src immunoprecipitates (results not shown). To examine further whether Src family members were present in FAK immunocomplexes under our experimental conditions, Triton lysates from control and bombesin-stimulated Swiss 3T3 cells were immunoprecipitated with the 2A7 mAb and the immunoprecipitates were incubated with [γ-32P]ATP and the synthetic peptide AEAEIY-GEFEAKKKK, which has been identified as a Src optimal peptide substrate (56). As shown in Fig. 4A, Src kinase activity was not detected in these FAK immunoprecipitates. In contrast, AEAEIYGEFEAKKKK phosphorylation was readily observed when the polyclonal anti-Src antibody SRC-2 was used to immunoprecipitate Src family members from lysates of parallel cell cultures treated with or without bombesin (Fig. 4A, right panel). The increase in Src kinase activity induced by...
FAK activity in Swiss 3T3 cells. A, quiescent and confluent cells were stimulated for the indicated times with 10 nM bombesin and lysed. The extracts were incubated with the C20 mAb and the FAK-associated tyrosine kinase activity in the resulting immunoprecipitates was measured by in vitro kinase reactions that were performed as described under "Experimental Procedures." Quantification of kinase activity was performed by densitometric scanning of FAK phosphorylation bands. The results shown are representative of five independent experiments and are expressed as fold-stimulation above control. Inset, a representative experiment is shown. Quiescent Swiss 3T3 cells were stimulated for the indicated times. Cells were lysed and half of the lysates were immunoprecipitated with the C20 polyclonal antibody and the resulting immunocomplexes were subjected to in vitro kinase reactions carried out as specified under "Experimental Procedures" (A-C, ivk). The other half of the lysates were also immunoprecipitated with C20 and analyzed by SDS-PAGE followed by transfer of proteins to Immobilon membranes and Western blotting with anti-FAK mAb (A-C, wb). The results shown are representative of three identical experiments. The position of FAK is indicated by an arrow.

B, confluent and quiescent cells were stimulated for the indicated times with 20 nM vasopressin (VAP, A), 2 μM LPA (B), and 5 μM SPC (C). Cells were lysed and half of the lysates were immunoprecipitated with the C20 polyclonal antibody and the resulting immunocomplexes were subjected to in vitro kinase reactions carried out as specified under "Experimental Procedures" (A-C, ivk). The other half of the lysates were also immunoprecipitated with C20 and analyzed by SDS-PAGE followed by transfer of proteins to Immobilon membranes and Western blotting with anti-FAK mAb (A-C, wb). The results shown are representative of three identical experiments. The position of FAK is indicated by an arrow.

Treatment with bombesin is in agreement with our recent results (55).

In order to verify that the increase in FAK activity in the in vitro kinase assays was not mediated by the presence of Src family members in the immunoprecipitates, we examined the effect of the pyrazolopyrimidine PP-1, a novel inhibitor of the Src family kinase members (59). Lysates of Swiss 3T3 cells treated with 10 nM bombesin were incubated with either C20 or SRC-2 to immunoprecipitate FAK and Src family members, respectively. Then, the immune complexes were incubated with [γ-32P]ATP with either the Src optimal peptide substrate or Raytide in the absence or in the presence of 1 μM PP-1, a concentration previously shown to inhibit Src kinase activity (59). Addition of PP-1 caused a marked inhibition of Src kinase activity (Fig. 4B). In striking contrast, PP-1 had only a slight effect on FAK kinase activity, as measured by the phosphorylation of Raytide (Fig. 4C).

To substantiate the differential inhibition of Src and FAK by PP-1 presented in Fig. 4, we examined the effect of increasing concentrations of this compound on the autophosphorylation of FAK and Src. As shown in Fig. 5A, addition of PP-1 caused a marked inhibition of immunoprecipitated Src autokinase activity. Densitometric scanning of these experiments show that PP-1 inhibited Src family kinase activity in a concentration-dependent manner; half-maximal inhibition was obtained at 0.09 μM PP-1 and almost complete inhibition of Src activity was achieved at a concentration of 1 μM (Fig. 5B). In contrast, PP-1 even at concentrations as high as 10 μM, had only a slight effect on FAK autophosphorylating activity (Fig. 5A and B).

Similar results were obtained in Swiss cells stimulated with LPA instead of bombesin (Fig. 5C). Addition of 1 μM PP-1 to the in vitro kinase reaction inhibited completely the stimulation of Src kinase activity induced by LPA, but it had only a slight effect on FAK activation induced by this agonist (Fig. 5C). These results indicate that PP-1 dissociates the increase in the kinase activity induced by the 7-transmembrane domain recep-
cent Swiss 3T3 cells were stimulated for the indicated times with 10 nM bombesin. In vitro kinase reactions were performed in the FAK immunoprecipitates in the presence of the highly specific Src substrate AEEEIYGEFEAKKKK. Phosphorylation of the Src substrate was carried out after a 5-min incubation with [γ-32P]ATP. In vitro kinase reactions were performed in the Src immunoprecipitates in the presence of the highly specific Src substrate AEEEIYGEFEAKKKK. Phosphorylation of the Src substrate was carried out after a 5-min incubation with [γ-32P]ATP as described under “Experimental Procedures.” The results shown are representative of three identical experiments. Right, parallel cultures of quiescent cells (−) or cells treated for 40 s with bombesin (Bom) were lysed. Anti-Src family immunoprecipitates were obtained with SRC-2 antibody and in vitro kinase reactions using AEEEIYGEFEAKKKK as a substrate were carried out as described previously. The results shown are representative of three identical experiments. B, quiescent and confluent Swiss 3T3 cells were stimulated with 10 nM bombesin (Bom) for 40 s to activate FAK family members. Cells were then lysed and Src kinases were immunoprecipitated with the anti-Src family antibody SRC-2. In vitro kinase reactions were performed in the Src immunoprecipitates in the presence of AEEEIYGEFEAKKKK. Phosphorylation of this highly specific Src substrate was carried out after a 5-min incubation with [γ-32P]ATP in the absence or presence of 1 μM PP-1. C, quiescent and confluent Swiss 3T3 cells were stimulated with 10 nM bombesin (Bom) for 2.5 min, to activate FAK. Cells were then lysed and FAK was immunoprecipitated with C-20 anti-FAK antibody. In vitro kinase reactions were performed in the FAK immunoprecipitates in the presence of Raytide. Phosphorylation of Raytide was carried out during a 30-min incubation with [γ-32P]ATP in the absence or presence of 1 μM PP-1.

FIG. 4. Effect of Src inhibitor on bombesin-stimulated activation of FAK and Src family members. A, left, confluent and quiescent Swiss 3T3 cells were stimulated for the indicated times with 10 nM bombesin. Cells were lysed and FAK was immunoprecipitated with the 2A7 mAb. In vitro kinase reactions were performed in the FAK immunoprecipitates in the presence of the highly specific Src substrate AEEEIYGEFEAKKKK. Phosphorylation of the Src substrate was carried out after a 5-min incubation with [γ-32P]ATP as described under “Experimental Procedures.” The results shown are representative of three identical experiments. Right, parallel cultures of quiescent cells (−) or cells treated for 40 s with bombesin (Bom) were lysed. Anti-Src family immunoprecipitates were obtained with SRC-2 antibody and in vitro kinase reactions using AEEEIYGEFEAKKKK as a substrate were carried out as described previously. The results shown are representative of three identical experiments.

Role of PKC and Ca2+ on Stimulation of FAK Activity—Next, we examined the signaling pathways leading to FAK activation in response to bombesin in Swiss 3T3 cells. As a rapid activation of PKC is a prominent early event elicited by bombesin in these cells, we examined the role of PKC in bombesin-stimulated FAK activation. Direct stimulation of PKC with PDBu for 2.5 min increased FAK autophosphorylation and PKC activity stimulated by PDBu was inhibited by pretreatment with the PKC inhibitor PP-1 (also known as GF 109203X), an inhibitor of PKC (60), prior to stimulation with either PDBu or bombesin. Treatment with GF 109203X abrogated PDBu-induced stimulation of immunoprecipitable PKC activity but did not prevent bombesin stimulation of PKC autophosphorylation (Fig. 6). Similarly, pretreatment with GF 109203X did not affect LPA stimulation of PKC autophosphorylation (not shown). These results indicated that activation of PKC could not account for

FIG. 5. PP-1 dissociates the increase in FAK phosphorylation induced by bombesin or LPA from the presence of Src kinase family members in the FAK immunoprecipitates. A, quiescent and confluent Swiss 3T3 cells were stimulated with 10 nM bombesin (Bom) for 2.5 min, to activate FAK, or for 40 s, to activate Src family members. Cells were then lysed and FAK and Src kinases were immunoprecipitated with the C-20 polyclonal antibody, and SRC-2 anti-Src family antibody, respectively. In vitro kinase reactions were performed as described under “Experimental Procedures” in the presence of the indicated concentrations of PP-1 inhibitor. The positions of FAK and Src family proteins are indicated by the arrows. B, quantification of the effect of PP-1 inhibitor on the kinase activity of FAK (open circles) and Src family kinases (closed circles) was determined by densitometric scanning. Values shown are the mean ± S.E. of five independent experiments, and are expressed as the percentage of inhibition with respect to the value of kinase activity in the absence of PP-1. C, quiescent and confluent Swiss 3T3 cells were stimulated with 2 μM LPA (LPA) for 2 min, to activate FAK, or for 40 s, to activate Src family members. Cells were then lysed and FAK and Src kinases were immunoprecipitated with C-20 anti-FAK antibody, and SRC-2 anti-Src family antibody, respectively. In vitro kinase reactions were performed as described under “Experimental Procedures” in the presence of 1 μM PP-1. The positions of FAK and Src family proteins are indicated by the arrows.
Bombesin-mediated Focal Adhesion Kinase Activation

The stimulation of FAK activity by bombesin or LPA.

To investigate whether an increase in intracellular Ca\(^{2+}\) mediates FAK activation by bombesin, quiescent cells Swiss 3T3 cells were treated with the tumor promoter thapsigargin. This agent specifically inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase and thereby depletes Ca\(^{2+}\) from intracellular compartments (61). Treatment with 30 nM thapsigargin for 30 min abolished the increase in cytosolic Ca\(^{2+}\) induced by subsequently added bombesin (results not shown), but did not block the increase in FAK kinase activity induced by bombesin (Fig. 6). In addition, treatment of the cells either with thapsigargin or the Ca\(^{2+}\)-ionophore A23187, at concentrations that induced a marked increase in cytosolic Ca\(^{2+}\) concentrations, had no effect on FAK autophosphorylation (not shown). These results indicate that bombesin stimulates FAK kinase activation through a signal transduction pathway that is independent of either PKC stimulation or Ca\(^{2+}\) mobilization.

Treatment with Cytochalasin D and Suspension of Cells Inhibit Bombesin-stimulated FAK Activity—Given the localization of FAK to focal adhesions which form at the end of actin stress fibers (20, 21), we examined whether the stimulation of FAK activity induced by bombesin depends on the integrity of the actin cytoskeleton. Quiescent cultures of Swiss 3T3 cells were treated for 2 h with cytochalasin D at 2.5 \(\mu M\), conditions known to depolymerize the actin cytoskeleton in these cells (23). The cultures, treated with or without cytochalasin D, were subsequently stimulated with bombesin. As illustrated by Fig. 7 (upper panel), treatment with cytochalasin D completely blocked the increase in FAK activity in response to bombesin.

To examine whether the stimulation of kinase activity of FAK by vasopressin and SPC was also dependent on the integr...
ivity of the actin cytoskeleton, quiescent Swiss 3T3 cells were treated with or without 2.5 μM cytochalasin D, as above, and subsequently stimulated with these agonists. Treatment with cytochalasin D also blocked FAK activation in response to either vasopressin or SPC (Fig. 7B).

To further substantiate the results obtained with cytochalasin D, we determined whether bombesin induces FAK activation in Swiss 3T3 cells placed in suspension, a condition that causes disruption of the actin filament network, disassembly of focal adhesion plaques, and dephosphorylation of FAK. Quiescent cultures of Swiss 3T3 cells were gently detached, left in suspension for 30 min, and subsequently stimulated with bombesin. As shown in Fig. 7C, FAK activation in response to bombesin was completely blocked in suspended cells. Taken together, these results indicate that bombesin-mediated FAK activation depends on the integrity of the actin cytoskeleton.

**DISCUSSION**

An increase in the tyrosine phosphorylation of the non-receptor tyrosine kinase FAK has been extensively documented as an early event in the action of multiple agonists that modulate cell growth, differentiation, and motility in a variety of cell types. In contrast, little is known about the regulation of FAK activity in response to these agents. The results presented here demonstrate that bombesin rapidly increases FAK-associated tyrosine kinase activity in Swiss 3T3 cells. Other agonists that act via 7-transmembrane domain receptors including vasopressin, LPA, and SPC also elicited a rapid increase in FAK-associated kinase activity in these cells.

Previous studies demonstrated that integrin engagement or v-Src transformation not only induces FAK tyrosine phosphorylation but also enhances kinase activity of FAK immunoprecipitates (33, 36). However, subsequent studies from several laboratories demonstrated that the major site of FAK auto-phosphorylation (i.e. Tyr-397) is a high-affinity binding site for the SH2 domain of members of the Src kinase family. Association of Src to phosphorylated FAK has been demonstrated in v-Src transformed cells and in cells replated on fibronectin (48, 50, 51, 53). Consequently, the increase in kinase activity measured in FAK immunoprecipitates in previous studies could be due to the presence of co-precipitating Src rather than to FAK activation. The agonist-mediated increases in FAK-associated kinase demonstrated in the present study could also reflect the presence of Src in the immunoprecipitates rather than an increase in the activity of FAK. Assays of FAK phosphorylation, as performed here do not exclude this possibility because active Src has been shown to phosphorylate FAK at multiple sites in vitro (62, 63). Consequently, it was important to elucidate whether increases in the tyrosine kinase activity detected in FAK immunoprecipitates were due to FAK activation or reflected co-precipitating Src.

To determine the contribution of Src kinases to the activity of FAK immunoprecipitates, we used PP-1, a novel inhibitor of Src kinase family members, in the *in vitro* phosphorylation assay. This compound inhibits Src kinases at nanomolar concentrations but does not affect other tyrosine kinases such as Zap70 or JAK-1 (59). Our results demonstrate that PP-1 does not inhibit FAK-associated tyrosine kinase activity but, at similar concentrations, virtually abolishes bombesin-stimulated Src kinase activity measured in parallel Src immunoprecipitates. Furthermore, under the conditions of cell lysis used here (i.e. Triton only buffer) we could not detect Src kinase activity in FAK immunoprecipitates from bombesin-stimulated Swiss 3T3 cells. Our results dissociate the increase in FAK-associated tyrosine kinase induced by bombesin from the presence of Src kinases in the immunoprecipitates. We conclude that neuropeptides and bioactive lipids not only elevate FAK tyrosine phosphorylation but also stimulate FAK kinase activation.

Bombesin is known to stimulate the rapid hydrolysis of inositol phospholipids to generate the second messengers diacylglycerol and inositol 1,4,5-trisphosphate which activate PKC and mobilize Ca\(^{2+}\), respectively. The results presented here demonstrate that direct activation of PKC by treatment of intact cells with biologically active phorbol esters increases FAK tyrosine kinase activity and imply that PKC stimulation is a potential signaling pathway leading to FAK kinase activation. However, our results also show that bombesin rapidly stimulates FAK activation through a signal transduction pathway that is independent of PKC.

In view of the rapid kinetics of neuropeptide-stimulated Ca\(^{2+}\) mobilization and FAK kinase activation, we also tested whether Ca\(^{2+}\) could be responsible for the effect of bombesin on FAK activation. We found that neither Ca\(^{2+}\)-ionophore nor thapsigargin caused a Ca\(^{2+}\)-dependent increase in FAK kinase activity. Furthermore, depletion of the intracellular Ca\(^{2+}\) pool by treating cells with thapsigargin did not prevent bombesin-induced FAK stimulation. We conclude that increases in the cytoplasmic Ca\(^{2+}\) concentration do not mediate bombesin-induced FAK kinase activation. Thus, neither of the two major signals generated by activation of phospholipase C is responsible for neuropeptide stimulation of FAK kinase activation. These findings distinguish the regulation of FAK activity by G protein-coupled receptor agonists from that of the FAK homologue Pyk2/CAKb/FAFTK/CaDTK, which is believed to be a Ca\(^{2+}\)-regulated tyrosine kinase (64, 65).

Agonist-mediated increase in FAK tyrosine phosphorylation is accompanied by profound alterations in the organization of the actin cytoskeleton and in the assembly of focal adhesions (26, 27, 29, 66–68), the distinct areas of the plasma membrane where FAK is localized (20, 21). Treatment of the cells with cytochalasin D, which disrupts actin filaments, prevents the increase in FAK tyrosine phosphorylation in response to multiple agents, suggesting a mechanism involving the actin cytoskeleton and the focal adhesion plaques (23, 27–29, 31, 32, 55). The small G protein p21\(^{Rho}\), a member of the Ras superfamily of small GTP-binding proteins (68), has been implicated in mitogen-stimulated formation of focal adhesions and actin stress fibers as well as in the tyrosine phosphorylation of FAK (28, 66, 69, 70). It is thought that translocation of FAK into nascent focal adhesions would induce its activation and auto-phosphorylation, as a result of clustering and/or conformational change (39, 71).

Here we show that cytochalasin D, which disrupts the organization of the actin cytoskeleton, profoundly inhibits FAK activation induced by bombesin and other agonists. Previous studies demonstrated that treatment with cytochalasin D does not inhibit production of inositol phosphates, Ca\(^{2+}\) mobilization, and stimulation of PKC, Src, and p42MAPK/p44MAPK activation (23, 27, 44, 54, 55), indicating that disruption of the actin cytoskeleton prevents FAK activation in a selective manner. Furthermore, bombesin also failed to induce FAK activation in Swiss 3T3 cells placed in suspension, a condition that also causes disruption of the actin cytoskeleton and disassembly of focal adhesion plaques (55) but does not interfere with bombesin receptor signaling, as judged by assays of Ca\(^{2+}\) mobilization (9, 23, 72), p42MAPK activation (44), and Src kinase activation (55). These findings are consistent with a model in which FAK activation, like tyrosine phosphorylation, requires the recruitment of FAK into intact focal adhesion plaques.

In conclusion, our results demonstrate that stimulation of intact cells with bombesin, vasopressin, LPA, or SPC induces a rapid increase in the tyrosine kinase activity of FAK. Our studies demonstrate that the increase in FAK activity can be
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dissociated from the presence of Src kinase family members in the immunoprecipitates by the use of PP-1, a compound that potently inhibited Src but not FAK. Bombesin induces FAK activation, like tyrosine phosphorylation, through a PKC- and Ca²⁺-independent pathway that is critically dependent on the integrity of the focal adhesions and the actin cytoskeleton.

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