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Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation in intact Swiss 3T3 cells

(signal transduction/neuropeptides/growth control/protein phosphorylation)

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ABSTRACT **The mitogenic neuropeptides bombesin and vasopressin markedly increased tyrosine and serine phosphorylation of multiple substrates in quiescent Swiss 3T3 fibroblasts, including two major bands of M_r 90,000 and 115,000. Tyrosine phosphorylation of these proteins was increased as judged by immunoprecipitation of $^{32}P_i$-labeled cells and immunoblotting of unlabeled cells with monoclonal anti-phosphotyrosine antibodies, elution with phenyl phosphate, and phospho amino acid analysis. Phosphotyrosyl proteins generated by bombesin and vasopressin did not correspond either by apparent molecular weight or by immunological and biochemical criteria to several known tyrosine kinase substrates, including phospholipase Cγ, the microtubule-associated protein 2 kinase, GTPase-activating protein, or phosphatidylinositol kinase. The effect was rapid (within seconds), concentration dependent, and inhibited by specific receptor antagonists for both bombesin and vasopressin. The endothelin-related peptide, vasoactive intestinal contractor, also elicited a rapid and concentration-dependent tyrosine/serine phosphorylation of a similar set of substrates. These results demonstrate that neuropeptides, acting through receptors linked to GTP-binding proteins, stimulate tyrosine phosphorylation of a common set of substrates in quiescent Swiss 3T3 cells and suggest the existence of an additional signal transduction pathway in neuropeptide-induced mitogenesis.**

Neuropeptides are increasingly recognized to act as cellular growth factors and have been implicated in a variety of normal and abnormal biological processes, including embryogenesis, tissue regeneration, and tumorigenesis (1). A number of neuropeptides, including bombesin, vasopressin, and the endothelin-related peptides, are potent mitogens for Swiss 3T3 cells (2–5). These peptides bind to their distinct receptors (6, 7) and trigger a common set of early signaling events in these cells, including rapid production of inositol 1,4,5-trisphosphate, mobilization of Ca^{2+} from intracellular stores, and sustained activation of protein kinase C (8, 9).

Bombesin and vasopressin initiate early biochemical events and DNA synthesis through GTP-binding protein (G protein) signal transduction pathways (10–17). Thus, receptor-mediated responses to both these peptides in permeabilized cells (10, 11) and ligand affinity in membrane and solubilized receptor preparations (12–16) are modulated by guanine nucleotides, indicating a close functional association with a G protein(s). Recently, the bombesin receptor has been cloned from Swiss 3T3 cells and shown to belong to the family of seven hydrophobic-domain, G-protein-linked neuropeptide receptors (17). In contrast to mitogenic neuropeptides, growth factors such as platelet-derived growth factor (PDGF) signal through receptors endowed with intrinsic tyrosine kinase activity (18). Tyrosine phosphorylation is fundamental to the signal transduction process for this class of mitogen (18, 19), and intense interest has recently focused on the identity of cellular targets for the receptor tyrosine kinases (20–26). It is not known, however, whether tyrosine phosphorylation plays a role in the mitogenic action of G-protein transducing peptides.

In the present study, we demonstrate that the mitogenic neuropeptides, bombesin, vasopressin, and the endothelin-related peptide, vasoactive intestinal contractor (VIC), stimulate rapid tyrosine and serine phosphorylation of multiple substrates in quiescent Swiss 3T3 cells. Furthermore, we show that these proteins are unrelated to previously identified tyrosine kinase substrates and may therefore represent additional targets for neuropeptide-stimulated tyrosine/serine kinase(s).

MATERIALS AND METHODS

Cell culture of Swiss 3T3 cells, phospho amino acid analysis, and assays of phosphatidylinositol (PI) kinase activity were performed as described (2, 27, 28).

Immunoprecipitations. Quiescent cultures of Swiss 3T3 cells were incubated in phosphate-free Dulbecco's modified Eagle's medium containing 100–500 μCi of carrier-free $^{32}P_i$ per ml (1 Ci = 37 GBq) at 37°C for 16 h to label the endogenous ATP pool. After addition of peptide factors, cell lysis and immunoprecipitation either of phosphotyrosyl proteins using a monoclonal anti-phosphotyrosine [anti-Tyr(*P*)] antibody coupled to agarose (29), or of GTPase-activating protein (GAP) with a polyclonal affinity-purified antibody to GAP (J. Downward and P. Warne, personal communication) were performed as described (30). Immunoprecipitates were analyzed by one-dimensional polyacrylamide gel electrophoresis (SDS/PAGE) with 7.5% acrylamide. In some experiments, immunoprecipitated phosphotyrosyl proteins were eluted from the matrix with 50 mM phenyl phosphate for 15 min at 4°C prior to SDS/PAGE.

Western Blot Analysis. Anti-Tyr(*P*) immunoprecipitates of unlabeled cells were fractionated by SDS/PAGE, transferred to Immobilon membranes according to the manufacturer's instructions (Millipore), and immunoblotted using the Py20 anti-Tyr(*P*) monoclonal antibody as described (31). Immunoreactive bands were detected by using ^{125}I-labeled sheep anti-mouse IgG followed by autoradiography.

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Abbreviations: GAP, GTPase-activating protein; GRP, gastrin-releasing peptide; PI, phosphatidylinositol; PDGF, platelet-derived growth factor; VIC, vasoactive intestinal contractor; G protein, GTP-binding protein.

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Materials. Bombesin, vasopressin, albumin-agarose, PI, and PI phosphate were obtained from Sigma. Endothelin-1 and VIC were from Peninsula Laboratories. Agarose-linked anti-Tyr(*P*) monoclonal antibody was purchased from Oncogene Sciences (Mineola, NY) and Py20 anti-Tyr(*P*) antibody was from ICN. Carrier-free $^{32}P_i$ (10 mCi/ml), [γ-^{32}P]ATP (5000 Ci/mmol), ^{125}I-labeled sheep anti-mouse IgG (15 μCi/μg), and recombinant PDGF (BB homodimer) were from Amersham. Anti-GAP antibody was raised against a fragment of GAP comprising amino acid residues 169–449 and was the generous gift of Julian Downward (Imperial Cancer Research Fund). All other reagents used were of the purest grade available.

RESULTS

Bombesin and Vasopressin Stimulate Tyrosine Phosphorylation of Multiple Substrates in Intact Swiss 3T3 Cells. Quiescent cultures of Swiss 3T3 cells metabolically labeled with ^{32}P were treated with bombesin and lysed, and phosphotyrosyl proteins were then immunoprecipitated with an anti-Tyr(*P*) monoclonal antibody. Analysis of immunoprecipitates from bombesin-treated cells consistently showed an increase in the phosphorylation of two major components migrating with apparent molecular weights (M_r) of 115,000 (p115) and 90,000 (p90), as well as additional bands of M_r 81,000 and 75,000 (Fig. 1*A*). Other peptides of the bombesin family, including gastrin-releasing peptide (GRP) at 20 nM, neuromedin B at 50 nM, and GRP-(14–27) at 20 nM, also stimulated phosphorylation of the same bands (results not shown). In contrast, the biologically inactive fragment of GRP [GRP-(1–16)] had no effect on phosphorylation. Vasopressin produced an identical pattern of phosphorylated bands in anti-Tyr(*P*) immunoprecipitates of $^{32}P_i$-labeled Swiss 3T3 cells, although it was consistently weaker than bombesin in stimulating phosphorylation (Fig. 1*A*). Immunoprecipitation of lysates with a different monoclonal anti-Tyr(*P*) antibody (Py20) produced results identical to those shown in Fig. 1.

Immunoprecipitation of phosphorylated components from lysates of Swiss 3T3 cells treated with either bombesin (Fig. 1*B*) or vasopressin (Fig. 1*C*) was specifically blocked by an excess of competing phosphotyrosine, but not by either phosphothreonine or phosphoserine. Furthermore, the phosphorylated bands induced by bombesin and vasopressin were specifically eluted from the anti-Tyr(*P*)/agarose matrix by the competing hapten phenyl phosphate (Fig. 1*D*).

Phospho Amino Acid Analysis and Immunoblotting. The increase in phosphotyrosine content of the phosphoproteins generated in response to bombesin and vasopressin was determined directly by phospho amino acid analysis of the $^{32}P_i$-labeled bands. Bombesin and vasopressin markedly increased the phosphotyrosine content of p90 (5- to 7-fold) and p115 (3- to 5-fold). The phosphoserine content of both bands was also strikingly increased (3- to 7-fold). Representative autoradiograms of up to four independent experiments are shown in Fig. 2*A*. Phosphotyrosine represented a considerably larger proportion of total phospho amino acids in p115 (25–30%) than in p90 (8–14%). Bombesin and vasopressin also increased the phosphoserine and phosphotyrosine content of the M_r 81,000 band (results not shown).

Neuropeptide-induced tyrosine phosphorylation was assessed independently of metabolic labeling by Western blotting anti-Tyr(*P*) immunoprecipitates of unlabeled cells using a different anti-Tyr(*P*) antibody. As shown in Fig. 2*B*, bombesin and vasopressin both increased the amount of phosphotyrosine in a major band of M_r 115,000 (≈4-fold), as well as bands of M_r 81,000 and 75,000, in Western blots of anti-Tyr(*P*) immunoprecipitates. The neuropeptides also increased phosphotyrosine in the M_r 90,000 band (3- to 4-fold) but was detected more weakly by the anti-Tyr(*P*) antibody. Similar results were obtained when cells were rapidly extracted and analyzed by direct Western blotting without first immunoprecipitating phosphotyrosyl proteins (results not shown). The components detected in Western blots exactly comigrated with the bands found in immunoprecipitates of parallel $^{32}P_i$-labeled cells.

Relationship of Neuropeptide-Induced Phosphotyrosyl Proteins to GAP and PI Kinase. Several cellular proteins have recently been identified as substrates for the PDGF and epidermal growth factor receptor tyrosine kinases, including phospholipase Cγ (20), PI kinase (21, 22), GAP (23–25), and microtubule-associated protein 2 kinase (26). Analysis of anti-Tyr(*P*) immunoprecipitates of $^{32}P_i$-labeled Swiss 3T3 cells treated with PDGF showed increased labeling of several components including the PDGF receptor; bands of M_r 150,000, 110,000, and 85,000; and bands comigrating with the M_r 115,000 and 81,000 bands induced by bombesin; PDGF also increased the level of a M_r 42,000 band in immunoblots of anti-Tyr(*P*) immunoprecipitates (results not shown). The

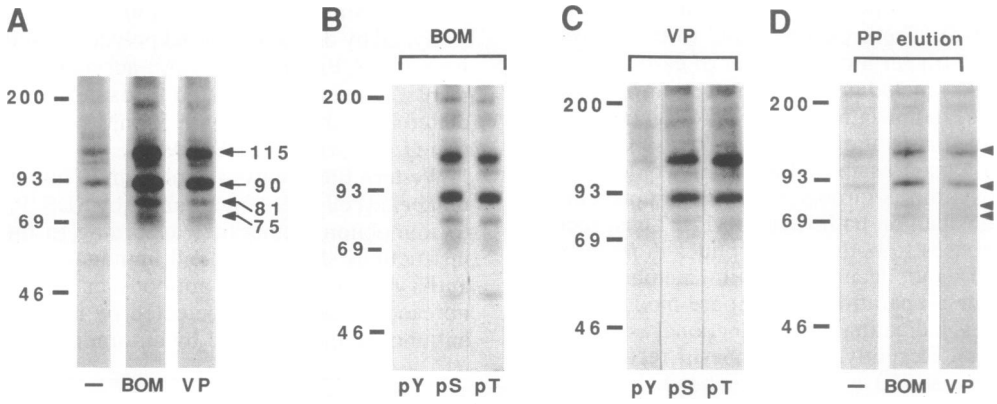

FIG. 1. Stimulation of tyrosine phosphorylation by bombesin and vasopressin in intact Swiss 3T3 cells. Confluent and quiescent Swiss 3T3 cells were labeled with $^{32}P_i$ and incubated for 10 min with either solvent (lanes −), 6 nM bombesin (lanes BOM) (*A*, *B*, and *D*), or 20 nM vasopressin (lanes VP) (*A*, *C*, and *D*). The cells were lysed, and the lysates were immunoprecipitated with an anti-Tyr(*P*) monoclonal antibody; immunoprecipitates were analyzed by SDS/PAGE (*A* and *D*). (*B* and *C*) Immunoprecipitation of bombesin- (*B*) and vasopressin- (*C*) treated cell lysates with anti-Tyr(*P*) was also performed in the presence of either 4 mM phosphotyrosine (lanes pY), 4 mM phosphothreonine (lanes pT), or 4 mM phosphoserine (lanes pS). (*D*) Phosphotyrosyl proteins were eluted from anti-Tyr(*P*) immunoprecipitates of bombesin- and vasopressin-treated cells with 50 nM phenyl phosphate (PP). Arrowheads indicate the positions of the p115, p90, and M_r 81,000 and 75,000 protein bands. Other experimental details were as described in *Materials and Methods*. In this and subsequent figures, representative autoradiograms are shown of experiments repeated 3–10 times.

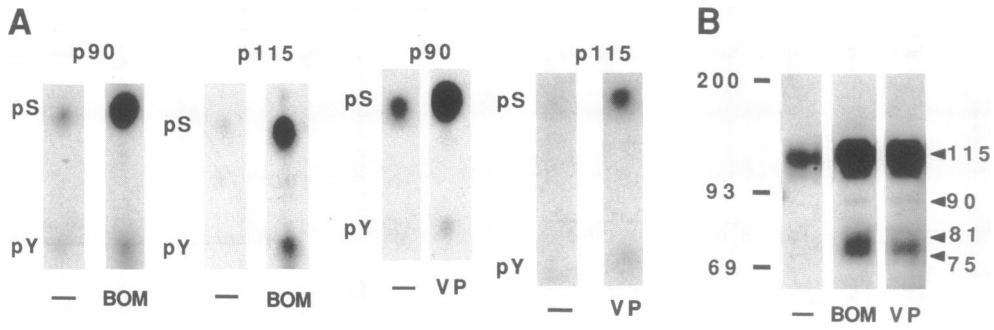

FIG. 2. Phospho amino acid and immunoblot analysis of the M_r 115,000 and 90,000 phosphotyrosyl proteins induced by bombesin and vasopressin. (*A*) Quiescent cells labeled with $^{32}P_i$ were treated for 10 min with either 6 nM bombesin (lanes BOM) or 20 nM vasopressin (lanes VP), or an equivalent volume of solvent (lanes −). Cell lysis, anti-Tyr(*P*) immunoprecipitation, and SDS/PAGE were performed as described in Fig. 1. After autoradiographic localization and excision of the p90 and p115 bands, phospho amino acid analysis was performed as described in *Materials and Methods*. Autoradiograms were scanned with an LKB Ultroscan XL densitometer to quantify phospho amino acids or phosphoproteins in terms of peak area. Bombesin increased the phosphotyrosine content of p90 by 7.0 ± 1.4-fold (n = 4) and that of p115 by 5.4 ± 1.8-fold (n = 4). The increases in phosphotyrosine induced by vasopressin were 4.8 ± 0.8-fold for p90 (n = 3) and 3.0 ± 0.1-fold for p115 (n = 2). (*B*) Quiescent, unlabeled cultures were treated for 10 min with either 6 nM bombesin (lane BOM) or 20 nM vasopressin (lane VP) or with an equivalent volume of solvent (lane −). The cells were then lysed, and immunoprecipitation and SDS/PAGE were performed. After transfer to Immobilon membranes, phosphotyrosyl proteins were immunoblotted with Py20 monoclonal anti-Tyr(*P*) antibody and immunoreactive bands were visualized with ^{125}I-labeled sheep anti-mouse IgG followed by autoradiography. Transfer of all major phosphorylated bands was equally efficient as shown by transfer of $^{32}P_i$-labeled bands to Immobilon. The positions of p115, p90, and bands of M_r 81,000 and 75,000 are indicated by arrowheads. Bombesin increased the immunoreactivity of p90 by 3.6 ± 0.6-fold (n = 5) and that of p115 by 4.1 ± 0.7-fold (n = 6). Corresponding increases for vasopressin were 2.8 ± 0.4-fold (n = 3) and 3.5 ± 0.6-fold (n = 5). No other neuropeptide-stimulated bands were detected below the molecular weights shown. Other experimental details are as described in *Materials and Methods*.

similarity in molecular weights between p115 and GAP (M_r 120,000), and between both p90 and p115 and M_r 81,000–85,000 and 110,000 components of the purified PI kinase (32), suggested that these proteins might be more closely related. In accord with data from other cell lines, PDGF specifically increased phosphorylation of a M_r 120,000 band in anti-GAP immunoprecipitates of $^{32}P_i$-labeled Swiss 3T3 cells (Fig. 3*A*). Likewise, PI kinase activity was markedly increased in anti-Tyr(*P*) immunoprecipitates of PDGF-treated Swiss 3T3 cells (Fig. 3*B*). In contrast, neither GAP phosphorylation nor PI kinase activity was stimulated by bombesin or vasopressin (Fig. 3). Similarly, neither bombesin nor vasopressin increased the labeling of bands comigrating with either phospholipase Cγ (M_r 150,000) or microtubule-associated protein 2 kinase (M_r 42,000) in immunoprecipitates of labeled cells or immunoblots (Figs. 1 and 2).

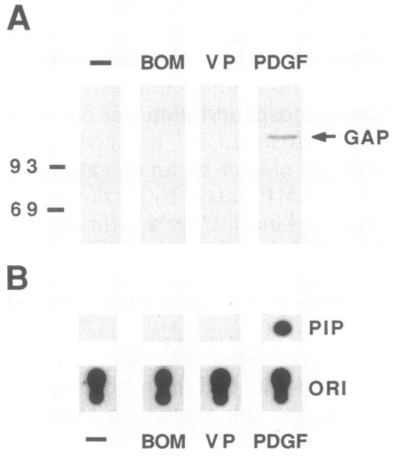

FIG. 3. Effect of bombesin and vasopressin on GAP phosphorylation and PI kinase activity. (*A*) Quiescent, $^{32}P_i$-labeled Swiss 3T3 cells were treated for 10 min with either 6 nM bombesin (lane BOM), 20 nM vasopressin (lane VP), recombinant PDGF (25 ng/ml), or with an equivalent volume of solvent (lane −); lysed; and immunoprecipitated with an affinity-purified anti-GAP antibody as described. (*B*) Quiescent, unlabeled cultures were treated as described in *A*, lysed, and immunoprecipitated with anti-Tyr(*P*). PI kinase activity in the immunoprecipitates was assayed as described. The origin (ORI) and the position of the PI phosphate (PIP) standard are indicated.

PDGF and epidermal growth factor stimulate a rapid autophosphorylation of their receptors in Swiss 3T3 cell membranes (results not shown). In contrast, bombesin failed to stimulate tyrosine phosphorylation in the same membrane preparations in which the bombesin receptor remains functional, as judged by binding activity and modulation of binding by guanine nucleotides. Similarly, vasopressin also had no effect on phosphorylation in the same membranes (results not shown). In addition, the M_r 75,000–85,000 cell-surface receptor for bombesin (9, 12, 33), which had been affinity-labeled with ^{125}I-GRP, was not immunoprecipitated by anti-Tyr(*P*) antibody. These results indicate that stimulation of tyrosine phosphorylation by bombesin and vasopressin is not a consequence of receptor-associated kinase activity.

Concentration Dependence, Receptor Specificity, and Time Course. Bombesin stimulated the labeling of p115 and p90 in a concentration-dependent manner (Fig. 4*A*). Half-maximal effect was obtained at 0.3 nM, and the maximum stimulation was achieved at 2 nM. Very similar results were obtained when the increase in tyrosine phosphorylation was monitored by immunoblotting anti-Tyr(*P*) immunoprecipitates from unlabeled cells (Fig. 4*B*). Furthermore, the specific bombesin antagonist [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin prevented the labeling of the three components induced by bombesin in a competitive manner (Fig. 4*C*). Vasopressin-stimulated tyrosine phosphorylation was also concentration dependent with half-maximal and maximum effects at 1 and 5 nM, respectively (Fig. 4*D*). The effect of vasopressin was blocked by a specific V_1 receptor vasopressin antagonist (Fig. 4*D*). We verified that [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin did not block vasopressin-stimulated phosphorylation and, reciprocally, that the pressor-type vasopressin antagonist had no effect on the response to bombesin (results not shown). The broad spectrum neuropeptide antagonist [D-Arg^1,D-Trp^{7,9},Leu^{11}]substance P (4) inhibited both bombesin- and vasopressin-induced tyrosine phosphorylation. Thus, the increase in tyrosine phosphorylation induced by bombesin and vasopressin is mediated through the same receptors previously shown to mediate mitogenic responsiveness to these peptides.

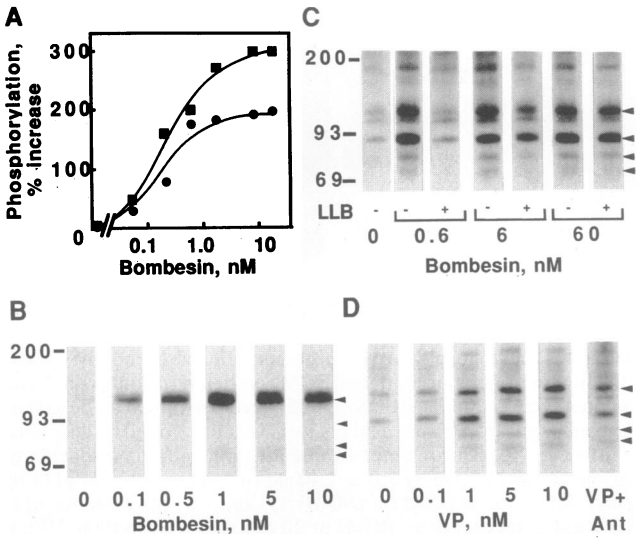

FIG. 4. Concentration dependence and receptor specificity of bombesin- and vasopressin-stimulated tyrosine phosphorylation. (*A*) Quiescent, $^{32}P_i$-labeled cells were challenged for 10 min with the concentration of bombesin indicated and then lysed and immunoprecipitated with anti-Tyr(*P*). Autoradiograms were scanned as described in the legend to Fig. 2*A* and values correspond to the phosphorylation of p90 (■) and p115 (●) expressed as percentage increase above the control, unstimulated values. (*B*) Immunoprecipitates were prepared from lysates of unlabeled cells that had been treated with the indicated concentrations of bombesin. After transfer to Immobilon, the immunoprecipitates were Western blotted with Py20 as described. Identical results were obtained when immunoblotting was performed after phenyl phosphate elution of phosphotyrosyl proteins. (*C*) Cells were labeled with $^{32}P_i$ and treated for 10 min with various concentrations of bombesin, as indicated, either in the absence (lanes −) or presence (lanes +) of the specific bombesin antagonist [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin (LLB) at 1 μM. (*D*) Cells were labeled with $^{32}P_i$ and incubated for 10 min with the indicated concentrations of vasopressin. Parallel cells were treated with 10 nM vasopressin in the presence of 1 μM [1-(2-mercapto-2,2-cyclopentamethylenepropionic acid)-*O*-Me-Tyr^2,Arg^8]vasopressin, a specific anti-vasopressor antagonist (VP + Ant). Cell lysis and immunoprecipitation with anti-Tyr(*P*) were performed as described.

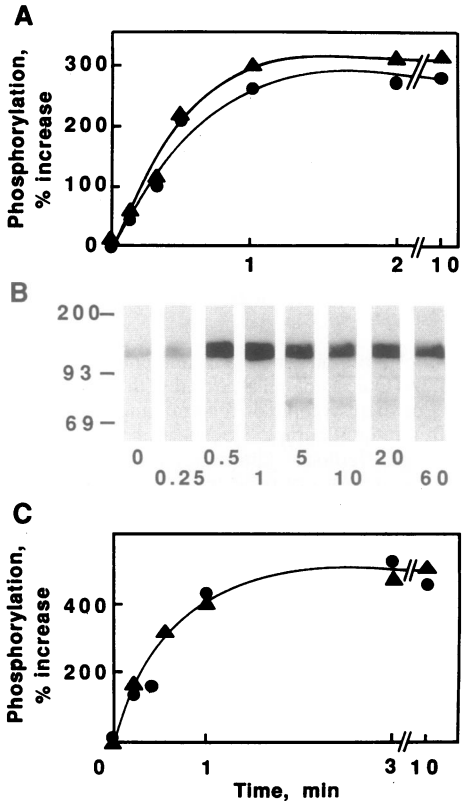

FIG. 5. Time course of bombesin- and vasopressin-stimulated phosphorylation of p90 and p115. $^{32}P_i$-labeled (*A* and *C*) or unlabeled (*B*) Swiss 3T3 cells were treated either with 6 nM bombesin (*A* and *B*) or with 20 nM vasopressin (*C*) for different times as indicated, lysed, and immunoprecipitated as described above. Immunoprecipitates prepared from unlabeled, bombesin-treated cells were eluted with 50 mM phenyl phosphate and immunoblotted with Py20 (*B*) as described. Autoradiograms were scanned and phosphorylation of p90 (▲) and p115 (●) is expressed as percentage increase above control values.

The stimulation of phosphorylation of p115 and p90 is one of the earliest events in the action of bombesin and vasopressin in quiescent 3T3 cells. An increase in tyrosine phosphorylation, as shown either by immunoprecipitation from lysates of ^{32}P-labeled cells (Fig. 5 *A* and *C*) or by Western blotting (Fig. 5*B*), was detected as early as 20 sec after addition of the peptides, reached a maximum after 60 sec, and persisted for up to 1 h thereafter.

Effect of Endothelin-Related Peptides and Other Agents. Recently, we reported that endothelin 1 and the closely related peptide VIC bind to specific, high-affinity receptors in Swiss 3T3 cells and stimulate mobilization of intracellular Ca^{2+} and DNA synthesis (5). As shown in Fig. 6*A*, VIC, like bombesin and vasopressin, caused a striking increase in phosphorylation of p115 and p90 and bands of M_r 81,000 and 75,000 in anti-Tyr(*P*) immunoprecipitates of Swiss 3T3 cells. All the bands generated by VIC comigrated in one-dimensional SDS/PAGE with the bands phosphorylated in response to bombesin and vasopressin. Endothelin 1 at 25 nM elicited an identical response. VIC-stimulated phosphorylation was concentration dependent, with a maximum effect at 10 nM, and extremely rapid: phosphorylation was detectable within seconds and reached a maximum after 1 min (Fig. 6*B*). VIC also generated a pattern of labeled bands similar to that found for bombesin and vasopressin, either in immunoblots of anti-Tyr(*P*) immunoprecipitates (Fig. 6*C*) or in direct immunoblots of VIC-treated cells (results not shown). Phospho amino acid analysis confirmed that VIC increased the phosphotyrosine and phosphoserine content of both p115 and p90.

In contrast to bombesin, vasopressin and VIC, neuropeptides that have no receptors on Swiss 3T3 cells including galanin and angiotensin II (4), did not increase phosphorylation in anti-Tyr(*P*) immunoprecipitates (results not shown). Likewise, tyrosine phosphorylation was not stimulated either by an increase in cytosolic Ca^{2+} induced by the Ca^{2+} ionophore A23187 at 100 nM, or by an elevation in the intracellular level of cyclic AMP caused by forskolin (25 μM). Direct activation of protein kinase C by a saturating concentration (100 nM) of phorbol 12,13-dibutyrate increased the labeling of a similar set of substrates, but the intensity of the bands was markedly lower than that induced by neuropeptides.

DISCUSSION

These results demonstrate that the mitogenic peptides bombesin, vasopressin, and endothelin stimulate tyrosine and serine phosphorylation of multiple substrates in quiescent Swiss 3T3 cells. In these experiments, vasopressin and endothelin-related peptides have been shown to promote tyrosine phosphorylation in intact cells. Cirillo *et al.* (34) previously reported bombesin-stimulated tyrosine phosphorylation of a M_r 115,000 putative bombesin receptor in Swiss 3T3 cells. However, this finding has remained controversial because other investigators have failed to detect significant bombesin-induced tyrosine phosphorylation in these cells (20, 35). Here we show that neuropeptides increased tyrosine phosphorylation of p90 and p115 as judged by multiple

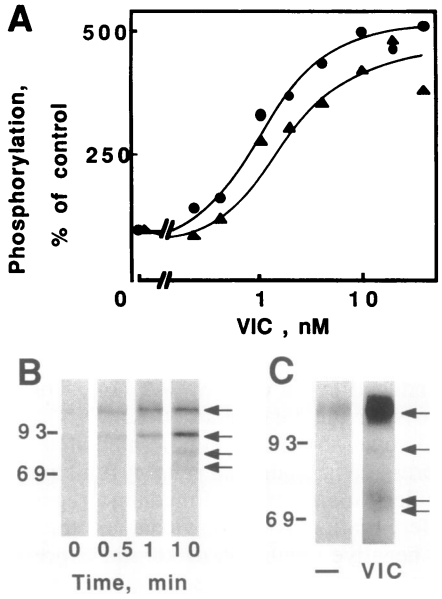

FIG. 6. Stimulation of tyrosine phosphorylation by VIC. (*A*) Quiescent, $^{32}P_i$-labeled Swiss 3T3 cells were treated for 10 min with various concentrations of VIC, as indicated. Cells were lysed, and the lysates were immunoprecipitated with anti-Tyr(*P*) as described. Autoradiograms were scanned and values correspond to phosphorylation of p90 (▲) and p115 (●) expressed as in Figs. 4*A* and 5. (*B*) $^{32}P_i$-labeled cells were treated with 10 nM VIC for various times as shown, lysed, and immunoprecipitated. (*C*) Immunoblot of immunoprecipitates prepared from lysates of unlabeled cells treated with either 10 nM VIC for 10 min or an equivalent volume of solvent (lane −).

procedures including immunoprecipitation and immunoblotting with anti-Tyr(*P*) antibodies and phospho amino acid analysis. Furthermore, there was a striking stimulation of serine phosphorylation of both bands, and in particular p90. The concentration dependence of bombesin, vasopressin, and VIC for induction of tyrosine/serine phosphorylation and the sensitivity of this effect to specific receptor antagonists indicate that neuropeptide-activated phosphorylation is mediated by the same receptors that induce other early biochemical responses and stimulation of DNA synthesis in Swiss 3T3 cells.

It is likely that neuropeptide-stimulated tyrosine phosphorylation plays a role in the transduction of the mitogenic response to neuropeptides, given the fundamental role of tyrosine kinases in the action of polypeptide growth factor receptors and oncogene products (18–26). The fact that an increase in tyrosine phosphorylation is one of the earliest events in the action of bombesin, vasopressin, and VIC is consistent with this possibility. However, the tyrosine kinase activity stimulated by PDGF and other growth factors is intrinsic to the receptor, and a major substrate is the receptor itself (18). In contrast, the receptors for bombesin and vasopressin are linked to G proteins (10–16), and cloning of the bombesin and endothelin receptors shows that they belong to the superfamily of receptors that contain seven hydrophobic domains (17, 36, 37). The present data show that bombesin, vasopressin, and VIC stimulate tyrosine phosphorylation of a common set of substrates in intact 3T3 cells but not in membranes. These findings suggest that neuropeptides and receptor kinases stimulate phosphorylation through fundamentally different mechanisms. Moreover, the lack of identity between neuropeptide-induced phosphotyrosyl proteins and known targets for the PDGF receptor (20–26) indicates significant differences between the substrate specificities of these two pathways.

Classically, tyrosine phosphorylation by polypeptides and the generation of second messengers via neuropeptide receptors linked to G proteins have been regarded as separate though complementary pathways in the mitogenic stimulation of cells (8, 38). The findings that neuropeptides can stimulate tyrosine phosphorylation of specific substrates suggests the existence of an additional signal transduction pathway in the mitogenic response to these agents. Elucidation of the putative components of such a pathway, as well as the identity and function of the substrates, warrants further experimental work.

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1. Zachary, I., Woll, P. J. & Rozengurt, E. (1987) *Dev. Biol.* **124**, 295–308.
2. Rozengurt, E. & Sinnett-Smith, J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2936–2940.
3. Rozengurt, E., Legg, A. & Pettican, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1284–1287.
4. Woll, P. J. & Rozengurt, E. (1988) *Growth Factors* **1**, 75–83.
5. Fabregat, I. & Rozengurt, E. (1990) *Biochem. Biophys. Res. Commun.* **167**, 161–167.
6. Collins, M. K. & Rozengurt, E. (1983) *Proc. Natl. Acad. Sci. USA* **72**, 1924–1928.
7. Zachary, I. & Rozengurt, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7616–7620.
8. Rozengurt, E. (1986) *Science* **234**, 161–166.
9. Rozengurt, E. & Sinnett-Smith, J. (1990) *Philos. Trans. R. Soc. London Ser. B* **327**, 209–221.
10. Erusalimsky, J., Friedberg, I. & Rozengurt, E. (1988) *J. Biol. Chem.* **263**, 19188–19194.
11. Erusalimsky, J. & Rozengurt, E. (1989) *J. Cell. Physiol.* **141**, 253–261.
12. Sinnett-Smith, J., Lehman, W. & Rozengurt, E. (1990) *Biochem. J.* **265**, 485–493.
13. Coffer, A., Fabregat, I., Sinnett-Smith, J. & Rozengurt, E. (1990) *FEBS Lett.* **263**, 80–84.
14. Fishman, J. B., Dickey, B. F. & Fine, R. E. (1987) *J. Biol. Chem.* **262**, 14049–14055.
15. Rozengurt, E., Fabregat, I., Gil, J. & Coffer, A. (1990) *J. Cell Sci.* Suppl. **13**, 43–56.
16. Dickey, B. F., Fishman, J. D., Fine, R. E. & Navarro, J. (1987) *J. Biol. Chem.* **262**, 8738–8742.
17. Battey, J. F., Way, J. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Mann, E. & Feldman, R. I. (1990) *Proc. Natl. Acad. Sci. USA* **88**, 395–399.
18. Williams, L. T. (1989) *Science* **243**, 1564–1570.
19. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61**, 203–212.
20. Meisenhelder, J., Suh, P.-G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57**, 1109–1122.
21. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. & Roberts, T. M. (1987) *Cell* **50**, 1021–1029.
22. Whitman, M., Kaplan, D., Roberts, T. & Cantley, L. (1987) *Biochem. J.* **247**, 165–174.
23. Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B. & Aaronson, S. A. (1989) *Nature (London)* **342**, 711–714.
24. Ellis, C., Moran, M., McCormick, F. & Dawson, T. (1990) *Nature (London)* **343**, 377–381.
25. Kaplan, D. R., Morrison, D. K., Wang, G., McCormick, F. & Williams, L. T. (1990) *Cell* **61**, 125–133.
26. Rossamando, A. J., Payne, D. M., Weber, M. J. & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6940–6943.
27. Kamps, M. P. & Sefton, B. M. (1989) *Anal. Biochem.* **176**, 22–27.
28. Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. & Roberts, T. M. (1985) *Nature (London)* **315**, 239–242.
29. Mehmet, H., Nånberg, E., Lehmann, W., Murray, M. & Rozengurt, E. (1990) *Growth Factors* **3**, 83–95.
30. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. & Cantrell, D. A. (1990) *Nature (London)* **346**, 719–723.
31. Glenny, J. R., Jr., Zokas, L. & Kamps, M. J. (1988) *J. Immunol. Methods* **109**, 277–285.
32. Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. & Cantley, L. C. (1990) *J. Biol. Chem.* **265**, 19704–19711.
33. Zachary, I. & Rozengurt, E. (1987) *J. Biol. Chem.* **262**, 3947–3950.
34. Cirillo, D. M., Gaudino, G., Naldini, L. & Comoglio, P. M. (1986) *Mol. Cell. Biol.* **6**, 4641–4649.
35. Isacke, C. M., Meisenhelder, J., Brown, K. D., Gould, K. L., Gould, S. J. & Hunter, T. (1986) *EMBO J.* **5**, 2889–2898.
36. Arai, H., Hori, S., Aramori, I., Ohkubo, H. & Nakanishi, S. (1990) *Nature (London)* **348**, 730–732.
37. Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. & Masaki, T. (1990) *Nature (London)* **348**, 732–735.
38. Seuwen, K., Magnaldo, I. & Pouyssegur, J. (1988) *Nature (London)* **335**, 254–256.