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A Novel Mitogenic Signaling Pathway of Bradykinin in the Human Colon Carcinoma Cell Line SW-480 Involves Sequential Activation of a $G_{q/11}$ Protein, Phosphatidylinositol 3-Kinase β, and Protein Kinase Cε*

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The signaling routes connecting G protein-coupled receptors to the mitogen-activated protein kinase (MAPK) pathway reveal a high degree of complexity and cell specificity. In the human colon carcinoma cell line SW-480, we detected a mitogenic effect of bradykinin (BK) that is mediated via a pertussis toxin-insensitive G protein of the $G_{q/11}$ family and that involves activation of MAPK. Both BK-induced stimulation of DNA synthesis and activation of MAPK in response to BK were abolished by two different inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin and LY 294002, as well as by two different inhibitors of protein kinase C (PKC), bisindolylmaleimide and Ro 31-8220. Stimulation of SW-480 cells by BK led to increased formation of PI3K lipid products (phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate) and to enhanced translocation of the PKCε isoform from the cytosol to the membrane. Both effects of BK were inhibited by wortmannin, too. Using subtype-specific antibodies, only the PI3K subunits p110β and p85, but not p110α and p110γ, were detected in SW-480 cells. Finally, p110β was found to be co-immunoprecipitated with PKCε. Our data suggest that in SW-480 cells, (i) dimeric PI3Kβ is activated via a $G_{q/11}$ protein; (ii) PKCε is a downstream target of PI3Kβ mediating the mitogenic signal to the MAPK pathway; and (iii) PKCε associates with the p110 subunit of PI3Kβ. Thus, these results add a novel possibility to the emerging picture of multiple pathways linking G protein-coupled receptors to MAPK.

G protein-coupled receptors mediate effects of peptide hormones and neurotransmitters on intermediary metabolism as well as play an important role in the regulation of cell growth and differentiation. Similar to receptor tyrosine kinases, they initiate signaling pathways that finally activate members of the mitogen-activated protein kinase (MAPK)[1] family. One MAPK subfamily, which includes the extracellular signal-regulated kinases Erk1 and Erk2, is stimulated via a consecutive activation of the protein kinases Raf and MEK. The MAPK cascade is initially switched on via activation of the low molecular mass GTP-binding protein Ras. GTP-bound Ras associates the proximal kinase Raf to the plasma membrane, resulting in its activation.

Several signal transduction pathways from G protein-coupled receptors to MAPK have been proposed that may be classified according to the type of G protein involved (for review, see Refs. 1 and 2). Thus, MAPK activation via pertussis toxin (PTX)-sensitive G_i protein-coupled receptor, such as the m_2 muscarinic receptor, was found to be mediated by $G_{\beta\gamma}$ subunits, phosphatidylinositol 3-kinase γ (PI3Kγ), and Ras (3). In contrast, receptors coupled to G proteins of the PTX-insensitive $G_{q/11}$ family, such as the m_1 muscarinic receptor, mediate MAPK activation via a G_α subunit that is Ras-independent and may involve PKC (4). Once activated, the different PKC isoforms, with the exception of PKCζ, activate the MAPK cascade at the level of Raf (5), but may also involve tyrosine kinases of the Src family (6, 7). MAPK activation by PTX-sensitive G_o proteins appears to be independent of $G_{\beta\gamma}$ and Ras, but requires PKC (8). G_s-coupled receptors such as the β-adrenergic receptor were found to exert a dual effect on MAPK involving $G_{\beta\gamma}$-mediated activation and cAMP-mediated inhibition (9). Alternatively, Ullrich and co-workers (10–12) have suggested an epidermal growth factor receptor transactivation by both G_i- and $G_{q/11}$-coupled receptors as an essential prerequisite for MAPK activation. They propose an epidermal growth factor receptor tyrosine phosphorylation by G protein-coupled receptors as the key event, which might be mediated by cytosolic tyrosine kinases such as Src and PYK2.

In addition to receptor tyrosine kinases and PKC, PI3Ks appear to be key signaling enzymes implicated in the regulation of receptor-stimulated mitogenesis. After activation, they preferentially utilize phosphatidylinositol 4,5-bisphosphate as substrate, which is phosphorylated to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), followed by rapid degradation to PtdIns(3,4)P_2. Both molecules have been proposed to act as second messengers. Recent studies indicate that both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 can directly activate certain PKC isoforms and the serine/threonine-protein kinase Akt/PKB (for review, see Refs. 13 and 14). In terms of mode of regulation, class I members are subdivided into receptor tyrosine kinase-associated (class I_A) or G protein-coupled receptor-activated (class I_B) PI3Ks (for review, see Ref. 15). The class I_A types have been structurally characterized as a heterodimer consisting of a 110-kDa catalytic subunit (p110) and an 85-kDa

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; PTX, pertussis toxin; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; BK, bradykinin; PVDF, polyvinylidene difluoride; CTX, cholera toxin; BSA, bovine serum albumin; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino}ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

regulatory subunit (p85). They are stimulated through receptors with intrinsic or associated tyrosine kinase activity that bind to the p85 subunit, thereby inducing PI3K activity. The only known class I_B member (termed PI3Kγ) consists of a p110 catalytic subunit that lacks the binding site for p85, but is associated with a p101 non-catalytic subunit (16). The p110γ catalytic subunit is directly stimulated by $\beta\gamma$-complexes of G proteins (17). G_α subunits of G_i (but not G_q or G_{12}) proteins only moderately activate p110γ (17, 18). The functional discrimination of class I_A and I_B members was questioned very recently since, *in vitro*, PI3Kβ has been shown to respond synergistically to both $G_{\beta\gamma}$ and a synthetic phosphotyrosyl peptide that binds to the SH2 domain of p85 (19). These and other studies (20, 21) suggest that also a p85/p110 PI3K may be regulated in the downstream region of pertussis toxin-sensitive G proteins.

In this report, we present evidence for the activation of p85/p110β by the G_q protein-coupled bradykinin receptor in intact human colon carcinoma SW-480 cells. In addition, we obtained results showing that protein kinase Cϵ is a mediator connecting PI3Kβ with the MAPK signaling cascade in this endothelial cell line.

EXPERIMENTAL PROCEDURES

Materials—[[3,4-$^3H_2$]Pro^{3,4}]Bradykinin ([^{3}H]BK; 102 Ci/mmol), *myo*-[2-^{3}H]inositol (20.5 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and $^{32}P_i$ (8500–9120 Ci/mmol) were obtained from (NEN Life Science Products). [^{3}H]Thymidine (2.0 Ci/mmol), the reagents for SDS-polyacrylamide gel electrophoresis, Hybond PVDF membranes, and the ECL Western blotting detection system were purchased from Amersham Pharmacia Biotech. BK, captopril, cholera toxin (CTX), PTX, forskolin, aprotinin, bacitracin, leupeptin, bovine serum albumin (BSA), myelin basic protein, sodium orthovanadate, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, dithiothreitol, EGTA, ATP, Nonidet P-40, protein A-Sepharose, peroxidase-conjugated goat anti-rabbit IgG, HEPES, TES, 3-isobutyl-1-methylxanthine, Tween 20, and diagnostic film (Biomax, Eastman Kodak Co.) were obtained from Sigma (Deisenhofen, Germany). Bisindolylmaleimide was purchased from Boehringer (Mannheim, Germany). Wortmannin and LY 294002 were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). Ammonium formate and sodium tetraborate were from Serva (Heidelberg, Germany). Polyclonal antibodies against p44 MAPK; the PI3K subunits p110α and p110β; and the PKC isoforms δ, ϵ, and ζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Generation of the monoclonal antibody against the PI3K p110γ subunit was detailed elsewhere (17). Ro 31-8220 was a generous gift from Dr. D. Bradhaw (Roche, Welwyn Garden City, United Kingdom). The bradykinin B_2 receptor antagonist FR 173657 was kindly provided by Dr. N. Inamura (Fujisawa Pharmaceutical Co., Osaka, Japan). Anti-p85α and anti-p85β antisera were gifts from Dr. B. Vanhaesebroeck (Ludwig Institute for Cancer Research, London, UK).

Cell Culture and Membrane Preparation—Human colon adenocarcinoma SW-480 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in humidified air with 5% CO_2 at 37 °C. For stimulation experiments, SW-480 cells were grown in 6-well dishes, and for [^{3}H]thymidine incorporation, they were grown in 24-well dishes and treated as indicated in the figure legends. An SW-480 particulate fraction (referred to as "membranes") was prepared by resuspending cells in 50 mM HEPES, pH 7.5, and centrifuging at 100,000 $\times$ *g* for 20 min at 4 °C. The pellets were resuspended in 50 mM HEPES, pH 7.5, containing bacitracin (100 μg/ml), phenylmethylsulfonyl fluoride (0.1 mM), and leupeptin (2 μg/ml) and were stored at −80 °C. Protein concentration was determined according to Bradford (22).

[^{3}H]Thymidine Incorporation—Subconfluent cells were deprived of serum for 24 h and then treated with bradykinin (10 nM) with or without the respective inhibitors as indicated. The cells were incubated for another 24 h, followed by the addition of [^{3}H]thymidine (1 μCi/ml, 2 μM) for 12 h. Finally, cells were filtered through Whatman GF/C glass-fiber filters using a Brandel harvester and washed three times with 5 ml of 10 mM HEPES, pH 7.4. The filters were dried, and the cells were counted for incorporated radioactivity by liquid scintillation counting.

[^{3}H]Bradykinin Binding—The bradykinin receptor binding assay was performed as described previously (23) with some modifications. SW-480 membranes (~400 μg of protein/tube) were incubated with [^{3}H]BK (0.6–0.8 nM) in 1 ml of a medium containing 25 mM TES, pH 6.8, 1 mM 1,10-phenanthroline, 140 μg/ml bacitracin, 1 mM dithiothreitol, 10 μM captopril, and 0.1% BSA. After incubation at 4 °C for 30 min (equilibrium conditions), the samples were quickly filtered through Whatman GF/B glass-fiber filters pretreated with 0.1% aqueous polyethyleneimine using a Brandel harvester. The filters were washed with 3 $\times$ 5 ml of ice-cold 10 mM TES, pH 6.8; dried; and counted for radioactivity (Quickszint 501, Zinsser, Frankfurt, Germany). Nonspecific binding was determined in the presence of 1 μM unlabeled bradykinin. Specific binding of [^{3}H]BK was in the range 40–60%.

Phosphatidylinositol Turnover—Determination of total inositol phosphates was performed as described previously (23). In brief, SW-480 cells in 24-well plates were prelabeled with 4 μCi/ml *myo*-[^{3}H]inositol for 24 h. At 2 h prior to stimulation, the cells were incubated in serum-free medium containing 20 mM HEPES, pH 7.4, and 1 μM captopril. The cells were stimulated with increasing concentrations of bradykinin, as indicated, in the presence of LiCl for 10 min. For termination, the medium was replaced by 1 ml of 10% trichloroacetic acid. After 10 min, the extracts were collected, and the trichloroacetic acid was removed by washing four times with 2 volumes of water-saturated diethyl ether. After neutralization by adding Tris base, the samples were diluted to 4 ml with distilled water. The inositol phosphate fractions containing inositol mono-, bis-, and trisphosphates were obtained by eluting five times with 2 ml of 1.0 M ammonium formate and 0.1 M formic acid from AG 1-X8 columns (200–400 mesh, formate form; Bio-Rad). Radioactivity of the inositol phosphate-containing fractions was determined by liquid scintillation counting.

Measurement of p44 MAPK (Erk1) Activity—SW-480 cells were preincubated in serum-free RPMI 1640 medium for 2 h and then treated with the different inhibitors and/or BK as indicated in the figure legends. After stimulation, cells were scraped off and centrifuged for 1 min at 5000 $\times$ *g*. The medium was removed, and the pellets were lysed in 1 ml of lysis buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). After a 30-min incubation on ice, the lysates were centrifuged (10 min, 15,000 $\times$ *g*, 4 °C) to pellet insoluble material. The supernatants were transferred into new tubes, and Erk1 was immunoprecipitated using a rabbit polyclonal antibody (1 μg/ml of lysate) from Santa Cruz Biotechnology. The immunoprecipitates were subsequently washed with phosphate-buffered saline containing 1% Triton X-100 and 2 mM orthovanadate; Tris-HCl, pH 7.5, containing 0.5 M LiCl; and kinase buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, and 0.5 mM orthovanadate). Phosphorylation of immunoprecipitates was performed in 30 μl of kinase buffer supplemented with 1 μCi of [γ-^{32}P]ATP, 20 μM ATP, 1.5 mg/ml myelin basic protein, and 3.3 μM dithiothreitol. After 20 min at 30 °C, the reaction was terminated by the addition of 10 μl of SDS-polyacrylamide gel electrophoresis buffer. The samples were boiled for 5 min and analyzed by SDS gel electrophoresis on 12% (w/v) gels. The dried gels were autoradiographed, and the radioactivity incorporated into myelin basic protein was quantified using a PhosphorImager (NIH Image Version 1.57).

PKC Translocation—For the measurement of PKC translocation, SW-480 cells were subjected to serum-free RPMI 1640 medium for 2 h before stimulation. The cells were then exposed to BK (100 nM) for 5 min at 37 °C. For several experiments, cells were pretreated with the PI3K inhibitor wortmannin for 30 min. The incubation was terminated by removing the cells and centrifuging at 20,000 $\times$ *g* for 1 min at 4 °C. The pellets were resuspended in 50 mM HEPES, pH 7.4, containing bacitracin (100 μg/ml), phenylmethylsulfonyl fluoride (0.1 mM), pepstatin A (1 μg/ml), and leupeptin (2 μg/ml) and were stored at −80 °C. Protein concentration was determined according to Bradford (22) with BSA as a standard. For Western blot analysis, these membranes were separated on 7.5% gels by SDS-polyacrylamide gel electrophoresis and transferred to Hybond PVDF membranes. After blocking in 1% (w/v) BSA and 1% (w/v) nonfat dried milk powder overnight, the PVDF strips were incubated with the PKC antibodies as indicated (1 μg/ml of the blocking solution). The strips were washed twice with Tris-buffered saline, pH 7.6, containing 0.05% (v/v) Tween 20; incubated for 45 min with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology); and washed again four times as described above. Secondary antibodies were detected using the ECL Western blotting detection system by exposure to Biomax films.

Immunoprecipitation of PKCϵ and Western Blot Analysis—Cell lysates prepared as described above were incubated with anti-PKCϵ an-

tibody (1 μg/ml) at 4 °C for 2 h on a rotating drive. Antigen-antibody complexes were recovered using protein A-Sepharose. The immunoprecipitates were washed three times with phosphate-buffered saline, pH 7.4, containing 1% Triton X-100 and 2 mM vanadate; resuspended in 50 μl of electrophoresis sample buffer, boiled for 3 min; and subjected to SDS-polyacrylamide gel electrophoresis using a 7.5% gel, followed by transfer to PVDF membranes. After blocking overnight with 3% nonfat dried milk in Tris-buffered saline and 0.5 M NaCl, PVDF blots were incubated with the appropriate primary antibodies (Santa Cruz Biotechnology), and horseradish peroxidase-conjugated anti-rabbit IgG was used for detection with the ECL system.

$^{32}P_i$ Labeling of SW-480 Cells and Analysis of Phosphatidylinositol Phosphates—PI3K lipid kinase activity was determined using the method of Stephens *et al.* (20) with minor modifications. Briefly, SW-480 cells were freshly isolated; washed two times with phosphate-free RPMI 1640 medium; and incubated for 1 h in phosphate-free RPMI 1640 medium containing 25 mM HEPES, pH 7.5, 1 mg/ml fatty acid-free BSA, and 10% fetal calf serum. The SW-480 cells were then labeled overnight with 100 μCi of $^{32}P_i$/dish (6×10^6 cells/2 ml). After labeling, cells were washed two times with 140 mM NaCl, 5 mM KCl, 2.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.06 mM $MgSO_4$, 15 mM HEPES, 5.6 mM glucose, and 0.1% BSA, pH 7.2, at 37 °C; centrifuged at 1200 rpm for 5 min; resuspended in 0.5 ml of the above buffer; and treated with BK as indicated. Reactions were terminated by the addition of 1 ml of ice-cold 2.4 N HCl. Then, 1 ml of chloroform/methanol/HCl (1:2:1), 0.75 ml of chloroform/phosphoinositide mixture (with 10 μg of phosphoinositide mixture/point; Sigma), and 1 ml of chloroform were added subsequently. The mixture was thoroughly vortexed, and phase separation was performed by a short centrifugation (2500 rpm, 4 min). The lower chloroform phase was transferred to a new vial, and the upper phase was re-extracted twice with 1.5 ml of chloroform. Pooled chloroform phases were dried, and the lipids were deacylated by incubation for 1 h in methylamine (33% (v/v) in ethanol; Fluka) at 50 °C. After removal of the methylamine, the samples were resuspended in 1 ml of water and extracted twice with 1 ml of 1-butanol. The aqueous phase containing the labeled lipid head group was analyzed by high pressure liquid chromatography as described (24).

RESULTS

Bradykinin B_2 Receptor-mediated Mitogenic Effects in SW-480 Cells—In the human colon carcinoma cell line SW-480, we detected an endogenously expressed bradykinin receptor. Binding studies with [^{3}H]BK (displacement experiments) revealed an IC_{50} value of ~1 nM (Fig. 1*A*). After prelabeling of SW-480 cells with *myo*-[^{3}H]inositol, BK induced a concentration-dependent increase in inositol phosphate formation with an EC_{50} value of ~3 nM (Fig. 1*B*). The phosphatidylinositol system represents the main signaling pathway of bradykinin B_2 receptors in most tissues or cells (25). Both the binding parameter and the dose-response curve are in a good agreement with those for other B_2 receptors (25). As in small cell lung cancer cells (26), BK exerted a mitogenic effect in SW-480 cells as measured with the thymidine incorporation assay. This effect of BK was completely blocked in the presence of the nonpeptidic bradykinin B_2 receptor antagonist FR 173657 (27), suggesting the involvement of the B_2 receptor subtype in the mitogenic action of BK (Fig. 1*C*).

Bradykinin-induced Cell Proliferation Is Mediated via the Extracellular Signal-regulated Protein Kinase/MAPK Pathway—Treatment of SW-480 cells with BK led to the immediate activation of p44 MAPK as determined using the myelin basic protein assay (Fig. 2). To investigate whether activation of the MAPK pathway is required for the induction of cell division by BK, we measured the effect of BK on thymidine incorporation in the presence of PD 098059, which inhibits the activation of MAPK by blocking the activity of MAPK kinase (MEK) (28). Under the conditions used, both the BK-induced cell proliferation and the MAPK activation by BK were completely abolished in the presence of PD 098059 (Fig. 2). It may be concluded that the proliferation of SW-480 cells in response to BK is dependent on the activation of the MAPK pathway.

Effects of CTX or PTX on MAPK Activation in Response to Bradykinin—In SW-480 cells, the BK-induced MAPK activation was insensitive to treatment with PTX (200 ng/ml) (Fig. 3*A*). The same PTX concentration was shown to effectively inhibit MAPK activation by lysophosphatidic acid in PC-12 cells (29).

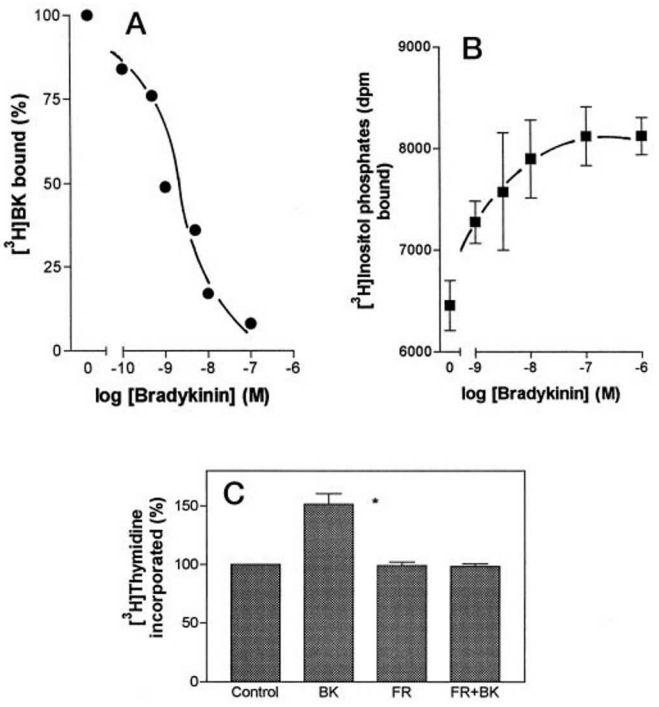

FIG. 1. **Bradykinin binding, bradykinin-induced inositol phosphate formation, and effect of bradykinin on [^{3}H]thymidine incorporation in SW-480 cells.** *A*, competition curve of unlabeled bradykinin for binding of [^{3}H]BK to SW-480 cell membranes. The calculated IC_{50} value was 1 nM. *B*, concentration-dependent stimulation of phosphatidylinositol metabolism in response to BK with a calculated EC_{50} value of 3 nM. Shown are representative experiments performed in duplicate (*A*) or quadruplicate (*B*) determinations and repeated once with similar results. *C*, for the [^{3}H]thymidine assay, serum-deprived cells were pretreated for 1 h either with vehicle (*Control*) or with the specific bradykinin B_2 receptor antagonist FR 173657 (*FR*; 1 μM), stimulated with 1 μM BK, and then assayed for [^{3}H]thymidine incorporation 36 h later as described under "Experimental Procedures." The results are expressed as the means ± S.E. from 12 wells in four separate experiments. *, significantly different compared with control values ($p < 0.05$; Student's *t* test).

Besides G_i and $G_{q/11}$ proteins, immunoblotting experiments with specific antibodies (Santa Cruz Biotechnology) revealed the presence of G_s, G_{12}, and G_{13} proteins, whereas G_o and G_z were not detected in SW-480 cells (data not shown). To investigate whether the PTX-insensitive G_s protein might play a role in the mitogenic signaling pathway of BK, SW-480 cells were treated with CTX. The effect of BK on MAPK activity was clearly abolished by CTX (Fig. 3*B*). In addition, treatment of SW-480 cells with forskolin also prevented the activation of MAPK by BK (Fig. 3*C*). Since cAMP has been reported to inhibit MAPK in smooth muscle cells and some fibroblast cell lines (30, 31), we conclude that the permanent activated adenylate cyclase in the presence of CTX counteracts the stimulation of MAPK activity in response to BK.

Effects of BK on DNA Synthesis and MAPK Are Blocked by Both Inhibitors of PI3K and PKC—Next we tested two different inhibitors of PI3K, wortmannin and LY 294002, for their ability to affect the mitogenic action of BK in SW-480 cells. When PI3K was blocked, neither DNA synthesis (Fig. 4) nor MAPK activity (Fig. 5) was stimulated by BK, suggesting an involvement of a PI3K in the BK signaling pathway in SW-480 cells. Furthermore, two different inhibitors of PKC, bisindolylmale-

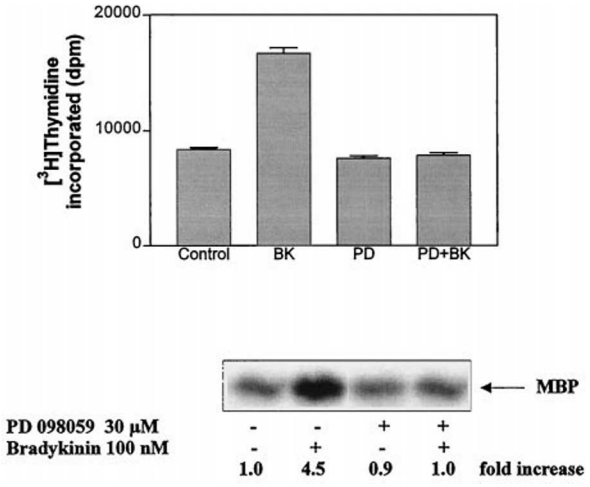

FIG. 2. **Effect of bradykinin on DNA synthesis dependent on MAPK activation.** *Upper*, SW-480 cells were treated for 36 h with 100 nM BK in the absence or presence of the MEK-1 inhibitor PD 098059 (*PD*; 30 μM). DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation as described under "Experimental Procedures." Each value is the mean ± S.E. of 12 wells representative of two independent experiments. *Lower*, lysates from SW-480 cells after stimulation with 100 nM BK in the absence or presence of PD 098059 were analyzed for MAPK activity using the myelin basic protein (*MBP*) assay. Results from one of two similar experiments are shown.

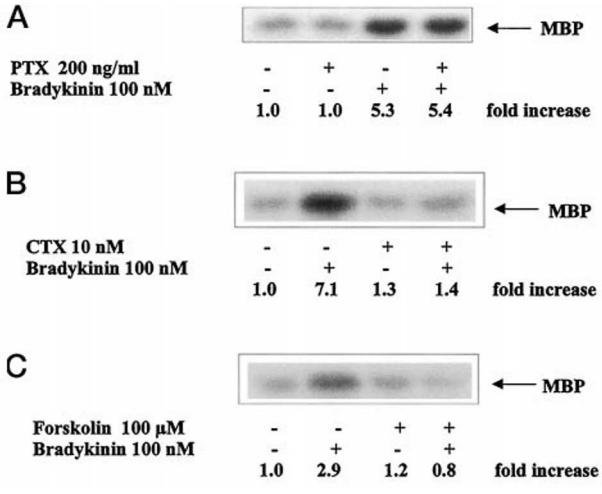

FIG. 3. **Effects of PTX, CTX, and forskolin on BK-induced activation of MAPK.** *A*, SW-480 cells were preincubated with PTX (200 ng/ml) for 24 h. The effects of PTX treatment on basal and BK-stimulated MAPK activities was determined after a 5-min exposure to BK (100 nM). *B*, SW-480 cells were treated with CTX (10 nM) for 30 min in the presence of 100 μM 3-isobutyl-1-methylxanthine, followed by the addition of BK (100 nM) for 5 min. *C*, SW-480 cells were stimulated with forskolin (100 μM, 5 min) prior to the incubation with 100 nM BK for another 5 min. MAPK activity was assessed using the myelin basic protein (*MBP*) phosphorylation assay as described under "Experimental Procedures." Shown are representative autoradiograms from two (*B* and *C*) or three (*A*) experiments with similar results.

imide and Ro 31-8220, were used to study the involvement of PKC in the mitogenic action of BK in SW-480 cells. As shown in Figs. 4 and 6, also in the presence of PKC inhibitors, BK failed to induce both stimulation of DNA synthesis and activation of MAPK, suggesting an involvement of protein kinase C in the mitogenic signaling pathway of BK in SW-480 cells as well. Taken together, these results obtained with different inhibitors and different experimental approaches indicate that a PI3K as well as a PKC are downstream mediators of the G_q protein-coupled bradykinin receptor in SW-480 cells.

Bradykinin Stimulates Accumulation of PI3K Products in

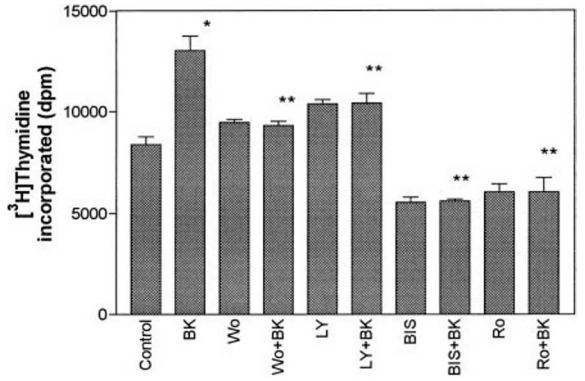

FIG. 4. **Effects of various inhibitors on BK-induced increases in [^{3}H]thymidine incorporation.** Serum-deprived SW-480 cells were preincubated for 1 h with the PI3K inhibitors wortmannin (*Wo*; 100 nM) and LY 294002 (*LY*; 3 μM) and with the PKC inhibitors bisindolylmaleimide (*BIS*; 5 μM) and Ro 31-8220 (*Ro*; 5 μM). Thereafter, BK (10 nM) was added for 36 h and [^{3}H]thymidine for 12 h of incubation. The rate of DNA synthesis was determined as described under "Experimental Procedures." The results represent the means ± S.E. from 12–15 wells in three independent experiments. *, significantly different from the control; **, significantly different compared with the BK effect in the absence of the inhibitor ($p < 0.05$; Student's t test).

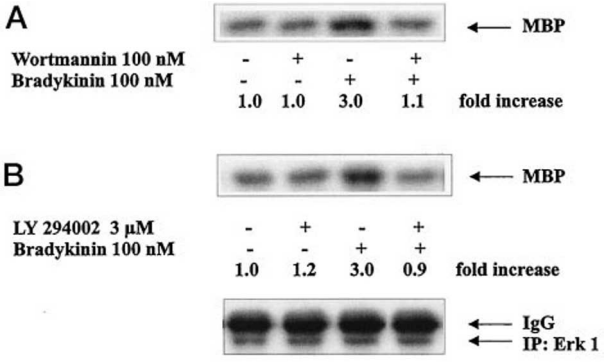

FIG. 5. **Inhibition of MAPK activation in SW-480 cells by wortmannin and LY 294002.** Serum-starved SW-480 cells were preincubated with either wortmannin (100 nM) (*A*) or LY 294002 (3 μM) (*B, upper panel*) for 30 min and then treated with BK (100 nM) for 5 min. After lysis of the cells, MAPK activity was assayed as described under "Experimental Procedures." Shown are representative autoradiograms of three to four independent experiments. A representative control blot of immunoprecipitated MAPK (*IP:Erk1*) after Western blotting with the anti-MAPK antibody is also shown (*B, lower panel*). *MBP*, myelin basic protein.

Intact SW-480 Cells—In SW-480 cells prelabeled with $^{32}P_i$, BK rapidly stimulated the accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Fig. 7). The levels of [^{32}P]PtdIns(3,4,5)P_3 and of its metabolite, [^{32}P]PtdIns(3,4)P_2, reached a maximum after 8–15 s and decreased after 1 min (data not shown). The quantity of accumulated [^{32}P]PtdIns(3,4,5)P_3 and of its metabolite, [^{32}P]PtdIns(3,4)P_2, is comparable to their pattern of accumulation in human neutrophils after stimulation with fMet-Leu-Phe (20). In SW-480 cells pretreated with wortmannin, BK failed to stimulate lipid kinase activity.

SW-480 Cells Contain p85/p110 PI3Kβ—To investigate which subtype of class I PI3Ks may be activated by BK we analyzed SW-480 cell lysates by Western blotting using specific antibodies against the catalytic subunits p110α, p110β, and p110γ and against the regulatory subunits p85α and p85β. Fig. 8 shows that in SW-480 cells, only p110β and the p85α and p85β subunits exhibited significant expression, whereas p110α and p110γ were not detectable by immunoblotting. Thus, heterodimeric PI3Kβ, but not monomeric PI3Kγ, appears to be the target of the bradykinin receptor-stimulated G_q protein.

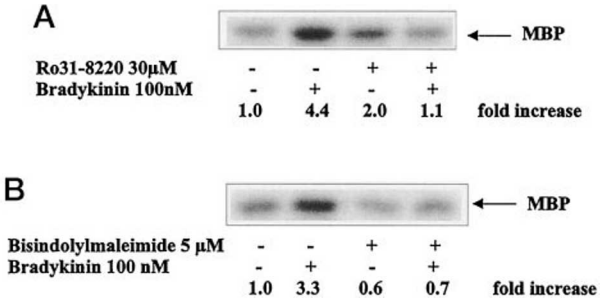

FIG. 6. **Activation of MAPK in SW-480 cells is inhibited by bisindolylmaleimide and Ro 31-8220.** SW-480 cells were pretreated with either 30 μM Ro 31-8220 (*A*) or 5 μM bisindolylmaleimide (*B*) for 30 min, followed by the addition of BK (100 nM) for 5 min. The cells were then lysed, and MAPK activity was determined as described under "Experimental Procedures." Shown are autoradiograms that are representative of the results obtained in three to four separate experiments. *MBP*, myelin basic protein.

PKCε May Be a Mediator Connecting PI3Kβ with the MAPK Cascade—Among the different PKC isoforms, the novel PKCε, PKCδ, and PKCη as well as the atypical PKCζ have been demonstrated to be activated by PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 *in vitro* (32, 33). Western blotting of whole cell extracts established that SW-480 cells express the PKC isoforms ε, δ, and ζ. For activation studies, we measured the stimulus-induced translocation of PKC from the cytosol to the plasma membrane. Following the kinetics of BK-induced translocation of PKC isoforms in other cells (29, 33) SW-480 cells were stimulated with 100 nM BK for 5 min. Throughout the repeated experiments, only PKCε showed an increased membrane association when cells were triggered with BK. The BK-induced translocation of PKCε was completely abolished in the presence of wortmannin (Fig. 9), suggesting that activation of PKCε is a downstream event of the BK-induced activation of PI3Kβ. The mechanism whereby PKC isoforms may be activated by PI3K *in vivo* is not yet clear. Recently, a specific association (co-immunoprecipitation) of PKCδ with PI3K after cytokine stimulation in human erythroleukemia cells was reported (34). Therefore, we examined a possible association of PKCε with p110β. Cell lysates from SW-480 cells were immunoprecipitated with anti-PKCε antibodies and analyzed with antibodies to p110β. Indeed, PI3Kβ and PKCε were found to co-immunoprecipitate in SW-480 cells in a specific manner as demonstrated by control experiments with non-immune serum (Fig. 10). There was no detectable increase in association of PKCε and PI3Kβ in BK-treated cells (data not shown).

DISCUSSION

In this study, we investigated the signaling pathway linking the endogenously expressed bradykinin receptor to MAPK in the human colon carcinoma cell line SW-480. We present evidence for the activation of p85/p110β PI3K downstream of the bradykinin B_2 receptor, which couples to a PTX-insensitive G protein. To our knowledge, this is the first demonstration that (i) a tyrosine kinase-associated PI3K is activated by a G protein-coupled receptor solely in an intact cell system and that (ii) the activation of a PI3K is mediated via a pertussis toxin-insensitive G protein of the $G_{q/11}$ family.

Recent studies have suggested that G_i-coupled receptor- and $G_{\beta\gamma}$-stimulated MAPK activation is attenuated by the PI3K inhibitors wortmannin and LY 294002 (21). Furthermore, the PI3Kγ isoform was identified as the target of $G_{\beta\gamma}$ complexes from PTX-sensitive G proteins and was suggested to link G_i-coupled receptors to the MAPK pathway (3, 17, 18).

In SW-480 cells, bradykinin was found to activate phospholipase Cβ, leading to production of inositol polyphosphates, and to exert a mitogenic action via the bradykinin B_2 receptor subtype. In addition, using two different experimental approaches, we obtained results indicating the involvement of a PI3K in the mitogenic bradykinin signaling. First, both BK-induced stimulation of DNA synthesis and activation of MAPK are inhibited by wortmannin or LY 294002. Activation of MAPK represents an essential step in the mitogenic action of BK in SW-480 cells because the effect of bradykinin on DNA synthesis was completely blocked by the MAPK inhibitor PD 098059. Second, bradykinin is capable of stimulating the lipid kinase activity of PI3K in SW-480 cells, resulting in the formation of the putative second messengers PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (14). Immunoblotting experiments revealed that SW-480 cells lack the p110γ and p110α isoforms, but express the heterodimeric isoform p85/p110β (PI3Kβ). Thus, PI3Kγ may be excluded from participating in the signaling pathway from the bradykinin receptor to MAPK in SW-480 cells. Recently, Kurosu *et al.* (19) reported that p85/p110β was stimulated by $G_{\beta\gamma}$ subunits from rat liver *in vitro*. Quite recently, this group demonstrated a potentiation of insulin-induced PtdIns(3,4,5)P_3 accumulation by adenosine and prostaglandin E_2 in rat adipocytes (35). Our results suggest that a G protein-coupled receptor is also capable of activating PI3Kβ in an intact cell system independently of simultaneous activation of a receptor tyrosine kinase.

In contrast to the hitherto existing idea that PI3K exclusively mediates the effect of βγ-complexes released from G_i proteins, the G protein involved in SW-480 cells is PTX-insensitive. Among the PTX-insensitive G proteins expressed in SW-480 cells, $G_{12/13}$ do not stimulate phosphatidylinositol hydrolysis (36) and may be excluded from linking the bradykinin receptor to phospholipase Cβ. The bradykinin receptor appears to be capable of interacting with multiple G proteins, including also G_s (23, 37). If the effect of bradykinin on MAPK is triggered by βγ-complexes released from a G_s protein as demonstrated for the β-adrenergic receptor (9), it might be expected that permanent activation of G_s in the presence of CTX simulates or potentiates the BK action on MAPK. Surprisingly, treatment of SW-480 cells with CTX completely prevented the activation of MAPK induced by BK. Furthermore, the BK-induced activation of MAPK was abolished in the presence of forskolin, which activates adenylate cyclase independently of the G_s protein. It may therefore be assumed that the inhibitory effect of CTX on the BK-induced stimulation of MAPK activity is due to cAMP triggered by CTX. We conclude that the G protein involved in both stimulation of phospholipase Cβ by BK and stimulation of MAPK in response to BK belongs to the $G_{q/11}$ family.

Our results suggest the involvement of a PKC upstream or downstream of PI3Kβ. One plausible candidate to play a role as a downstream effector of PI3K is PKCε since PKCε is activated by both lipid-derived second messengers of PI3K, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (33, 38). Overexpression of PKCε, but not that of PKCδ, another target of PI3K, has been shown to induce cell transformation (39) as well as activation of Raf-1 kinase (40) and MAPK (5). Both PKC δ and PKCε were found to associate with PI3K in TF-1 cells, a human erythroleukemia cell line (41). In addition, PKCε was suggested to be a mediator connecting PI3K with the MAPK pathway in erythroid progenitor cells (42).

We obtained two lines of evidence indicating a link between PKCε and PI3K in SW-480 cells. First, BK-induced translocation of PKCε is sensitive to wortmannin, and second, PKCε associates with p110β as demonstrated by co-immunoprecipitation. This association was not enhanced after stimulation of SW-480 cells with bradykinin. Similarly, in TF-1 cells, only the association of PI3K with PKCδ, but not that with PKCε, was

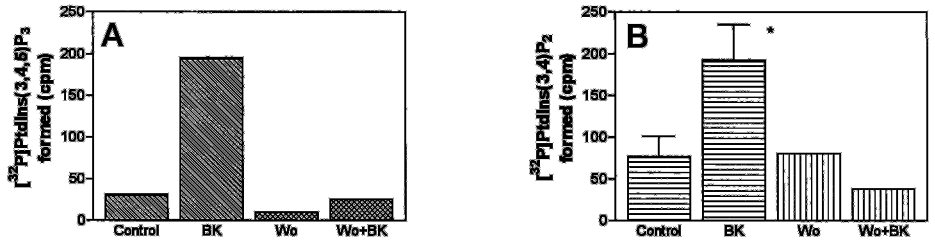

FIG. 7. **Effect of bradykinin on accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in intact SW-480 cells.** $^{32}P_i$-labeled human colon carcinoma SW-480 cells were challenged with 100 nM BK, and the levels of [^{32}P]PtdIns(3,4,5)P_3 (*A*) and [^{32}P]PtdIns(3,4)P_2 (*B*) were determined after 15 s as described under "Experimental Procedures." Data shown are mean cpm obtained from two or three independent experiments. *, significantly different from the control ($p < 0.05$). For some experiments, SW-480 cells were pretreated for 30 min with 100 nM wortmannin (*Wo*) before bradykinin was added.

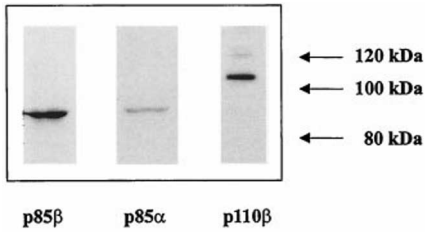

FIG. 8. **Endogenous expression of PI3K subunits in SW-480 cells.** Lysates from SW-480 cells were subjected to Western blot analysis using specific antibodies to p110α, p110β, p110γ, p85α, and p85β. No significant immunoreactivity was detected with antibodies to p110α and p110γ.

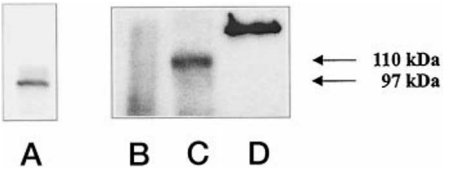

FIG. 10. **Anti-PKCε immunoprecipitates from SW-480 cells co-immunoprecipitate p110β PI3K.** Cell lysates from SW-480 cells were immunoprecipitated with anti-PKCε antibody and analyzed by immunoblotting with antibody to PKCε (*lane A*) or anti-p110β antibody (*lane C*). *Lane B* shows an immunoprecipitate with non-immune serum Western blotting with anti-p110β antibody. *Lane D* shows a control blot with p110β-glutathione *S*-transferase fusion protein (135 kDa), re-probed with anti-p110β antibody.

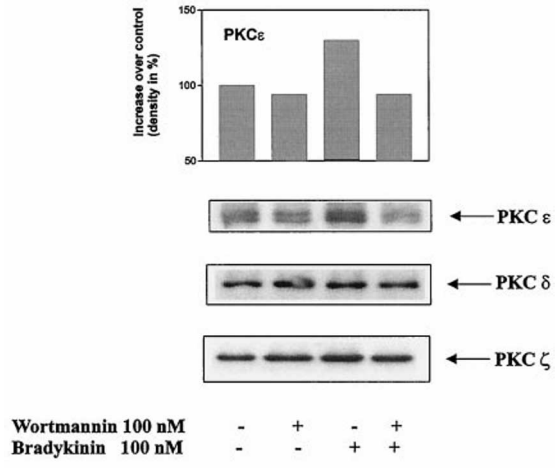

FIG. 9. **Effect of wortmannin on bradykinin-stimulated translocation of PKCε.** SW-480 cells were exposed to 100 nM BK for 10 min in the absence or presence of wortmannin (100 nM, 30-min preincubation). Membranes were prepared and analyzed by immunoblotting with antisera to the different PKC isoforms indicated (1 μg/ml) as described under "Experimental Procedures." Representative immunoblots are shown after background smoothing and quantification with the program NIH Image Version 1.57 of experiments repeated three times with similar results.

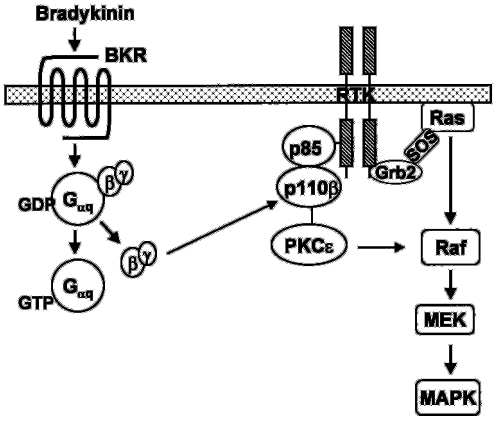

FIG. 11. **Model of bradykinin receptor-mediated PI3Kβ- and PKCεdependent MAPK activation in SW-480 cells.** Activation of a $G_{q/11}$ protein in response to BK leads to release of βγ-complexes, which probably mediate activation of PI3Kβ (19). By an unknown mechanism, p110β recruits and activates PKCε, which presumably precedes activation of Raf kinase (5) and, subsequently, MAPK. *BKR*, BK receptor.

found to be increased after cytokine stimulation (41). There are also contradictory results whether or not PI3K lipid products may be a prerequisite for the PI3K/PKC association. In TF-1 cells, wortmannin inhibited this association, whereas LY 294002 did not (41). In our case, the inhibitory effect of wortmannin on the BK-induced translocation of PKCε from the cytosol to the membrane favors an essential role of lipid kinase-generated second messengers and suggests a downstream position of PKCε related to PI3K.

In conclusion, we have shown that, in SW-480 cells, the mitogenic signaling of bradykinin involves the consecutive activation of a $G_{q/11}$ protein, PI3Kβ, PKCε, and MAPK (Fig. 11). Thus, this study defines a novel connection between a G_q protein-coupled receptor and the MAPK pathway with putative functional consequences for cell growth and carcinogenesis.

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