Tyrosine Phosphorylation of Gsα and Inhibition of Bradykinin-induced Activation of the Cyclic AMP Pathway in A431 Cells by Epidermal Growth Factor Receptor*

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An increasing amount of experimental data suggest that cross-talk exists between pathways involving tyrosine kinases and heterotrimeric G proteins. In a previous study, we demonstrated that bradykinin (BK) increases the intracellular accumulation of cAMP in the human epidermoid carcinoma cell line A431 by stimulating adenylate cyclase activity via a stimulatory G protein (Gsα) (Liebmann, C., Graneß, A., Ludwig, B., Adomeit, A., Boehmer, A., Boehmer, F.-D., Nürnberg, B., and Wetzker, R. (1996) Biochem. J. 313, 109–118). Here, we present several lines of evidence indicating the ability of epidermal growth factor (EGF) to suppress BK-induced activation of the cAMP pathway in A431 cells via tyrosine phosphorylation of Gsα. Gsα was specifically immunoprecipitated from A431 cells using the anti-αs antiserum AS 348. Tyrosine phosphorylation of Gsα was detectable in EGF-pretreated cells with monoclonal anti-phosphotyrosine antibodies. Additionally, A431 cells were labeled with [32P]orthophosphate in vivo and treated with EGF, and the resolved immunoprecipitates were subjected to amino acid analysis. The results clearly indicate that EGF induces tyrosine phosphorylation of Gsα in A431 cells. Treatment of A431 cells with EGF decreased BK-induced cAMP accumulation in intact cells as well as the stimulation of adenylate cyclase by BK, NaF, and guanyl nucleotides, but not by forskolin. Also, EGF treatment abolished both the BK- and isoproterenol-induced stimulation of guanosine 5′-O-(3′-[32S]thiotriphosphate) binding to Gsα. In contrast, the BK-evoked, Gsα-mediated stimulation of inositol phosphate formation in A431 cells was not affected by EGF pretreatment. Thus, EGF-induced tyrosine phosphorylation of Gsα is accompanied by a loss of its susceptibility to G protein-coupled receptors and its ability to stimulate adenylate cyclase via guanyl nucleotide exchange. We propose that Gsα may represent a key regulatory protein in the cross-talk between the signal transduction pathways of BK and EGF in A431 cells.

There is mounting evidence indicative of complex, probably cell-specific interactions between signaling pathways involving heterotrimeric G proteins and tyrosine kinases. For example, the stimulation of G protein-coupled receptors modulates key proteins of the mitogen-activated protein kinase pathway via protein kinase C- or protein kinase A-mediated phosphorylation on serine or threonine residues (1–3). Furthermore, several isomers of α subunits of G proteins were shown to be phosphorylated in vitro on tyrosine residues by tyrosine kinase receptors such as the epidermal growth factor (EGF)† receptor and the insulin receptor or by non-receptor tyrosine kinases of the Src kinase family. EGF was shown to activate cardiac adenylate cyclase via a mechanism requiring both Gsα and the EGF receptor tyrosine kinase (4, 5). EGF was also found to stimulate phospholipase C in rat hepatocytes (6) or phospholipase A2 in rat kidney (7) in a pertussis toxin-sensitive manner. It is thus probable that such phosphorylation might affect the function of the G protein. Thus, the results presented here may represent a key regulatory protein in the crosstalk between signaling pathways involving Gsα and tyrosine kinases.

† The abbreviations used are: EGF, epidermal growth factor; BK, bradykinin; GTP–G, guanosine 5′-O-(3′-thiotriphosphate); DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; Gpp(NH)p, 5′-guanylylimidodiphosphate; PIPES, pipericin-N,N′-bis(2-ethanesulfonic acid).

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of protein kinase C leads to an increased serine/threonine phosphorylation of EGFR receptors and, subsequently, to a reduced binding of EGF. These findings prompted us to investigate whether EGF is able to affect (vice versa) the BK signaling pathways in A431 cells.

In this paper, we present experimental evidence that EGF treatment of A431 cells results in both inhibition of BK-induced ([35S]GTP·S binding to Gs) and BK-induced stimulation of adenylate cyclase activity in A431 membranes as well as inhibition of BK-induced cAMP accumulation in intact A431 cells. Furthermore, we show that EGF stimulation leads to specific tyrosine phosphorylation, but also to an increase in serine/threonine phosphorylation of Gs. This is the first report demonstrating possible functional consequences of Gs tyrosine phosphorylation in intact cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A431 human epidermoid carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) supplemented with 4.5 g/liter glucose, 2 mM glutamine, 7.5% fetal calf serum, and antibiotics. Treatment of intact cells with BK and various agents as outlined in the figure legends was performed with nearly confluent cultures.

**Membrane Preparations**—An A431 particulate fraction (referred to as “serum-free” DMEM) was prepared as described before (13). Protein concentration was determined according to Lowry et al. (15) or Bradford (16) with bovine serum albumin as a standard.

**Adenylate Cyclase Assay**—The activity of adenylate cyclase in A431 membranes was determined according to Schultz and Jakobs (17) with slight modifications. Briefly, membranes (80 μg of protein/assay) were incubated for 20 min at 25°C in a standard mixture containing [α-32P]ATP (2–10 × 10^6 cpm/tube), 5 mM MgCl2, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM cAMP, 0.1 mM ATP, 5 mM creatine phosphate, 150 units of creatine phosphokinase, 1 mg/ml bovine serum albumin, and 50 mM HEPES, pH 7.2. To inactivate angiotensin-converting enzyme, which degrades BK, the incubation mixture was supplemented with 1 μM captopril. Variable additions such as NaF (as [αF32P]J), forskolin, GTP·S, and BK were added as indicated, giving a total assay volume of 100 μl. The samples were preincubated for 5 min at 25°C, and the reaction was started by adding the membranes. The incubation was terminated by the addition of 400 μl of ZnCl2 solution (125 mM) followed by 500 μl of Na2CO3 solution (125 mM). The samples were centrifuged for 5 min at 10,000 × g, and 800 μl of the supernatant were transferred to aluminum oxide (E. Merck, Darmstadt, Germany) containing columns. After draining of the sample, labeled cAMP was eluted by the subsequent addition of 2–3 ml of cold ethanol (96%, v/v), giving a final concentration of 65% (v/v). The cells were scraped off the plates, and the ethanol extract was centrifuged at 14,000 × g for 6 min at room temperature. The supernatants containing the extracted cAMP were removed, and the pellets were washed with 500 μl of ethanol (65% v/v) and centrifuged as described above. Supernatants were pooled, evaporated to dryness at 60°C, and resolved in 600 μl of 0.05 M acetic acid buffer. Two samples were taken for estimation of cAMP concentration using the cAMP 125I-labeled scintillation proximity (non-acetylation) assay from Amersham. For each well, the protein content was determined (16). ([35S]GTP·S Binding)—Binding of [35S]GTP·S to A431 membranes was determined as described previously (18). The reaction mixture contained [35S]GTP·S (106 cpm/mg), 1 μM GDP, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10 μM captopril, 50 mM HEPES, pH 7.2, and 15 μg of membrane protein in a total volume of 200 μl. Further details are provided in the figure legends. The incubation was started by the addition of the membranes and was carried out in quadruplicates for 45 min at 4°C (equilibrium conditions). The reaction was terminated by rapid filtration through Whatman GF/C glass-fiber filters under vacuum. The filters were washed with 3 × 2 ml of 50 mM HEPES, pH 7.2, containing 0.1 M NaCl, 1 μM EDTA, and 10 μM captopril and were dried and added for radioactivity. Non-specific binding was determined in the presence of 10 μM unlabeled GTP·S and represents ~40% of total binding.

**Determination of Total Inositol Phosphates—**BK-induced formation of inositol phosphates in A431 cells was determined as described by Tilly et al. (19). Briefly, cells were preincubated with 4 μCi/ml [3H]inositol for 24 h in inositol-free DMEM. 2 h prior to stimulation, the medium was changed to serum-free DMEM containing 20 mM HEPES, pH 7.2. Then, the cells were stimulated for 10 min with bradykinin at the concentrations indicated in the presence of 10 mM LiCl2. The reaction was terminated by replacing the medium with 1 ml of 10% trichloroacetic acid. Inositol phosphate-containing extracts were washed four times with 2 volumes of water-saturated diethyl ether, neutralized by addition of 5 ml of 5% sodium borate, and three times with phosphate-buffered saline. The samples were then freeze-dried, resuspended in 40 μl of 0.1 M NaOH, and 50 μl of 1 M formic acid for five times, yielding the inositol phosphate fraction. Radioactivity was measured using a FleScint IV scintillator (Packard Instrument Co.).

**Immunoprecipitation and Immunoblotting of Subunits—**Anti-serum AS 348 was raised against the peptide sequence RMML-RQYELL, corresponding to C-terminal region 385–394 of αs, and characterized as described elsewhere (20, 21). RC20 monoclonal anti-phosphotyrosine antibodies (Transduction Laboratories) were purchased from Dianova (Hamburg, Germany). Subconfluent A431 cells preincubated in serum-free DMEM overnight, and then the medium was changed to fresh serum-free DMEM, and the cells were stimulated with EGF (100 ng/ml) at 37°C in the absence or presence of other additions and for various lengths of time as indicated, followed by the preparation of membranes. Immunoprecipitation of Gs proteins was performed according to Laugwitz et al. (22). For several experiments, AS 348 antibodies were covalently coupled to protein A-Sepharose by means of dimethyl pimelimidate. Briefly, 200 μl of AS 348 antiserum (or nonimmune serum as a control) and 200 μl of protein A-Sepharose (12.5 mg of beads) were incubated for 2 h and subsequently washed three times with phosphate-buffered saline, pH 7.4, and twice with 0.2 M sodium borate, pH 9.0. The beads were resuspended in 0.1 M borate buffer. The antibodies were cross-linked to the beads by adding 5.2 mg of dimethyl pimelimidate and mixing for 30 min at room temperature. Thereafter, the beads were washed with 0.2 M ethanolamine, pH 8.0, and the incubation was continued with 0.2 M ethanolamine, pH 8.0, for 2 h at room temperature. Finally, the beads were washed two times with 0.1 M glycine, pH 3.0, and three times with phosphate-buffered saline. The covalently coupled antibodies were stored in phosphate buffered saline, pH 7.4, with 20% glycerol and 0.01% sodium azide. They were solubilized in 40 μl of 2% (w/v) SDS for 10 min at room temperature. Thereafter, 120 μl of precipitating buffer containing 1% (w/v) Triton X-100, 1% (w/v) deoxycholate, 150 mM NaCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 μM sodium orthovanadate, and 10 mM Tris-HCl, pH 7.4, were added. To remove insoluble material, the solubilized membranes were centrifuged at 4°C and 12,000 × g for 10 min. Covariantly coupled antiserum AS 348 (20 μl) or nonimmune serum as a control was added to the supernatants, and the samples were incubated at 4°C for 4 h under constant rotation. Thereafter, the beads were pelleted (14,000 × g, 10 s) and washed twice with 1 ml of washing buffer A containing 1% (w/v) Nonidet P-40, 0.05% (w/v) SDS, 600 mM NaCl, and 50 mM Tris-HCl, pH 7.4, and twice with 1 ml of washing buffer B containing 300 mM NaCl, 10 mM EDTA, and 100 mM Tris-HCl, pH 7.4. The Sepharose beads were resuspended in 40 μl of SDS sample buffer, heated for 10 min at 100°C, and centrifuged, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% (w/v) acrylamide gels and transferred onto nitrocellulose filters. The blots were then processed as described previously (22) for AS 348 antibodies and according to the instructions of the manufacturer in the case of RC20 anti-phosphotyrosine antibodies.

**Metabolic Labeling of A431 Cells and Immunoprecipitation of αs Subunits**—Subconfluent A431 cells in 6-well plates (Nunc) were depleted of serum for 24 h. Then, the medium was changed to phosphate-free DMEM containing 0.5 mM NaCl, 5 mM HEPES, and 1 mM EDTA, 1 mM dithiothreitol, 10 μM captopril, 50 mM HEPES, pH 7.2, and 15 μg of membrane protein in a total volume of 200 μl. Further details are provided in the figure legends. The incubation was started by the addition of the membranes and was carried out in quadruplicates for 45 min at 4°C (equilibrium conditions). The reaction was terminated by rapid filtration through Whatman GF/C glass-fiber filters under vacuum. The filters were washed with 3 × 2 ml of 50 mM HEPES, pH 7.2, containing 0.1 M NaCl, 1 μM EDTA, and 10 μM captopril and were dried and added for radioactivity. Non-specific binding was determined in the presence of 10 μM unlabeled GTP·S and represents ~40% of total binding.
deoxycollate, 0.5% (w/v) SDS, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 0.1 mM sodium vanadate, and 0.1% (w/v) bovine serum albumin. The lysate was cleared by centrifugation at 12,000 x g for 4°C for 10 min. Metabolically labeled \( G_{\alpha} \) phosphoprotein was immunoprecipitated by protein A-Sepharose-bound anti-\( \alpha \) antisera as described above. Immunocomplexes were washed twice with both buffers A and B, heated with SDS sample buffer, and subjected to 10% SDS-PAGE. The \( \alpha \) protein bands were localized in the fixed dried gels by exposure to Biomax film (Eastman Kodak Co.) for 4 h at −80°C using an intensifying screen.

Phosphoamino Acid Analysis—The section of the dried SDS-polyacrylamide gels corresponding to the position of \( G_{\alpha} \) (−100 to 200 cpm) was excised; the protein was precipitated and hydrolyzed; and phosphoamino acid analysis was performed by two-dimensional separation on thin-layer cellulose plates as described by Boyle et al. (23). The thin-layer plates were analyzed using a Bio-Rad Model GS250 Molecular Imager and prolonged exposure (3–4 days) on BI imaging screens.

Materials—[α-32P]ATP (800 Ci/mmol), [35S]GTPγS (1200–1400 Ci/ mmol), [3H]PI (5000–9120 Ci/mmol), and myo-[2-3H]inositol (20.5 Ci/ mmol) were purchased from Amersham (Braunschweig, Germany). Bradykinin, GTPγS, Gpp(NH)p, 3-isobutyl-1-methylxanthine, HEPES, PIPES, di-thiothreitol, bacitracin, captopril, phenylmethylsulfonyl fluoride, cAMP, ATP, creatine phosphate, creatine phosphokinase, bovine serum albumin, ovalbumin, peroxidase-conjugated goat anti-rabbit IgG, forskolin, lauryl sulfate, Nonidet P-40, deoxycholate, sodium orthovanadate, Tween 20 (polyoxyethylene sorbitan monolaurate), dimethyl pime-limidate, and protein A-Sepharose were obtained from Sigma (Deisenhofen, Germany). Leupeptin, pepstatin A, and Pefabloc were from Boehringer (Mannheim, Germany). Ammonium formate, sodium tetraborate, and Triton X-100 were purchased from SERVA (Heidelberg, Germany). Hoe 140 (α-Arg[ Hyp, Th i, p-Tic], Oic) BK, where Thi is β-Z-thienylamine, Tic is p-1,2,3,4-tetraoxoquinoline-3-carboxylic acid, and Oic is [3aS, 7aS]octahydroindol-2-carboxylic acid) was kindly provided by Prof. B. Scholakens (Hoechst AG, Frankurt, Germany). The EGF receptor-specific blocker AG 1478 was purchased from Calbiochem.

**RESULTS**

EGF Counteracts BK-induced Cyclic AMP Accumulation, but Not BK-induced Formation of Inositol Phosphates, in A431 Cells—In intact A431 cells, BK elicited a concentration-dependent increase in intracellular cAMP up to ∼140% of the basal level (100%) after 20 min of stimulation (Fig. 1). Half-maximal effects were seen at ∼3 nM BK. When the cells were pretreated with EGF (100 ng/ml, 5 min), the ability of BK to enhance cAMP accumulation was significantly inhibited (Fig. 1, inset, bar D versus bar B), whereas EGF pretreatment itself had no significant effect on the basal cAMP level (inset, bar C versus bar A). In contrast, in EGF-treated A431 cells, BK further stimulated inositol phosphate formation, but at a higher level. This was probably due to additional stimulation of the phospholipase C-γ isoform by EGF (Fig. 2). Thus, EGF pretreatment of A431 cells seems to inhibit the stimulatory BK effect on the cAMP system, but not phosphorylase breakdown, which are activated via the separate G proteins \( G_{\alpha} \) and \( G_{\gamma} \), respectively (13, 19).

**Effect of EGF on Stimulation of Adenylate Cyclase Activity in A431 Membranes—** Adenylate cyclase activity was measured in A431 membranes prepared from cells after treatment with EGF (100 ng/ml, 5 min). In these membranes, indeed, the stimulation of adenylate cyclase activity by NaF (0.1 mM), GTPγS (10 μM), Gpp(NH)p (10 μM), or BK (1 μM) was reduced compared with the effects in membranes prepared from A431 cells not treated with EGF (Fig. 3). The stimulatory effect of forskolin on adenylate cyclase did not significantly differ under either condition (Fig. 3). These results suggest that EGF interferes with activators of adenylate cyclase at the level of the \( G_{\alpha} \) protein, which is involved in the activation of adenylate cyclase activity by BK in A431 cells (13).

**Influence of EGF Pretreatment of A431 Cells on Functional Activation of \( G_{\alpha} \) by BK—** As shown in Fig. 4, under our assay conditions, BK caused a concentration-dependent and biphasic increase in [35S]GTPγS binding to A431 membranes. For the...
Compared with the effect of BK in the presence of anti-

$\text{p}$, diminished in the membranes from EGF-treated A431 cells (*, different cell preparations. The indicated effects were significantly di-

experiments in triplicate determinations with membranes from three

completely abolished the second phase of $^{35}$S binding to A431 membranes. Isoprenaline was found to stimulate

adenylate cyclase activity in A431 membranes prepared from EGF-pretreated cells (13). Pretreatment of A431 membranes with the anti-

the BK-induced stimulation of adenylate cyclase activity (13).

Stimulation of adenylate cyclase activity in A431 mem-

brane preparations from EGF-pretreated cells. Adenylate cyclase activity was measured as described under “Experimental Procedures” using membranes prepared from A431 cells pretreated with EGF (100 ng/ml, 5 min) (●) and membranes from untreated cells (□). Membranes were stimulated with 0.1 mM NaF, 10 $\mu$M GTPγS, 10 $\mu$M Gpp(NH)p, 1 $\mu$M BK, or 0.1 mM forskolin and compared with the basal activity of adenylate cyclase. Bars represent the means ± S.E. of three separate experiments in triplicate determinations with membranes from three different cell preparations. The indicated effects were significantly diminished in the membranes from EGF-treated A431 cells (*, $p < 0.05$; **, $p < 0.01$ (Student’s $t$ test)) compared with the respective controls.

first increase, half-maximal and maximal stimulations (up to 155% of control) were observed with −0.3 and 1 nM BK, respecti-

ly. At higher BK concentrations, $^{35}$S binding was continuously reduced, but was followed by a second increase at BK concentrations of −30 nM for half-maximal binding and 100 nM for maximal binding (up to 175% of control). The first as well as the second increase in $^{35}$S binding induced by BK were completely abolished in the presence of the bradyki-

nin B2 receptor antagonist Hoe 140, indicating that both effects are mediated via the same BK receptor type (Table I). The reasons for the biphasic curve shape in the BK-stimulated $^{35}$S binding to A431 membranes are not yet known. It should be noted that the basal binding of $^{35}$S binding to membranes prepared from EGF-treated cells was significantly enhanced (24.0 ± 1.0 fmol/mg of protein; $n = 6$) compared with that to membranes from untreated cells (18.5 ± 2.2 fmol/mg of protein). To identify the part of the complex pattern of BK-induced $^{35}$S binding to A431 membranes that may correspond to the activation of $\alpha_s$ subunits, we studied the effect of anti-$\alpha_s$ antiserum AS 348 in this assay. AS 348 was raised against the C-terminal region of $G_s$, corresponding to amino acids 385–394 (20), and has been successfully used in Western blots and to prevent

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TABLE I

| Additions       | Stimulation of [35S]GTP\(\gamma\)S binding (fmol/mg protein) |
|-----------------|-------------------------------------------------------------|
| None (basal)    | 18.5 ± 2.2                                                  |
| BK (0.5 nM)     | 27.8 ± 3.2*                                               |
| + Hoe 140       | 17.7 ± 1.9                                                |
| BK (100 nM)     | 33.5 ± 7.3*                                               |
| + Hoe 140       | 18.1 ± 1.2                                                |

*Significantly different from BK alone (p < 0.01; Student’s t test).

**Fig. 6. Identification of Gs\(\alpha\) protein after immunoprecipitation with AS 348.** A431 cell membranes were subjected to immunoprecipitation, SDS-PAGE, and immunoblotting. Immunoprecipitation with anti-\(\alpha\)s antisera AS 348 was performed as described under “Experimental Procedures.” The positions of the 40- and 50-kDa marker proteins are shown on the right. A431 membranes were lysed and subjected to immunoprecipitation with AS 348 (lane 2), with AS 348 in the presence of an excess of the antigenic peptide RMHLRQYELL (lane 1), or with nonimmune serum (lane 3) and subsequently blotted with AS 348. The strong band at ~55 kDa represents Ig heavy chains bleeding from the beads despite coupling.

**Fig. 7. Detection of tyrosine-phosphorylated Gs\(\alpha\) with anti-phosphotyrosine antibodies.** A431 cells were treated with EGF or not as indicated. Membrane preparation, immunoprecipitation with anti-\(\alpha\)s antisera AS 348 or nonimmune serum, and SDS-PAGE were performed as described under “Experimental Procedures.” After blotting, the nitrocellulose strips were developed with monoclonal anti-phosphotyrosine (PY) antibodies (RC20). Shown are the autoradiograms of control membranes and membranes prepared from EGF-pretreated A431 cells. A, immunoprecipitation with AS 348; B, immunoprecipitation with AS 348, but immunoblotting with RC20 in the presence of 1 mM phosphotyrosine (PY); C, immunoblotting with nonimmune serum (NIS).

class by BK at the level of Gs\(\alpha\), we investigated the possibility that the EGF effect is mediated by tyrosine phosphorylation of Gs\(\alpha\). At first, we checked whether anti-\(\alpha\)s antisera AS 348, which has been successfully used for immunoprecipitation of photolabeled \(\alpha\)s subunits in human thyroid membranes (24), may also be useful for immunoprecipitation of Gs\(\alpha\) in A431 cells. Western blots showed that AS 348 recognizes two forms of Gs\(\alpha\) in A431 cells with molecular masses of 45 and 52 kDa (13). Immunoprecipitation with protein A-Sepharose-coupled AS 348 antibodies followed by immunoblotting with AS 348 clearly detected the 45-kDa \(\alpha\)s subunit. The long splice variant of \(\alpha\)s (52 kDa) was only weakly present and could be superimposed by the heavy chain of the antibodies (Fig. 6, lane 2). AS 348 antibodies blocked by the antigenic peptide (Fig. 6, lane 1) as well as nonimmune serum (lane 3) did not precipitate the 45-kDa protein, demonstrating that immunoprecipitation with the employed antisera AS 348 is indeed specific. As shown in Fig. 7A, in membranes from EGF-pretreated cells, but not in those from untreated cells, the 45-kDa protein immunoprecipitated with AS 348 was also recognized on Western blots by monoclonal anti-phosphotyrosine antibodies. Anti-phosphotyrosine antibodies failed to detect the 45-kDa band in both membrane fractions after immunoblotting in the presence of an excess of unlabeled phosphotyrosine (Fig. 7B) or after immunoprecipitation with nonimmune serum (Fig. 7C), indicating the specificity of anti-phosphotyrosine detection. Another set of experiments demonstrated that EGF-induced tyrosine phosphorylation of Gs\(\alpha\) was not detectable when EGF receptor tyrosine kinase was inhibited by the EGF receptor-specific tyrosinase AG 1478 (Fig. 8). In an alternative approach, A431 cells were metabolically labeled with [32P]Pi, and then stimulated with EGF (100 ng/ml), followed by immunoprecipitation with anti-\(\alpha\)s antisera AS 348. In AS 348 immunoprecipitates, but not in control precipitates with nonimmune serum, a 45-kDa and a 52-kDa phosphoprotein were detectable, which most likely represent the two phosphorylated isoforms of Gs\(\alpha\). After treatment with EGF, immunoprecipitated Gs\(\alpha\) was more strongly phosphorylated (1.5-fold increase in radioactivity)
EGF-induced Tyrosine Phosphorylation of Gsα in A431 Cells

In this study, we present evidence that, in A431 cells, EGF induces tyrosine phosphorylation of Gsα. To our knowledge, this is the first demonstration of Gsα tyrosine phosphorylation in vivo. The use of two different approaches to detect tyrosine phosphorylation of Gsα produced complementary sets of data. First, monoclonal anti-phosphotyrosine antibodies are able to recognize Gsα immunoprecipitates prepared from EGF-pre-treated A431 cells. The specificity of this result is demonstrated by results from parallel experiments designed as controls: anti-phosphotyrosine antibodies failed to identify Gsα (i) in non-stimulated cells, (ii) in the presence of the EGF receptor tyrosine kinase inhibitor AG1478, (iii) in the presence of an excess of unlabeled phosphotyrosine, and (iv) after precipitation with non-immune serum. Second, phosphoamino acid analysis of immunoprecipitated Gsα after in vivo labeling of A431 cells with [32P]P, indicated that EGF pretreatment results in the specific appearance of phosphotyrosine, which was absent in control cells. In both control and EGF-treated cells, a relatively large amount of phosphorylated serine and threonine was detected in Gsα. The serine and threonine phosphorylation was somewhat enhanced in Gsα from EGF-pretreated A431 cells compared with the basal levels in nonstimulated cells.

Concomitantly with EGF-induced tyrosine phosphorylation of Gsα, we detected effects of EGF on Gsα function with three different approaches including the influence of EGF on BK-induced G protein activation, on cAMP accumulation, and on adenylylate cyclase activation. The quantitation of [35S]GTPγS binding to membranes accounts for receptor-induced activation of G proteins. In control cells, [35S]GTPγS binding to A431 membranes was stimulated in a concentration-dependent manner by both BK and isoprenaline. The effect of isoprenaline was completely and the effect of BK was partly abolished in membranes prepared from A431 cells pretreated with EGF. In the case of BK, two lines of evidence indicate that the αs-mediated

DISCUSSION

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Concomitantly with EGF-induced tyrosine phosphorylation of Gsα, we detected effects of EGF on Gsα function with three different approaches including the influence of EGF on BK-induced G protein activation, on cAMP accumulation, and on adenylylate cyclase activation. The quantitation of [35S]GTPγS binding to membranes accounts for receptor-induced activation of G proteins. In control cells, [35S]GTPγS binding to A431 membranes was stimulated in a concentration-dependent manner by both BK and isoprenaline. The effect of isoprenaline was completely and the effect of BK was partly abolished in membranes prepared from A431 cells pretreated with EGF. In the case of BK, two lines of evidence indicate that the αs-mediated
stimulation of \(^{[35S]}\)GTP\(\gamma\)S binding is prevented specifically by EGF. In A431 cells pretreated with EGF, only the G\(_{\alpha}\)-mediated stimulation of cAMP accumulation by BK was abolished, whereas the BK-induced stimulation of inositol phosphate formation via G\(_{\alpha}\) was not. These findings provide an indirect explanation that only one part of the biphasic stimulation of \(^{[35S]}\)GTP\(\gamma\)S binding by BK was prevented by EGF. Furthermore, using anti-\(\alpha\)-antiserum AS 348, we can show that the part of BK-stimulated \(^{[35S]}\)GTP\(\gamma\)S binding that is missing in EGF-pretreated A431 cells exactly corresponds to that part of \(^{[35S]}\)GTP\(\gamma\)S binding that is likewise missing in the presence of AS 348.

In general, higher BK concentrations are necessary for both stimulation of adenylate cyclase (EC\(_{50} \sim 180\) nM) and stimulation of \(^{[35S]}\)GTP\(\gamma\)S binding (EC\(_{50} \sim 30\) nM) compared with BK-induced cAMP accumulation in intact cells (EC\(_{50} \sim 2\) nM). On the one hand, this might be the result of very different in vitro assay conditions required for optimal BK receptor binding (e.g., pH 6.8) and optimal conditions for adenylate cyclase measurement or \(^{[35S]}\)GTP\(\gamma\)S binding (e.g., pH 8.0) as described previously (13, 25). On the other hand, there are well known examples of dual signaling elicited by a single receptor, but different agonist concentrations. Thus, the luteinizing hormone receptor requires a 25-fold higher ligand concentration for stimulation of inositol phosphate formation compared with that for stimulation of adenylate cyclase in membranes (26). The inverse relation has been described for tachykinin receptor-mediated stimulation of inositol phosphate formation compared with stimulation of adenylate cyclase in membranes (26). These data dem-

Taken together, we present evidence suggesting that, in A431 cells, a bidirectional cross-talk exists between the signal transduction pathways of BK and EGF. On the one hand, in a fast reaction, BK is able to activate protein kinase C via G\(_{\alpha}\)/phospholipase C\(\beta\), subsequently leading to serine/threonine phosphorylation of the EGF receptor and thereby its desensitization (14, 19). In a slow reaction, BK can counteract this protein kinase C activation via a G\(_{\alpha}\)-adenylate cyclase-mediated pathway (13), which might allow re sensitization of the EGF receptor. On the other hand, EGF is capable of inducing tyrosine phosphorylation of G\(_{\alpha}\) and of preventing the G\(_{\alpha}\)-mediated activation of the cAMP pathway by BK. Further examples of cross-talk between G protein-coupled receptor pathways and receptor tyrosine kinases have been described recently. EGF-induced tyrosine phosphorylation of protein kinase C\(\beta\) in keratinocytes resulting in its inactivation was reported (28), suggesting a cross-talk between the EGF receptor and the protein kinase C pathway. Also very recently, Daub et al. (29) reported that the EGF receptor in Rat-1 fibroblasts is rapidly tyrosine-phosphorylated in response to the G protein-coupled receptor agonists endothelin and thrombin. These authors postulated that tyrosine kinases contribute in a general or cell-specific way to G protein receptor-mediated mitogenic signaling. Our results emphasize a differential recruitment of G\(_{\alpha}\) by G protein-coupled receptors and the EGF receptor as a novel cross-talk mechanism.

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REFERENCES

1. Koich, W., Heidecker, G., Kochs, G., Hummel, B., Vahidi, H., Mischak, H., Chau, J., Sonnenberg, G., Winter, M., and Niekamp, U. R. (1995) Nature 376, 249–252
2. Blumer, K. J., and Johnson G. L. (1994) Trends Biochem. Sci. 18, 236–240
3. Cook, S. J., and McCormick, F. (1993) Science 262, 1069–1072
4. Nair, B. G., Parikh, B., Milligan, G., and Patel, T. B. (1996) J. Biol. Chem. 271, 21317–21322
5. Nair, B. G., and Patel, T. B. (1993) Biochem. Pharmacol. 46, 1239–1245
6. Liang, M., and Garrison, J. C. (1991) J. Biol. Chem. 266, 13342–13349
7. Teitelbaum, I. (1990) J. Biol. Chem. 265, 4218–4222
8. Krupinsky, J., Rajaram, R., Lakonishok, M., Benovic, J. L., and Cerione, R. A. (1988) J. Biol. Chem. 263, 12333–12341
9. Hazboun, M., P. W., Pitcher, J., Lettrill, R. D. K., Lindner, M. E., Kurro, H., Parsyns, S. C., Caron, M. G., and Lefkowitz, R. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5720–5724
10. Moyer, J. S., Lindner, M. E., Shannon, J. D., and Parsons, S. J. (1995) Biochem. J. 306, 411–417
11. Reus-Domiano, S., and Hamm, H. (1995) FASEB J. 10, 1056–1066
12. Poppleton, H., Sun, H., Fulgham, D., Bertsch, P., and Patel, T. B. (1996) J. Biol. Chem. 271, 6947–6951
13. Liebmann, C., Schnittler, M., Nawrath, S., and Jakobs, K. H. (1991) Eur. J. Biochem. 207, 67–71
14. Tilly, B. C., van Paridou, P. A., Verlaan, I., Wirtz, K. W. A., de Laat, S. W., and Molenaar, W. H. (1987) Biochem. J. 244, 129–135
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Schiltz, G., and Jakobs, K. H. (1983) in Methods in Enzymatic Analysis, (Bergmeyer, H. H., Bergmeyer, J., and Gill, M., eds) Vol. IV, pp. 369–388, Verlag Chemie, Weinheim, Germany
18. Liebmann, C., Schnittler, M., Nawrath, S., and Jakobs, K. H. (1991) Eur. J. Pharmacol. Mol. Pharmacol. Sect. 207, 67–71
19. Tilly, B. C., van Paridou, P. A., Verlaan, I., Wirtz, K. W. A., de Laat, S. W., and Molenaar, W. H. (1987) Biochem. J. 244, 129–135
20. Spicher, K., Kalkbrenner, F., Zobel, A., Herhammer, R., Nürnberg, B., Söhlke, A., and Schultz, G. (1994) *Biochem. Biophys. Res. Commun.* 198, 906–914
21. Nürnberg, B., Spicher, K., Herhammer, R., Besserhoff, A., Frank, R., Hilz, H., and Schultz, G. (1994) *Biochem J.* 300, 387–394
22. Laugwitz, K.-L., Spicher, K., Schultz, G., and Offermanns, S. (1994) *Methods Enzymol.* 237, 283–294
23. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* 201, 110–149
24. Allgeier, A., Offermanns, S., Van Sande, J., Spicher, K., Schultz, G., and Dumont, J. E. (1994) *J. Biol. Chem.* 269, 13733–13735
25. Liebmann, C., Mammery, K., and Granell, A. (1994) *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* 288, 35–43
26. Gudermann, T., Birnbaumer, M., and Birnbaumer, L. (1992) *J. Biol. Chem.* 267, 4479–4486
27. Nakajima, Y., Touchida, K., Negishi, M., Ito, S., and Nakanishi, S. (1992) *J. Biol. Chem.* 267, 2437–2442
28. Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T., and Yuspa, S. H. (1996) *J. Biol. Chem.* 271, 5325–5331
29. Daub, H., Weise, F. U., Wallasch, C., and Ullrich, A. (1996) *Nature* 379, 557–560