Treatment of quiescent Swiss 3T3 cells with bombesin induces a rapid (40 s) and transient increase in the kinase activity of the Src family of tyrosine kinases, as determined by autophosphorylation in immune complex kinase assays (4.6 ± 0.2-fold stimulation, n = 44) and phosphorylation of exogenous substrates. Phorbol 12,13-dibutyrate increased the activity of Src family kinases with similar kinetics but was less effective than bombesin. However, Src family kinase activation by bombesin is not dependent either on protein kinase C or Ca2⁺. Bombesin stimulation of Src family kinase activity could also be dissociated from p125 focal adhesion kinase tyrosine phosphorylation. Neither treatment with cytochalasin D nor placement of the cells in suspension prevented the stimulation of Src family kinase activity induced by bombesin, but both abolished bombesin-induced tyrosine phosphorylation of p125 focal adhesion kinase. The stimulation of the Src family kinase activity by bombesin was completely prevented by treatment with vanadate, a potent inhibitor of protein-tyrosine phosphatases. Bradykinin and vasopressin also stimulated Src family kinase activity transiently, and this stimulation was also inhibited by vanadate. Our results dissect two separate pathways that lead to protein tyrosine phosphorylation in neuropeptide-stimulated Swiss 3T3 cells.

Tyrosine phosphorylation has recently been implicated in the intracellular signaling of neuropeptides that act as potent cellular growth factors through receptors with seven transmembrane helices (1–10). Bombesin and other mitogenic neuropeptides stimulate tyrosine phosphorylation of multiple proteins in Swiss 3T3 cells, a useful model system for the elucidation of signal transduction pathways leading to cell proliferation (11). The focal adhesion-associated protein p125FAK is a cytosolic tyrosine kinase that lacks Src homology domains 2 and 3 (12, 13), has been identified as a prominent tyrosine-phosphorylated protein stimulated by neuropeptides in Swiss 3T3 cells (8, 9, 14). Bombesin stimulates p125FAK tyrosine phosphorylation through a PKC- and Ca²⁺-independent pathway that requires the integrity of the actin filament network and functional p21rho (14, 15). A variety of signals that modulate cell growth and differentiation, including extracellular matrix proteins, polypeptide growth factors, bioactive lipids, and bacterial toxins (12, 13, 16–21), also induce tyrosine phosphorylation of p125FAK. Recent gene disruption experiments indicate a role for p125FAK in focal adhesion turnover and cell locomotion (22), but the precise downstream targets of p125FAK remain unclear.

It has been hypothesized that p125FAK activation and tyrosine autophosphorylation create a high affinity binding site for the SH2 domain of members of the Src kinase family, which could therefore play a role as signal transducers of tyrosine-phosphorylated p125FAK. The kinase activity of Src family kinase members (such as Src, Yes, and Fyn) is repressed when a key tyrosine residue in the carboxyl-terminal region (corresponding to Tyr-527 of the chicken protein) is phosphorylated by Csk (23, 24). Phosphorylation at Tyr-527 creates a binding site for the Src SH2 domain and allows an intramolecular interaction that locks the Src kinase domain in an inactive conformation (25–27). Two mechanisms that may “unlock” and activate Src family members are currently considered. In one case, dephosphorylation of Tyr-527 by a tyrosine phosphatase may destabilize the complex, releasing the SH2 domain and thereby activating the kinase activity (28–30). An alternative mechanism, involving competition for the SH2 domain of Src by a high affinity allosteric ligand, would also lead to enzymatic activation of this kinase. In this context, autophosphorylation of p125FAK at Tyr-527 appears to create a putative competing binding site for the SH2 domain of Src and thus would lead to the formation of a signaling complex in which Src kinases are active (27, 31–33). These considerations prompted us to examine the effect of bombesin and other neuropeptides on the activity of Src family kinases.

In the present study we demonstrate that bombesin induces a very rapid and transient stimulation of Src family members in Swiss 3T3 cells. Our results show that the activation of Src family kinases can be dissociated from p125FAK tyrosine phosphorylation and imply that at least two separate pathways lead to tyrosine phosphorylation in bombesin-stimulated cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM, supplemented with 10% fetal bovine serum, in a humidified atmosphere containing 10% CO₂ and 90% air at 37°C. For experimental purposes, 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 (34).

**Immunoprecipitation**—Quiescent cultures of Swiss 3T3 cells (four dishes for each experimental condition, 4 × 10⁵ cells/dish) were washed
twice with DMEM, equilibrated in the same medium at 37°C for at least 1 h. The cells were washed with 1 ml of DMEM for the times indicated. The stimulation was terminated by aspiration of medium and solubilizing the cells in 1 ml of ice-cold lysis buffer (10 mM Tris/HCl, 7.35, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM Na3VO4, 1% Triton X-100, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM phenylmethylsulfonfluoride). Lysates were clarified by centrifugation at 14,000 rpm for 10 min and the pellets, in which we did not detect any Src protein, were discarded. After this step, the lysates were preincubated with anti-mouse IgG-agarose or protein A-agarose and normal rabbit serum for 1 h at 4°C. After centrifugation, supernatants were transferred to fresh tubes, and proteins were immunoprecipitated at 4°C for 3–4 h with protein A-agarose-linked anti-mouse IgG antibody or with anti-mouse IgG-agarose-linked mAb directed against p125FAK (mAb 2A7) or anti-Tyr(P) proteins (mAb PY72) as described previously (8, 35, 36). Immunoprecipitates were washed three times with lysis buffer, extracted in 2 × SDS-PAGE sample buffer (200 mM Tris/HCl, 0.1 mM Na3VO4, 1 mM EDTA, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 8.6) by boiling 5 min, fractionated by one-dimensional SDS-PAGE, and further analyzed as described under "Results" and "in vitro" kinase reactions.

Src Family Kinase Autophosphorylation—Src family kinase immunoprecipitates were washed and pelleted (2,500 rpm, 10 min in the cold) three times in lysis buffer and two times in Src kinase buffer (20 mM Hepes, pH 7.35, 7.5, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 30 mM NaF, 20% methanol). After transfer, membranes were blocked using 5% nonfat dried milk as indicated. After incubating with secondary antibodies (horseradish peroxidase-conjugated goat antibodies to rabbit or mouse immunoglobulin), immunoreactive bands were visualized using ECL reagents.

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 polyvinylidene difluoride Immobilon membranes for 2 h at 400 mA in transfer buffer (192 mM glycine, 25 mM Tris base, 0.075% SDS, 6.6 mM Na3VO4, and 20% methanol). 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 with Src-2 antibodies (1:3000 dilution), the monoclonal anti-p125FAK antibody (Transduction Laboratories, 1:500), or the monoclonal anti-pTyr(P) antibody (PY20 and 4G10; 1:500). Blots were washed three times with lysis buffer as above and twice with a buffer solution containing 50 mM Hepes, pH 7.5, 0.1 mM EDTA, and 0.01% Brij and resuspended in 20 μl of this buffer. Kinase reactions were initiated by the addition of 100 μM of ATP, 10 mM MgCl2, and 2 μCi of (γ-32)P]-ATP in the presence of 100 μg Src kinase in a total volume of 30 μl. After incubation at 30°C for 5 min, 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, washed once with acetone, dried, and then counted in a scintillation counter.

Results

Bombesin Stimulates Kinase Activity of Src Family Members in Swiss 3T3 Fibroblasts—To examine the effect of bombesin on the kinase activity of Src family members, quiescent cultures of Swiss 3T3 cells were treated with 10 nM bombesin for various times and lysed. The lysates were incubated with the polyclonal SRC-2 antibody, which recognizes the C-terminal sequence (residues 509–533) of the family members Src, Yes, Blk and NCK, enolase phosphatase, and agarose-linked anti-mouse IgG were obtained from Sigma (St. Louis, MO), Enzo Life Sciences (Farmingdale, NY), and Calbiochem. The PKC inhibitor GF109203X, thapsigargin, and Ca2+ ionophore A23187 were obtained from Calbiochem. Src family affinity-purified antipeptide polyclonal antibody (SRC-2) and its cognate blocking peptide were from Santa Cruz Biotechnology Inc. mAb 2A7 directed against p125FAK and 4G10 anti-Tyr(P) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p125FAK monoclonal antibody, used in Western blotting, was from Transduction Laboratories (Lexington, KY). PY20 anti-Tyr(P) antibody was from ICN. Anti-Tyr(P) mAb clone PY72 was obtained from the Hybridoma Development Unit, Imperial Cancer Research Fund (London, UK). All other reagents used were of the purest grade available.

RESULTS

FIG. 1. Time course of bombesin-stimulated Src family kinase activity in Swiss 3T3 cells. A, quiescent and confluent Swiss 3T3 cells were stimulated for the indicated times with 10 nM bombesin. Cell lysis and immunoprecipitation with SRC-2 antibody were performed as described under "Experimental Procedures." Protein kinase assays in immune complexes were performed by a 15-min incubation with (γ-32)P]-ATP (top). Immunoprecipitates were also analyzed by SDS-PAGE, followed by transfer of proteins to Immobilon membranes and Western blotting with SRC-2 antibodies (bottom). The position of Src family proteins is indicated by an arrow. The broad band under the Src band is immunoglobulin heavy chain. B, quantification of kinase activity was determined by densitometric scanning of Src kinase family autophosphorylation bands. Values shown are the mean ± S.E. of five independent experiments (except the value corresponding to the point at 40 s, which is the mean ± S.E. of 4 independent determinations) and are expressed as -fold stimulation above control. C, enolase phosphorylation time course. Cell lysis and SRC-2 immunoprecipitation were performed as described above. Western blotting of SRC-2 kinase activity was carried out as described. Kinase assays in the presence of the highly specific Src substrate were performed as described under "Experimental Procedures." The results shown are representative of four independent experiments. 2A7 directed against p125FAK and 4G10 anti-Tyr(P) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p125FAK monoclonal antibody, used in Western blotting, was from Transduction Laboratories (Lexington, KY). PY20 anti-Tyr(P) antibody was from ICN. Anti-Tyr(P) mAb clone PY72 was obtained from the Hybridoma Development Unit, Imperial Cancer Research Fund (London, UK). All other reagents used were of the purest grade available.

Materials—Bombesin, vasopressin, bradykinin, PDB, TPA, methylionophore A23187 were obtained from Calbiochem. Src family proteins are expressed as -fold stimulation above control. C, enolase phosphorylation time course. Cell lysis and SRC-2 immunoprecipitation were performed as described above. Western blotting of SRC-2 kinase activity was carried out as described. Kinase assays in the presence of the highly specific Src substrate were performed as described under "Experimental Procedures." The results shown are representative of four independent experiments. 2A7 directed against p125FAK and 4G10 anti-Tyr(P) antibodies were obtained from Sigma (St. Louis, MO), Enzo Life Sciences (Farmingdale, NY), and Calbiochem. The PKC inhibitor GF109203X, thapsigargin, and Ca2+ ionophore A23187 were obtained from Calbiochem. Src family affinity-purified antipeptide polyclonal antibody (SRC-2) and its cognate blocking peptide were from Santa Cruz Biotechnology Inc. mAb 2A7 directed against p125FAK and 4G10 anti-Tyr(P) antibodies were...
were recovered after different times of bombesin treatment (Fig. 1A, lower panel). Densitometric scanning showed that the autophosphorylation of the Src family kinases was markedly increased as early as 20 s after the addition of bombesin to intact cells, reached a maximum of 4.6 ± 0.2-fold (mean ± S.E., n = 44) between 30 and 40 s, and returned to almost baseline levels by 2.5 min (Fig. 1B). No kinase activity was detected when the SRC-2 antibody was preincubated with the immunizing peptide prior to the addition to the lysates or when the lysates were immunoprecipitated with nonimmune serum (results not shown).

A rapid increase in the activity of Src family members after bombesin treatment of intact Swiss 3T3 cells was also demonstrated when the immunoprecipitable kinase activity was determined at the earliest time point examined (20 s) after bombesin stimulation (Fig. 1, C and D).

Role of PKC and Ca2+ in Bombesin-stimulated Src Family Kinase Activity—A rapid activation of PKC is a prominent early event elicited by bombesin in Swiss 3T3 cells (44–46). To determine whether direct activation of PKC increases the kinase activity of Src family members, quiescent cultures of Swiss 3T3 cells were treated with PDB for various times. As shown in Fig. 2A, PDB induced a rapid and transient increase in the autophosphorylation of Src family members. An increase in Src kinase activity was detected 20 s after the addition of PDB to intact cells, peaked after 40 s, and was no longer evident 2.5 min after PDB stimulation (Fig. 2A). An increase in Src family activity in response to PDB was also demonstrated when the immunoprecipitable kinase activity was determined by its ability to phosphorylate the optimal Src substrate (not shown). TPA, added for 40 s to intact cells, also increased the Src family kinase activity, whereas the biologically inactive methyl-TPA or phorbol had no effect on Src family kinase activity (Fig. 2B).

The results presented in Fig. 2, A and B, suggested that PKC activation could provide a potential mechanism by which bombesin stimulates Src family members. Consequently, we examined whether PKC was required for stimulation of Src family members in response to bombesin. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with or without 3.5 μM of bisindolylmaleimide GF 109203X, a selective inhibitor of PKC (47, 48) prior to stimulation with either PDB or bombesin for 40 s. As shown in Fig. 2C, treatment of the cells with GF 109203X prevented PDB-induced stimulation of Src family kinase activity. In contrast, this inhibitor did not affect bombesin stimulation of Src family kinase activation (Fig. 2C). Similar results were obtained when the immunoprecipitable Src family kinase activity was measured by phosphorylation of the optimal Src substrate (not shown).

The results shown above indicated that activation of PKC could not account for the stimulation of Src family activity by bombesin. To investigate whether an increase in intracellular Ca2+ mediates Src family activation by bombesin, quiescent Swiss 3T3 cells were treated with the tumor promoter thapsigargin. This agent specifically inhibits the endoplasmic reticulum Ca2+ -ATPase and thereby depletes Ca2+ from intracellular compartments (49). Treatment with 30 nM thapsigargin for 30 min abolished the increase in cytosolic Ca2+ induced by subsequently added bombesin (results not shown) but did not block the Src stimulation induced by bombesin or PDB (Fig. 2D).

Pretreatment with a combination of 30 nM thapsigargin and 3.5 μM GF 109203X did not prevent bombesin-induced Src family activation (results not shown). In addition, treatment of the cells for 40 s with either thapsigargin or the Ca2+ ionophore A23187, at concentrations that induced a marked increase in the cytosolic Ca2+ concentration in Swiss 3T3 cells (14), had no effect on Src family activity (Fig. 2D).

These results indicate that bombesin stimulates Src family kinase activation through a signal transduction pathway that is independent of PKC stimulation and Ca2+ mobilization.

Bombesin-stimulated Src Family Kinase Activity Is Dissociable from p125FAK Tyrosine Phosphorylation—Recently it has been proposed that tyrosine-phosphorylated p125FAK plays a role in promoting allosteric activation of Src family kinases (27, 31, 39). Since bombesin increases Src kinase activity and tyrosine-phosphorylation of p125FAK through a PKC and Ca2+-independent pathway in Swiss 3T3 cells, we tested whether p125FAK tyrosine phosphorylation is directly involved in Src family kinase activation by bombesin.

Bombesin-stimulated tyrosine phosphorylation of p125FAK depends on the integrity of the actin filament network (14, 17). If tyrosine-phosphorylated p125FAK lies upstream of Src kinases in a linear signal transduction pathway, treatment with cytochalasin D should abolish the activation of Src kinases induced by bombesin. To test this possibility, quiescent cul-

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**Fig. 2. Role of PKC and Ca2+ in bombesin-induced Src family kinase activation.** A, quiescent Swiss 3T3 cells were treated for the indicated times with 800 nM PDB. Cells were lysed and immunoprecipitated with SRC-2 antibody, and kinase reactions were carried out as specified under “Experimental Procedures.” The position of Src family kinases is indicated by an arrow. B, cells were treated for 40 s with 800 nM PDB, 400 nM TPA, or 400 nM phorbol, or for 400 nM methyl-TPA (MeTPA). Cells were lysed and immunoprecipitated with SRC-2 antibody, and kinase reactions were carried out as specified under “Experimental Procedures.” Quantification of kinase activity was carried out by densitometric scanning of autophosphorylated bands. Values shown are the mean ± S.E. of five independent experiments, expressed as -fold stimulation above control. C (top), quiescent Swiss 3T3 cells were pretreated in DMEM containing either the selective inhibitor of PKC GF 109203X (GF) or 3.5 μM GF 109203X (GF) for 1 h. Cells were then challenged for 40 s with bombesin (B) or PDB (P). The position of Src family proteins is indicated by an arrow. C (bottom), quantification of kinase activity was performed by densitometric scanning of the Src family autophosphorylated bands. Values shown are the mean ± S.E. of four independent experiments and are expressed as -fold stimulation above control. D (left), quiescent Swiss 3T3 cells were pretreated in the presence of either solvent MeSO (DMSO) or 30 μM thapsigargin (TG) for 30 min. Cells were then challenged for 40 s either with 10 nM bombesin (B) or 800 nM PDB (P). The results shown are representative of three independent experiments. The position of Src family kinases is indicated by an arrow. D (right), parallel culture cells were treated for 40 s with 30 μM thapsigargin (TG), 100 μM A23187 (A23187), or an equal volume of MeSO. The results shown are representative of three identical experiments. The position of Src family kinases is indicated by an arrow.
Fig. 3. Cytochalasin D prevents p125FAK tyrosine phosphorylation but does not block Src family kinase activation in response to bombesin. A, quiescent Swiss 3T3 cells were treated for 2 h either in DMEM containing either MeSO4 (DMSO) (-) or 2.5 μM of cytochalasin D (Cyt D) (+) and challenged by the indicated times with 10 nM bombesin (Bom). Cells were lysed, and the lysates were incubated with mAb 2A7 for 3 h. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to Immobilon membranes and Western blotting with anti-TyrP125 (mAb upper gel) or with a monoclonal anti-p125FAK antibody (lower gel). The results shown are representative of three identical experiments. The position of p125FAK (FAK) is indicated by an arrow. B, quiescent Swiss 3T3 cells were pretreated for 2 h in the presence of either MeSO4 (DMSO) or 2.5 μM cytochalasin D (Cyt D) (as in A). After this period, cells were challenged for 40 s with either solvent (−) or 10 nM bombesin (+). Cells were lysed and immunoprecipitated with SRC-2 antibody, and in vitro kinase autophosphorylation reactions were carried out as described under “Experimental Procedures.” Cyt D, a representative autoradiogram. The position of Src family kinases (Src) is indicated by an arrow. B (bottom), in vitro kinase autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify peak areas. Values shown, corresponding to the phosphorylation of Src family kinases from cells pretreated with either MeSO4 or cytochalasin D (Cyt D) and exposed to bombesin (closed bars) or solvent (open bars) are the mean ± S.E. of four independent experiments and are expressed as -fold stimulation with respect to control quiescent cells in DMEM containing the solvent MeSO4.

Fig. 4. Suspension of Swiss 3T3 cells prevents p125FAK tyrosine phosphorylation but does not block Src family kinase activation in response to bombesin. A, quiescent and confluent Swiss 3T3 cells were left on the dishes (attached), or they were detached from the plastic dishes gently with a rubber policeman, and an equal number of cells were incubated for 30 min in serum-free DMEM (susp). After this period both attached and suspended cells were challenged with 10 nM bombesin for the indicated times. Cells were lysed, and the lysates were incubated with mAb 2A7 for 3 h. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to Immobilon membranes and Western blotting with anti-TyrP125 (mAb upper gel) or with a monoclonal anti-p125FAK antibody (lower gel). The position of p125FAK (FAK) is indicated by an arrow. The results shown are representative of three identical experiments. B, quiescent Swiss 3T3 cells either attached (attached) or suspended for 30 min as before (susp) were challenged with 10 nM bombesin (Bom) for 40 s. Cells were lysed and immunoprecipitated with SRC-2 antibody, and in vitro kinase reactions were carried out as specified under “Experimental Procedures.” Cyt D (top), a representative autoradiogram. The position of Src family kinases (Src) is indicated by an arrow. B (bottom), in vitro kinase autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify peak areas. Values shown, corresponding to the phosphorylation of Src family kinases from cells either attached (attached) or suspended (susp) and stimulated with bombesin (closed bars) or solvent (open bars), are the mean ± S.E. of four independent experiments and are expressed as -fold stimulation above control.

To further substantiate the results obtained with cytochalasin D, we examined whether bombesin induces Src family 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 p125FAK. Quiescent cultures of Swiss 3T3 cells were gently detached, left in suspension for 30 min, and subsequently stimulated with bombesin. As shown in Fig. 4A, p125FAK tyrosine phosphorylation in response to bombesin was completely blocked in suspended cells (Fig. 4A). In striking contrast, bombesin still induced Src family kinase activation in suspended cells (Fig. 4B).

Taken together, the results presented in Figs. 3 and 4 indicate that bombesin-mediated activation of Src family kinases is independent of p125FAK tyrosine phosphorylation. Vanadate Inhibits Bombesin-mediated Stimulation of Src Family Kinases—A mechanism that could account for bombesin stimulation of Src family kinase activity independent of p125FAK tyrosine phosphorylation would involve the activation of a tyrosine phosphatase that dephosphorylates Tyr-527 of Src or a similar Tyr at the carboxyl terminus of other family members (50), leading to the activation of these kinases (28–30). In order to test this possibility, Swiss 3T3 cells were treated for 4 h with different concentrations of sodium orthovanadate (Na3V04), a potent inhibitor of phosphotyrosine-specific protein phosphatases (51, 52) and subsequently challenged with bombesin for 40 s. As shown in Fig. 5, A and B, Na3V04 inhibited the increase in Src family kinase autophosphorylation induced by bombesin in a dose-dependent manner. At 60 μM, Na3V04 completely blocked bombesin-mediated stimulation of Src family kinases. It is known that hydrogen peroxide (H2O2) potentiates the ability of vanadate to inhibit tyrosine phosphatases (53, 54). Treatment of the cells for 1 h with an equimolar mixture of...
Fig. 5. Vanadate inhibits Src family kinase stimulation induced by bombesin. A, quiescent Swiss 3T3 cells were either pretreated for 4 h with Na₃VO₄ (Van) at the indicated concentrations or for 1 h with either 12 μM Na₃VO₄ and 12 μM H₂O₂ (Van/H₂O₂) or with 0.5 μM okadaic acid (OKD). After this period, cells were incubated for 40 s without (−) or with (+) 10 nM bombesin (Bom). Cells were lysed and immunoprecipitated with SRC-2 antibody, and in vitro kinase reactions were carried out as specified under “Experimental Procedures.” The results shown are representative of four identical experiments. The position of Src family proteins is indicated by an arrowhead. B, autoradiograms corresponding to kinase reactions of immunoprecipitates obtained from cells pretreated with Na₃VO₄ (Van) or Na₃VO₄ and H₂O₂ (Van/H₂O₂) as in A were scanned with an LKB Ultrascan XL densitometer to quantify peak areas. Values are expressed as the percentage of the maximum stimulation above control unstimulated cells and are representative of five independent experiments. C, vanadate blocked Src family kinase activity at various times after bombesin stimulation and did not inhibit tyrosine phosphorylation of p125FAK. Quiescent Swiss 3T3 cells preincubated in the absence (−) or presence (+) of a mixture of 12 μM Na₃VO₄ and 12 μM H₂O₂ (Van/H₂O₂) as in A were stimulated with 10 nM bombesin for the indicated times. Cells were lysed, half of the lyse was immunoprecipitated with SRC-2 antibody, and in vitro kinase reactions were carried out as specified under “Experimental Procedures” (C, upper autoradiogram). The other half of the lysate was immunoprecipitated with mAb 2A7 for 3 h. These p125FAK immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to Immobilon membranes and Western blotting with anti-Tyr(P) mAb. The results shown are representative of three identical experiments. The positions of p125FAK (FAK) and Src family kinases are indicated with arrowheads.

12 μM Na₃VO₄ and 12 μM H₂O₂ also inhibited bombesin-induced stimulation of Src family kinases (Fig. 5, A and B). In contrast, okadaic acid, a specific inhibitor of serine/threonine phosphatases (55), did not inhibit the activation of Src family kinases induced by bombesin. In fact, it had a slight stimulatory effect on Src kinase activity (Fig. 5A).

Treatement with 12 μM Na₃VO₄/H₂O₂ for 60 min markedly inhibited the stimulation of Src family kinase activity observed after different time periods of bombesin stimulation (Fig. 5C). Similar results were obtained when 60 μM Na₃VO₄ was used instead of 12 μM Na₃VO₄/H₂O₂ (not shown). The results obtained in the autophosphorylation studies with Na₃VO₄/H₂O₂ were confirmed by studying the ability of the immunoprecipitable Src family kinase activity to phosphorylate the highly specific Src substrate. Pretreatment of the cells with 12 μM Na₃VO₄/H₂O₂ for 60 min markedly attenuated the increase in the phosphorylation of the Src peptide induced by bombesin (not shown).

It was important to establish that the inhibitory effect of Na₃VO₄ on Src family activation induced by bombesin was specific. Pretreatment with Na₃VO₄ did not inhibit bombesin stimulation of PKC-mediated 80-kDa/myristoylated alanine-rich protein kinase C substrate phosphorylation (results not shown). We also examined the effect of Na₃VO₄ or Na₃VO₄/H₂O₂ treatment on the stimulation of p125FAK tyrosine phosphorylation induced by bombesin. As shown in Fig. 5C, these tyrosine phosphatase inhibitors did not prevent bombesin-induced tyrosine phosphorylation of p125FAK, again indicating a clear dissociation between the stimulation of Src family kinase activity and p125FAK tyrosine phosphorylation in bombesin-treated cells.

Vasopressin and Bradykinin Stimulate Kinase Activity of Src Family Members in Swiss 3T3 Fibroblasts—To examine whether activation of other G-protein-coupled neuropeptide receptors also induces Src family kinase activation, quiescent cultures of Swiss 3T3 cells were treated with either 20 nM vasopressin or 20 nM bradykinin for various times. As shown in Fig. 6, A and B, vasopressin and bradykinin induced a rapid and transient increase in the autophosphorylation of Src family members. The maximum increase in the Src kinase activity was reached 60 s after neuropeptide stimulation (Fig. 6, A and B).
In agreement with the results obtained with bombesin, disruption of the microfilament network by pretreatment of the cells with 2.5 μM cytochalasin D for 2 h did not inhibit the stimulation of Src family kinases by either vasopressin or bradykinin, again dissociating Src family kinase activation from p125FAK tyrosine phosphorylation (results not shown).

Bombesin induced Src kinase activation through a vanadate-sensitive signal transduction pathway. To examine if this is the case with other neuropeptides, quiescent Swiss 3T3 cells were treated for 1 h with 12 μM Na3V04 and 12 μM H2O2, and they were subsequently stimulated with vasopressin and bradykinin. As shown in Fig. 6, C and D, treatment with Na3V04/H2O2 profoundly inhibited Src family kinase activation induced by either vasopressin or bradykinin in Swiss 3T3 cells.

**DISCUSSION**

The results presented here demonstrate that bombesin induces a rapid and transient increase in the activity of Src family tyrosine kinases in Swiss 3T3 cells as judged by autophosphorylation assays or by phosphorylation of exogenous substrates. The increase in Src kinase activity is one of the earliest events induced by bombesin in Swiss 3T3 cells.

Bombesin is known to induce the rapid hydrolysis of inositol phospholipids to generate the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which activate PKC and mobilize Ca++ respectively. The results presented here demonstrate that direct activation of PKC by biologically active phorbol esters stimulates the activity of Src family kinase members in a rapid and transient fashion. These results contrast with an earlier report in which TPA failed to increase Src kinase activity (56). However, in the previous study Src kinase activity was measured 10 min after phorbol ester addition, whereas the peak of Src family kinase activation by PDB that we observed occurred at 30–40 s, and the activation was no longer evident after 2.5 min of stimulation. To our knowledge, this is the first time that phorbol esters have been shown to induce rapid Src family kinase activation. These results indicate that stimulation of PKC in intact cells is a potential signaling pathway leading to Src family kinase activation. It seemed plausible, therefore, that PKC could mediate bombesin stimulation of Src family kinase members. However, our results indicate that bombesin rapidly stimulates Src family kinase activation through a signal transduction pathway that is independent of PKC.

In view of the rapid and transient kinetics of neuropeptide-stimulated Ca++ mobilization and Src family kinase activation, we tested whether Ca++ is responsible for the effect of bombesin on Src family kinase activation. Our results indicate that increases in the cytoplasmic Ca++ concentration do not mediate bombesin-induced Src kinase activation. Thus, the two major signals generated by activation of phospholipase C are not responsible for neuropeptide stimulation of Src family kinases.

The activity of Src is normally inhibited in vivo by extensive phosphorylation of Tyr-527, resulting in an intramolecular interaction between phosphorylated Tyr-527 and the SH2 domain (the close conformation). The activity of other members of the Src family is also down-regulated by phosphorylation of a corresponding carboxyl-terminal tyrosine residue (50). Deletion of Tyr-527 (as in v-Src), mutation of this residue to phenylalanine, or targeting of the gene encoding Csk disrupts this intramolecular interaction and leads to the enzymatic activation of Src kinase (24–26). Allosteric effectors that interfere with the binding of the SH2 domain to Tyr-527 also activate Src. The sustained activation of Src family members (41, 56) induced by platelet-derived growth factor is thought to act through this mechanism (27). In this context it has also been proposed that tyrosine-phosphorylated p125FAK could also play a physiological role in promoting allosteric activation of members of the Src family (27, 31–33). Given that bombesin induces a marked increase in tyrosine phosphorylation of p125FAK, also through a Ca++ and PKC-independent pathway, it was important to determine whether the activation of Src observed in this study was mediated by tyrosine-phosphorylated p125FAK.

We previously demonstrated that bombesin-induced p125FAK tyrosine phosphorylation is completely blocked by treatment with cytochalasin D, which disrupts the actin filament network and the assembly of focal adhesion plaques (14, 17, 36). Here we show that cytochalasin D, at concentrations that profoundly inhibited p125FAK tyrosine phosphorylation, does not impair the striking increase in Src family kinase activity induced by bombesin. Furthermore, bombesin also induces Src kinase family activation in Swiss 3T3 cells placed in suspension, a condition that also prevents the tyrosine phosphorylation of p125FAK induced by bombesin. In addition, we did not detect p125FAK in Src immunoprecipitates. Our findings indicate that Src family kinase activation can be dissociated from p125FAK tyrosine phosphorylation in bombesin-treated Swiss 3T3 cells and demonstrate, for the first time, that two distinct signal transduction pathways lead to protein tyrosine phosphorylation in bombesin-stimulated Swiss 3T3 cells.

Recently, it has become evident that tyrosine phosphatases not only act as antagonists of tyrosine kinases, but also can participate positively in cellular signaling (29, 57). Specifically, the negative regulation of Src family kinases by phosphorylation of Tyr-527 can be opposed by a cellular tyrosine phosphatase(s) (28, 58, 59). Thus, stimulation of a tyrosine phosphatase(s) provides a putative mechanism leading to Src family kinase activation independent of p125FAK tyrosine phosphorylation in bombesin-treated cells. Here we demonstrate that treatment of Swiss 3T3 cells with Na3V04 or Na3V04/H2O2, well known inhibitors of tyrosine phosphatases (51–54), prevents Src family kinase activation induced by bombesin. We also found that the neuropeptides vasopressin and bradykinin also induce transient Src family kinase activation through a vanadate-sensitive pathway. These results suggest the involvement of a tyrosine phosphatase in Src family kinase activation induced by neuropeptides and further dissociate this response from p125FAK tyrosine phosphorylation. Interestingly, recent evidence has suggested the existence of G-protein-activated tyrosine phosphatases (60, 61). In view of our results, it will be of interest to examine whether the bombesin, vasopressin, and bradykinin receptors are coupled to a G protein that stimulates tyrosine phosphatases and thereby induces Src kinase activation.

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Note: The reference number 7 is incorrect; it is likely a typographical error as it is not found in the list of references.
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