Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3204–3208, May 1989
Cell Biology

Heterologous desensitization of bombesin-induced mitogenesis by prolonged exposure to vasopressin: A post-receptor signal transduction block

(neuropeptides/growth control/bombesin receptor/c-*fos*/DNA synthesis)

JONATHAN B. A. MILLAR AND ENRIQUE ROZENGURT*

Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom

Communicated by Leon A. Heppel, January 30, 1989 (received for review November 20, 1988)

ABSTRACT **Prolonged exposure of quiescent Swiss 3T3 cells to vasopressin prevents mitogenic stimulation on subsequent addition of bombesin. This heterologous desensitization is selective and can be mimicked by vasopressin agonists, including [Lys^8]vasopressin and oxytocin but not by the V_1-type-specific vasopressin receptor antagonist [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin [where Pmp is 1-(β-mercapto-β,β-cyclopenthamethylene propionic acid)]. Furthermore, vasopressin-induced loss of responsiveness to bombesin can be blocked by addition of this antagonist, indicating that heterologous desensitization is mediated through the vasopressin receptor. Desensitization requires prolonged incubation (half-maximal desensitization occurring after ≈20 hr of pretreatment) and continuous protein synthesis. Bombesin responsiveness is restored by incubation in the absence of vasopressin. Pretreatment does not alter the number, affinity, or internalization capacity of the bombesin receptors. However, the induction of the protooncogene c-*fos* by bombesin is profoundly inhibited after vasopressin pretreatment. We suggest that the coupling of the activated bombesin receptor to the generation of its early signals is impaired in desensitized cells.**

Neuropeptides have been implicated in a number of physiological and pathological processes, including embryogenesis, tissue regeneration, immune regulation, and tumorigenesis (1). An important development in the study of these compounds has been the discovery that many of the neuropeptides, including vasopressin and bombesin, can act as mitogens for cells in culture (2–5). Indeed certain cell types, such as murine Swiss 3T3 fibroblasts, express receptors for several mitogenic neuropeptides and are thus capable of multiple neuropeptide regulation. Since Swiss 3T3 cells have been extensively used to analyze the mechanisms of mitogenic stimulation by these and other peptide growth factors (6), they provide an ideal model system for investigating the integration of multiple neuropeptide activities on the control of cell proliferation.

Exposure of cells to many peptide hormones or neurotransmitters decreases the subsequent response of target cells to further challenge with the same ligand (homologous desensitization) or with a structurally unrelated ligand that elicits responses through a separate receptor (heterologous desensitization). Desensitization has been well-documented for hormones that elicit short-term metabolic responses, such as those mediated through adenylate cyclase-coupled receptors (7–10). However, little is known about desensitization of long-term responses, such as cellular growth and differentiation. This laboratory has described the homologous desensitization to mitogenic stimulation by the neuropeptide vasopressin in Swiss 3T3 cells (11, 12). In contrast, long-term heterologous desensitization of cellular mitogenic responsiveness has not been characterized.

The purpose of the present study was to determine the effects of prolonged neuropeptide exposure on subsequent mitogenic stimulation by heterologous neuropeptides in Swiss 3T3 cells. In the course of this work, we found that prolonged pretreatment with vasopressin can induce a selective heterologous desensitization of the mitogenic activity of bombesin and structurally related peptides, including gastrin-releasing peptide (GRP), in Swiss 3T3 cells. Our findings suggest that the block to bombesin-stimulated mitogenesis occurs at a post-receptor locus and may involve an uncoupling of ligand-bound bombesin receptor for the generation of its early signals.

MATERIALS AND METHODS

Cell Culture and Method of Vasopressin Pretreatment. Maintenance of Swiss 3T3 stocks and production of confluent and quiescent cultures were carried out as described (13). Vasopressin pretreatment of cells was carried out as follows. Confluent and quiescent cultures were washed twice with fresh Dulbecco's modified Eagle's medium (DMEM) and then incubated as required in a mixture of 20% conditioned medium, 40% DMEM, and 40% Waymouth's medium (vol/vol) in the presence or absence of 50 nM [Arg^8]vasopressin at 37°C and 10% CO_2/90% air. Conditioned medium was taken from cells that were grown in 10% (vol/vol) fetal bovine serum (FBS) and had attained quiescence and thus was essentially devoid of growth-promoting activity. The cultures remained quiescent during the pretreatment.

Thymidine Incorporation Assay. Determinations of DNA synthesis were performed by incubating the cultures in 2 ml of a 1:1 mixture (vol/vol) of DMEM and Waymouth's medium containing [^{3}H]thymidine (1 μCi/ml; 1 μM; 1 Ci = 37 GBq) under various conditions as indicated. The incorporation of radioactivity into acid-precipitable material was measured as described (13).

Measurement of ^{125}I-Labeled GRP Binding and Cross-Linking of ^{125}I-Labeled GRP to Intact Cells. Binding and cross-linking of ^{125}I-labeled GRP to cultures of intact Swiss 3T3 cells were performed essentially as described (14, 15) with minor modifications as described in the figure legends.

Northern Blot Analysis. Isolation of total cellular RNA and transfer of agarose gel-separated RNA samples to Hybond-N membranes was performed as described (16). Membranes were probed for c-fos mRNA with a random-primed *Pvu* II–*Bgl* II fragment of pFBH containing sequences encoding the

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Abbreviations: GRP, gastrin-releasing peptide; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Pmp, 1-(β-mercapto-β,β-cyclopenthamethylene propionic acid); FBS, fetal bovine serum.
*To whom reprint requests should be addressed.

c-fos protein (a generous gift from N. Teich, Imperial Cancer Research Fund, U.K.). After boiling in 0.1% SDS/0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 15 min, the membranes were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a random-primed *Pst* I fragment of plasmid FM564 encoding the human GAPDH gene sequence (a generous gift of C. Williams and L. Lim, Institute of Neurology, London) as a control for the amount of total cellular RNA loaded per lane. Induction of c-*fos* is expressed as a fraction relative to the level of GAPDH mRNA observed.

Materials. Bombesin, GRP, cholera toxin, phorboldibutyrate, bovine insulin, cycloheximide, murine epidermal growth factor (EGF), and vasoactive intestinal peptide were purchased from Sigma. Litorin, neuromedin B, [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin, where Pmp is 1-(β-mercapto-β,β-cyclopenthamethylene propionic acid), and [Lys^8]vasopressin were from Peninsula Laboratories. [Arg^8]vasopressin, oxytocin, GRP-(14–27), and [Leu^{13},ψ(CH$_2$NH)Leu^{14}]bombesin were obtained from Bachem (Saffron Walden, U.K.). FBS was from GIBCO Europe. Ro 20-1724 was obtained from Roche Diagnostics. ^{125}I-labeled GRP (2000 Ci/mmol), [^{3}H]-thymidine, and the recombinant product of c-*sis* [platelet-derived growth factor (PDGF)] were from the Radiochemical Centre (Amersham). Ethylene glycolbis(succinimidyl succinimate) was obtained from Pierce.

RESULTS

Desensitization of Swiss 3T3 Cells to Mitogenic Stimulation by Bombesin: An Intracellular Effect. To determine whether prior exposure of 3T3 cells to vasopressin can influence the subsequent mitogenic stimulation by bombesin, confluent and quiescent cultures of Swiss 3T3 cells were treated in the presence or absence of 50 nM [Arg^8]vasopressin for 40 hr. After extensive washing to remove residual vasopressin (11), the incorporation of [^{3}H]thymidine promoted by various concentrations of bombesin was measured. Fig. 1 demonstrates that vasopressin pretreatment profoundly reduced bombesin-stimulated mitogenesis. In 36 individual determinations bombesin-stimulated mitogenesis was inhibited by 93.8 ± 0.6% (Table 1). Under the same conditions the mitogenic stimulation by a maximal dose of vasopressin was also attenuated by 90%, in agreement with that observed for homologous desensitization of vasopressin-induced responses (11). A similar result was obtained when the number of [^{3}H]thymidine-labeled nuclei was measured by autoradiography after a 40-hr incubation with bombesin (Table 2).

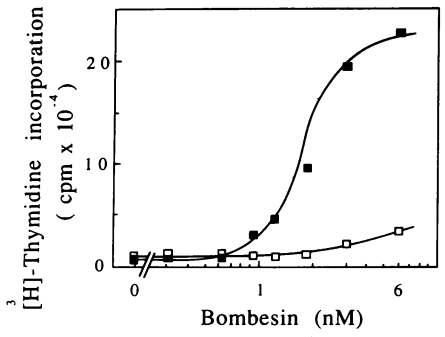

FIG. 1. Vasopressin pretreatment prevents bombesin-stimulated mitogenesis. Cells were pretreated for 40 hr in the presence (open squares) or absence (closed squares) of 50 nM [Arg^8]vasopressin. The cells were then washed three times with DMEM, challenged with various concentrations of bombesin, and assayed for DNA synthesis after a further 40-hr incubation. Each point represents the mean of triplicate determinations. Maximal stimulation by bombesin in control cells was 42% of that induced by 10% FBS.

Table 1. Selectivity of vasopressin-induced densensitization of Swiss 3T3 cells

Addition	Insulin, 200 ng/ml	% [^{3}H]thymidine incorporation in control cells	*n*
Bombesin	−	6.3 ± 0.8	36
Litorin	−	12.2 ± 2.8	3
Neuromedin B	−	11.1 ± 0.8	3
GRP	−	8.0 ± 0.9	3
GRP-(14–27)	−	6.2 ± 1.1	3
Vasopressin	+	11.2 ± 3.8	27
EGF	+	95.6 ± 6.7	18
VIP + Ro 20-1724	+	107.6 ± 3.7	3
Cholera toxin	+	84.0 ± 9.4	3
PBt_2	+	91.2 ± 11.3	21
PBt_2 + EGF	−	87.4 ± 15.3	9
PDGF	−	114.2 ± 13.3	9
FBS	−	93.7 ± 6.4	9

Cells were pretreated with 50 nM [Arg^8]vasopressin for 40 hr. Then, all cultures were washed twice with fresh DMEM after the pretreatment, then incubated for an additional 40 hr with [^{3}H]-thymidine and the above additions at the following concentrations: bombesin (6 nM), vasopressin (20 nM), EGF (1 nM), vasoactive intestinal peptide (3 nM), FBS (10%), cholera toxin (100 ng/ml), PDGF (0.7 nM), phorbol dibutyrate (PBt_2; 200 nM), litorin (10 nM), GRP (7 nM), GRP-(14–27) (6 nM), 4-(3-butoxy-4-methoxybenzoyl)-2-imidazolidine (Ro 20-1724) (10 μM), and neuromedin B (45 nM). The values shown are expressed as a percentage of the thymidine incorporation by each addition in control cells. The number of individual determination (*n*) is also shown. −, Absence of insulin; +, presence of insulin in the DNA synthesis assay.

The possibility that the loss of responsiveness to bombesin observed above could be due to the release into the medium of an inhibitory factor was discounted, since it was found that medium from cells that had been exposed to vasopressin for 40 hr supported bombesin stimulation of DNA synthesis in fresh quiescent cultures of Swiss 3T3 cells (data not shown).

These results suggest that pretreatment with vasopressin causes a heterologous desensitization of the mitogenic action of bombesin in Swiss 3T3 cells.

Heterologous Desensitization with Vasopressin Is Selective. A number of mammalian peptides structurally related to bombesin including GRP, litorin, and neuromedin B have been shown to interact with the bombesin receptor in Swiss 3T3 cells and, like bombesin, are also mitogenic (14). Pretreatment of cells with vasopressin reduced the mitogenic activity of these bombesin-related peptides by ≈90% (Table 1). Under the same conditions however, the cells were fully responsive to serum and a number of other mitogens, such as PDGF or combinations of mitogenic ligands such as EGF, phorbol esters, and cholera toxin in the presence of insulin (Table 1). Similar specificity was observed when the number of [^{3}H]thymidine-labeled nuclei was counted by autoradiog-

Table 2. Selectivity of vasopressin-induced desensitization in Swiss 3T3 cells demonstrated by autoradiography

Addition	Percentage of labeled nuclei: Control	Percentage of labeled nuclei: Vasopressin pretreated
None	1.3 ± 0.3	1.4 ± 0.2
Bombesin	29.6 ± 1.9	3.2 ± 0.3
Vasopressin + insulin	61.9 ± 3.3	6.9 ± 1.0
EGF + insulin	60.5 ± 3.4	60.7 ± 1.1

Control cultures or those pretreated with 50 nM [Arg^8]vasopressin for 40 hr were incubated for an additional 40 hr with [^{3}H]thymidine (2 μCi/ml) and no addition, 6 nM bombesin, 20 nM vasopressin, EGF (5 ng/ml), or insulin (200 ng/ml), as indicated. The number of labeled nuclei was then determined.

raphy after a 40-hr incubation in the presence of various ligands (Table 2). These results are in accordance with the selectivity of vasopressin pretreatment described for homologous desensitization of vasopressin-induced responses (11) and further suggest that exposure of 3T3 cells to vasopressin causes a selective heterologous desensitization to mitogenic stimulation through the bombesin receptor.

Time Course of Onset and Recovery from the Desensitization State and the Requirement for Continuous Protein Synthesis. Vasopressin-induced heterologous desensitization develops gradually and is reversible. The time course of heterologous desensitization begins at ≈10 hr, reaches a maximum after 30 hr (Fig. 2*A*), and is virtually superimposable to the time course of homologous desensitization. In contrast, the response of the cells to a combination of EGF and insulin is unchanged throughout this period. Thus, selective mitogenic desensitization can be distinguished from homologous and heterologous desensitizations of acute responses, such as Ca^{2+} mobilization, which require only a short exposure to ligand (<2 hr) to induce desensitization (17–25). Mitogenic responsiveness to bombesin was substantially recovered after a further 32 hr in the absence of vasopressin. Recovery was blocked by the continued presence of hormone (Fig. 2*B*).

The prolonged period of pretreatment necessary to induce desensitization (Fig. 2*A*) prompted us to assess the role of protein synthesis in this process. Table 3 shows that cycloheximide, at a concentration that causes a 70% inhibition of protein synthesis (26), blocked the ability of vasopressin to induce either homologous or heterologous desensitization. This suggests that the synthesis of certain, as yet unidentified, protein(s) may be involved in the induction of desensitization.

Induction of Heterologous Desensitization Is Mediated Through the Vasopressin Receptor. Heterologous desensitization is dependent on the concentration of vasopressin present during pretreatment. Half-maximal desensitization was achieved with ≈0.6 nM [Arg^8]vasopressin (Fig. 3*A*), a similar concentration to that required for half-maximal stimulation of DNA synthesis in the presence of insulin. The ability to induce heterologous desensitization to bombesin is also shared by two vasopressin-related peptides, [Lys^8]-vasopressin and oxytocin, but not by a specific V_1-type vasopressin receptor antagonist [Pmp^1,*O*-Me-Tyr^2,Arg^8]-vasopressin (Fig. 3*A*). This demonstrates that refractoriness is elicited by hormone agonists but not by a specific antagonist. Bombesin and related peptides have been shown (27), by competition binding assays, to bind to a distinct receptor from vasopressin. In addition another bombesin antagonist, [Leu^{13},ψ(CH$_2$NH)Leu^{14}]bombesin, has been described that specifically inhibits binding of bombesin to its receptor in Swiss 3T3 cells (28). Addition of this peptide in the pretreatment period with vasopressin did not affect the ability of vasopressin to induce heterologous desensitization of bombesin-mediated mitogenic response (Fig. 3*B*). In contrast, the specific V_1-type antagonist [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin completely prevented the induction of the desensitized state by vasopressin (Fig. 3*B*). These results indicate that heterologous desensitization is mediated through the vasopressin receptor.

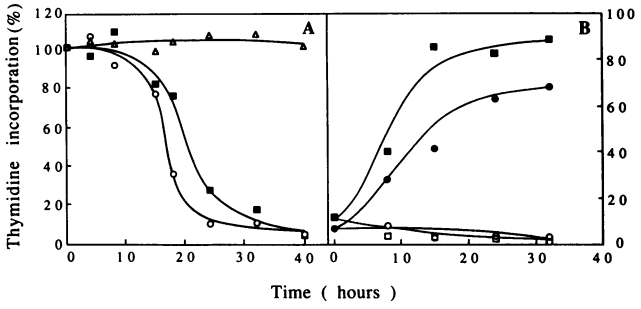

FIG. 2. Time course of onset and recovery from the desensitized state. (*A*) Cells were pretreated with 50 nM [Arg^8]vasopressin for various lengths of time. The cells were then washed three times with fresh DMEM and challenged with 6 nM bombesin (closed squares), 20 nM vasopressin + insulin (200 ng/ml) (open circles), or EGF (5 ng/ml) + insulin (200 ng/ml) (open triangles) and assayed for DNA synthesis after a further 40-hr incubation. Each point represents the mean of triplicate determinations. (*B*) Cells were pretreated in the presence or absence of 50 nM [Arg^8]vasopressin for 40 hr. The cells were then washed and placed in recovery medium (closed symbols) consisting of 1% FBS in a mixture of DMEM and Waymouth's medium or in the continued presence of 50 nM [Arg^8]vasopressin (open symbols) for various lengths of time. The cells were then washed and challenged with either 6 nM bombesin (circles) or 20 nM vasopressin + insulin (200 ng/ml) (squares) in the presence of [^{3}H]thymidine and assayed for incorporation of radioactive material into acid-precipitable fraction after 40 hr. Incorporation is presented as a percentage of incorporation into control cells that had not been pretreated with [Arg^8]vasopressin. Each point represents the mean of triplicate determinations.

Table 3. Requirement for protein synthesis for vasopressin-induced desensitization

Pretreatment	[^{3}H]Thymidine incorporation, cpm × 10^{-4}		
	No addition	Bombesin	Vasopressin + insulin
None	1.7 ± 0.4	13.7 ± 0.6	62.1 ± 0.5
Vasopressin	0.8 ± 0.1	1.0 ± 0.2	4.5 ± 0.9
Cycloheximide	0.3 ± 0.1	9.8 ± 1.2	49.4 ± 8.2
Vasopressin + cycloheximide	1.1 ± 0.1	12.0 ± 1.6	52.7 ± 3.8

Cells were pretreated with 50 nM [Arg^8]vasopressin or cycloheximide (100 ng/ml) or a combination of the two for 40 hr. All cultures were washed with fresh DMEM after the pretreatment and incubated for an additional 40 hr with [^{3}H]thymidine and no addition, bombesin (6 nM), or vasopressin (20 nM) plus insulin (200 ng/ml). Data represent the mean of triplicate determinations (± SD).

Vasopressin Pretreatment Does Not Alter the Bombesin Receptor. The induction of desensitization of short-term

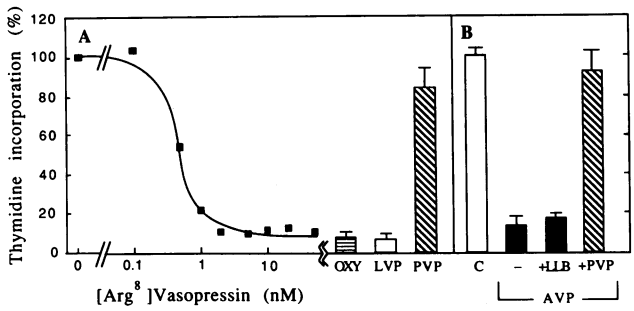

FIG. 3. Effect of pretreatment with various vasopressin agonists and antagonists on bombesin-stimulated mitogenesis. (*A*) Cells were pretreated for 40 hr in the presence of various concentrations of [Arg^8]vasopressin or with the related agonists oxytocin (OXY) at 10 μM, [Lys^8]vasopressin (LVP) at 50 nM, or with the vasopressin antagonist [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin (PVP) at 1 μM. The cells were then washed and challenged with 6 nM bombesin (squares) in the presence of [^{3}H]thymidine and assayed for DNA synthesis after a further 40-hr incubation. Each point represents the mean of triplicate determinations (+ SD). (*B*) A similar pretreatment was performed in the absence of any additions (open bar) or in the presence of 50 nM [Arg^8]vasopressin (AVP). The [Arg^8]vasopressin-containing dishes received no further addition (solid bar) or were supplemented with the selective bombesin antagonist [Leu^{13}, ψ(CH$_2$NH)Leu^{14}]bombesin (LLB) at 1 μM (stippled bar) or with [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin (PVP) at 1 μM (hatched bar). The cells were then washed and challenged with 6 nM bombesin and assayed for DNA synthesis after a further 40-hr incubation. Each point represents the mean of triplicate determinations (+ SD).

responses has been correlated with down-regulation, sequestration, or phosphorylation-mediated inactivation of the receptor (for review, see ref. 10). Peptides of the bombesin family bind to specific and saturable high-affinity binding sites on the surface of intact Swiss 3T3 cells (14). In addition ^{125}I-labeled GRP can be cross-linked in these cells to a M_r 75,000–85,000 glycoprotein identified as the putative bombesin receptor (29, 30). To test the possibility that the number or affinity of the bombesin receptors may have been altered by vasopressin pretreatment, Scatchard analysis of cellular binding sites was performed using ^{125}I-labeled GRP in control and pretreated cultures. As the results in Fig. 4*A* demonstrate, the affinity and total number of receptor binding sites was unaffected. A time course of ^{125}I-labeled GRP binding to intact control and pretreated cells performed at 37°C shows that the rate of association of radiolabeled ligand to cell surface receptors and the rate of internalization of the receptor–ligand complex was similarly unaffected (Fig. 4*B*). Finally pretreatment with vasopressin did not alter the ability of ^{125}I-labeled GRP to be cross-linked to the M_r 75,000–85,000 band nor did it alter its apparent molecular weight (Fig. 4*C*). We conclude that exposure to vasopressin does not affect any measurable parameter of the bombesin receptor. We thus suggest that the block induced by vasopressin must occur at some post-receptor locus.

Induction of the Protooncogene c-*fos* by Bombesin was Greatly Inhibited by Vasopressin Treatment. The rapid induction of the protooncogene c-*fos* is one of the earliest nuclear events after stimulation of quiescent fibroblasts by bombesin and other peptide growth factors (for review, see ref. 31). Induction of c-*fos* by such growth factors requires the production of receptor-mediated early signals (32). We have examined whether bombesin is able to induce the expression of c-*fos* in desensitized cells, as an indication of its ability to induce transmembrane signaling. In three independent experiments, the induction of c-*fos* by a maximal dose of bombesin was inhibited 69.1 ± 7.8% by vasopressin pretreatment. Under the same conditions the ability of vasopressin to induce c-*fos* was totally abolished (Fig. 5), further supporting the proposal that the early events induced by vasopressin are abrogated by homologous vasopressin desensitization (11).

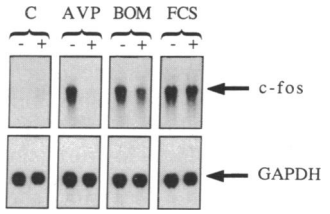

FIG. 5. Vasopressin pretreatment decreases the induction of the c-*fos* protooncogene by bombesin. Confluent and quiescent cells in 175-cm^2 flasks were incubated for 40 hr in the presence (+) or absence (−) of 50 nM [Arg^8]vasopressin. The cells were then washed and challenged with either no addition (lanes C), 6 nM bombesin (lanes BOM), 20 nM vasopressin (lanes AVP) in the presence of insulin, (200 ng/ml), or 10% FBS (lanes FCS), as indicated in 20 ml of DMEM for 30 min at 37°C and 10% CO_2/90% air. Total RNA was prepared, and Northern blot analysis was performed with c-fos probe and then the blot was stripped and reprobed with a GAPDH gene fragment. The level of c-*fos* induction was taken as a fraction over the level of GAPDH mRNA by scanning densitometry. Pretreatment with vasopressin did not alter the time course of c-*fos* expression.

In contrast the induction of c-*fos* by either serum (Fig. 5) or PDGF (data not shown) was completely unaffected by the pretreatment, showing that the effect was selective. Thus, vasopressin pretreatment may uncouple ligand-bound bombesin receptor from the generation of its early signals in desensitized cells.

DISCUSSION

The results presented in this paper demonstrate that pretreatment with vasopressin can induce a dose- and time-dependent heterologous desensitization to the mitogenic action of bombesin in Swiss 3T3 cells. As described for vasopressin-induced homologous desensitization (11), the effect is selective, reversible, and inducible by several vasopressin agonists but not by a specific vasopressin antagonist. Moreover addition of a saturating dose of a specific vasopressin antagonist, [Pmp^1,*O*-Me-Tyr^2,Arg^8]vasopressin, during pretreatment abolishes the ability of vasopressin to induce desensitization, whereas addition of a specific bomb-

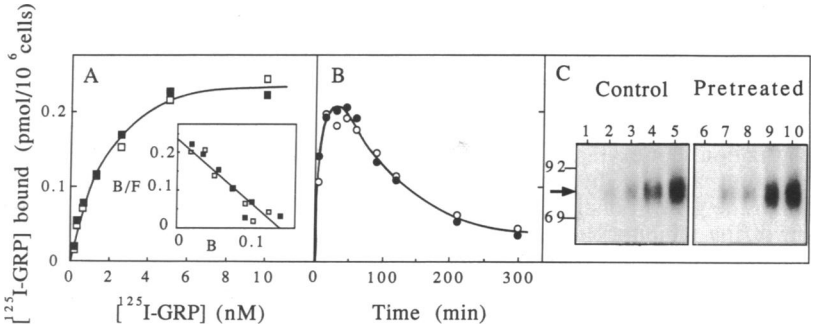

FIG. 4. Vasopressin pretreatment does not alter the ability of ^{125}I-labeled GRP ([^{125}I-GRP]) to bind to and affinity-label intact Swiss 3T3 cells. (*A*) Scatchard analysis. Confluent and quiescent cultures of Swiss 3T3 cells were pretreated for 40 hr in the presence (open squares) or absence (closed squares) of 50 nM [Arg^8]vasopressin for 40 hr. The cells were then washed and incubated in the presence of various concentrations of ^{125}I-labeled GRP in binding medium (DMEM containing bovine serum albumin at 1 mg/ml and 50 mM *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid pH 7.0) for 30 min at 37°C and 10% CO_2/90% air. After this time the cultures were washed and extracted and total cell-associated radioactivity was determined. Each point represents the mean of triplicate determinations. (*B*) Time course of ^{125}I-labeled GRP binding. Confluent and quiescent cultures of Swiss 3T3 cells were pretreated for 40 hr in the presence (open circles) or absence (closed circles) of 50 nM [Arg^8] vasopressin for 40 hr. The cells were then washed and incubated in the presence of 1 nM ^{125}I-labeled GRP in binding medium for various times at 37°C and 10% CO_2/90% air. Cell-associated radioactivity was determined. Each point represents the mean of triplicate determinations. (*C*) Effect of vasopressin pretreatment on the ability to affinity label a M_r 75,000–85,000 protein with ^{125}I-labeled GRP in Swiss 3T3 cells. Confluent and quiescent cultures of Swiss 3T3 cells were pretreated for 40 hr in the presence (pretreated) or absence (control) of 50 nM [Arg^8]vasopressin. The cells were then washed with DMEM and incubated in the presence of 1 nM ^{125}I-labeled GRP in binding medium for 10 min at 37°C and 10% CO_2/90% air. After this time the cultures were washed with PBS and incubated for 0 min (lanes 1 and 6), 2.5 min (lanes 2 and 7), 5 min (lanes 3 and 8), 7.5 min (lanes 4 and 9), or 10 min (lanes 5 and 10) in cross-linking medium [PBS containing bovine serum albumin at 1 mg/ml and 25 mM Hepes (pH 7.4)] in the presence of 6 mM ethylene glycol bis(succinimidyl succinimate) at 15°C. Cells were then extracted and samples were analyzed by one-dimensional SDS/PAGE. The arrow indicates the position of the M_r 75,000–85,000 protein.

esin antagonist, [Leu^{13},ψ(CH$_2$NH)Leu^{14}]bombesin, is ineffective. We conclude that induction of desensitization is mediated through the vasopressin receptor.

Loss of responsiveness to bombesin-stimulated DNA synthesis is also induced by chronic exposure of the cells to phorbol esters, which causes down-regulation of protein kinase C (33, 34). Vasopressin pretreatment does not induce down-regulation of this kinase as assayed by Western blot analysis (unpublished data) or loss of responsiveness to phorbol esters (Table 1). Thus, the two treatments mediate a loss of responsiveness to bombesin by fundamentally different mechanisms.

Homologous desensitization of many short-term responses has been correlated with either down-regulation or phosphorylation-mediated inactivation of the receptor leading to a concomitant alteration in either the availability or affinity of the receptor for ligand (for review, see ref. 10). However, a post-receptor lesion, or uncoupling, was reported to be partially responsible for homologous desensitization of vasopressin-induced responses in Swiss 3T3 fibroblasts and WRK$_1$ cells, derived from a rat mammary tumor (11, 35). Here we demonstrate that heterologous desensitization of bombesin-mediated mitogenesis cannot be ascribed to alteration of the bombesin receptor since the number, affinity, or internalization capacity of the receptors was not affected. Thus we conclude that the inhibition to mitogenic stimulation by bombesin occurs at or after the level of transmission of early signals.

One of the earliest responses of gene activation that can be measured after stimulation of quiescent cells by peptide growth factors, including bombesin, is the transient induction of the protooncogene c-*fos* (for review, see refs. 31 and 32). The inability of bombesin to cause full induction of c-*fos* expression in desensitized cells suggests (*i*) that vasopressin pretreatment induces a block to mitogenic stimulation within the first 30 min after challenge with bombesin and (*ii*) that the pretreatment may uncouple ligand-bound bombesin receptor from the generation of its early signals. Two possible models might explain the apparent loss of responsiveness to bombesin in desensitized cells: (*i*) one or more signaling pathways activated by bombesin may be abolished or (*ii*) all measurable early signals may be partially reduced. Either model could explain the residual expression of c-*fos* observed in desensitized cells (Fig. 5) in the absence of mitogenic stimulation.

Neuropeptides have been implicated in the unrestrained growth of certain tumors (1, 36) and consequently the mechanisms by which these peptides stimulate cell growth are attracting considerable attention. For example, bombesin and its structurally related counterparts have been implicated as autocrine growth factors in the growth of small cell lung cancer (37, 38). Disruption of the inhibitory mechanism(s) that the cell employs to regulate mitogenic stimulation by neuropeptides (desensitization) could result in the development of unrestrained cell growth.

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