Cross Talk between β -Adrenergic and Bradykinin B_2 Receptors Results in Cooperative Regulation of Cyclic AMP Accumulation and Mitogen-Activated Protein Kinase Activity

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Received 6 October 2000/Returned for modification 15 December 2000/Accepted 21 September 2001

Costimulation of G protein-coupled receptors (GPCRs) may result in cross talk interactions between their downstream signaling pathways. Stimulation of GPCRs may also lead to cross talk regulation of receptor tyrosine kinase signaling and thereby to activation of mitogen-activated protein kinase (MAPK). In COS-7 cells, we investigated the interactions between two particular mitogenic receptor pathways, the endogenously expressed β -adrenergic receptor (β -AR) and the transiently transfected human bradykinin (BK) B_2 receptor (B_2 R). When β -AR and B_2 R are costimulated, we found two different cross talk mechanisms. First, the predominantly G_q protein-coupled B_2 R is enabled to activate a G_i protein and, subsequently, type II adenylyate cyclase. This results in augmentation of β -AR-mediated cyclic AMP (cAMP) accumulation by BK, which alone is unable to increase the cAMP level. Second, independently of BK-induced superactivation of the cAMP system, costimulation of β -AR leads to protein kinase A-mediated blockade of phospholipase C activation by BK. Thereby, the pathway from B_2 R to MAPK, which essentially involves protein kinase C activation, is selectively switched off. The MAPK activation in response to isoproterenol was not affected due to costimulation. Furthermore, in the presence of isoproterenol, BK lost its ability to stimulate DNA synthesis in COS-7 cells. Thus, our findings might establish a novel paradigm: cooperation between simultaneously activated mitogenic pathways may prevent multiple stimulation of MAPK activity and increased cell growth.

Receptors and their downstream signaling pathways do not work in isolation. They are connected via many fold interactions (cross talk) and associated in signaling networks. Thus, stimulation of a particular receptor leads to activation of a signaling pathway that can subsequently interact with those activated by other receptors. This cross talk ensures the exchange of information between the individual signaling pathways and provides the molecular basis for their cooperation (2, 15, 16). Cross talk between different G protein-coupled receptors (GPCRs) is well known and results mostly in synergistic effects and the amplification of cellular responses (30). In addition, stimulation of various GPCRs may also lead to cross talk activation of extracellular signal-regulated kinases (ERK1 and/or -2), which belong to the family of mitogen-activated protein kinases (MAPKs) (15, 23, 31) and represent key enzymes of receptor tyrosine kinase (RTK) signal transduction. The biochemical routes coupling GPCRs to MAPK cascades are highly complex and cell specific. Although the details are not yet fully understood, at least two principal pathways of GPCR-induced activation of MAPKs are postulated: transactivation of RTKs such as the epidermal growth factor receptor (EGFR) and/or the protein kinase C (PKC)-Raf kinase pathway (5, 10, 11, 27). In some cases, a role for phosphoinositide

3-kinase γ (20) or the calcium-sensitive kinase PYK2 (12) has been demonstrated.

The majority of models describing pathways from GPCRs to the MAPK cascade are founded upon experimental data obtained by individual stimulation of a particular GPCR that is coexpressed with epitope-tagged MAPK in a transfectable cell line such as COS-7, Rat-1, or HEK-293 cells (8–11, 20). Under physiological conditions, in contrast, cells are permanently costimulated by various agonists. Although much is known about how stimulation of a particular GPCR activates MAPK in isolation, much less is understood about how MAPK activity is regulated when two or more GPCRs are activated simultaneously.

In COS-7 cells, the mitogenic pathways of two GPCRs, the endogenously expressed β -adrenergic receptor (β -AR) and the transiently expressed human bradykinin (BK) B_2 receptor (B_2 R), are relatively well investigated. The hitherto existing knowledge of MAPK activation via β -ARs may be summarized as follows. (i) Stimulation of β -AR initially leads to G_{α_s} -mediated increase in cyclic AMP (cAMP) level. (ii) Protein kinase A (PKA) then phosphorylates the β -AR (heterologous desensitization), which can subsequently switch from G_s to G_i protein. (iii) In turn, MAPK is activated via G_i -derived $\beta\gamma$ -complexes and Ras. (iv) Finally, MAPK activation by β -AR additionally requires the formation of a multireceptor complex consisting of β -AR, EGFR, and Src kinase, which induces transactivation of EGFR (8, 9, 24).

Recently, we demonstrated that in COS-7 cells, BK activates MAPK via a dual or bifurcated pathway involving the indepen-

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dent and $G_{\alpha q}$ -mediated activation of the PKC pathway as well as EGFR transactivation. Both pathways appear to converge at the level of the Ras-Raf complex. (1).

Here we investigated the cross talk between β -AR and B_2R and their downstream signaling when both receptors are simultaneously activated. We found that costimulation of COS-7 cells with BK and isoproterenol chiefly results in two fundamental changes in BK signaling. On the one hand, the B_2R becomes enable to activate a G_i protein, and, subsequently, adenylate cyclase type II (AC II), whereby the β -AR-mediated rise in cAMP is augmented. On the other hand, PKA activated in response to stimulation of β -AR selectively turns off the phospholipase C (PLC)-PKC part of the bifurcated pathway from B_2R to MAPK. Thereby, BK becomes unable to stimulate MAPK activity and cell growth. Thus, cooperation of two simultaneously activated mitogenic pathways may prevent multiple stimulation of MAPK activity and additive effects on cell proliferation.

MATERIALS AND METHODS

Cell culture, transfections, and preparation of cell lysates. COS-7 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. For determination of cAMP and inositol phosphates, confluent cells were transfected in 24-well plates with 1 μ g (per well) of pcDNA3 (Invitrogen) expressing human kidney B_2R (pcDNA3- B_2R) and, as indicated, pcDNA3-CD8- β ARK, encoding the adrenergic receptor kinase fused to the transmembrane protein CD8 by using the DEAE-dextran technique. For EGFR transactivation experiments, cells were transfected in 10-cm-diameter plates with 6 μ g of pcDNA3- B_2R per plate and for measurement of MAPK activity in 10-cm plates with 6 μ g of pcDNA3- B_2R and 0.5 μ g of pcDNA3 of hemagglutinin (HA)-tagged MAPK p42 (HA-MAPK). pcDNA3-HA-MAPK and pcDNA3-CD8- β ARK were generously provided by R. Wetzker (Research Group Molecular Cell Biology, Jena, Germany). For preparation of lysates, cells were washed in cold phosphate-buffered saline (PBS) and lysed at 4°C in a buffer containing 20 mM HEPES (pH 7.5), 10 mM EGTA, 40 mM β -glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μ g of aprotinin per ml, and 20 μ g of leupeptin per ml. The lysates were centrifuged at $14,000 \times g$ for 20 min at 4°C. Equivalent expression levels of cDNA constructs were verified by Western blotting with the respective antibodies.

MAPK assay. MAPK activity was measured with the myelin basic protein (MBP) assay after immunoprecipitation of HA-p42 MAPK (ERK2) with monoclonal antibody (MAb) to HA 12CA5 (Babco, Berkeley, Calif.) as previously described (1). [γ - ^{32}P]ATP was obtained from NEN Life Science Products (Boston, Mass.). Phosphorylated MBP was visualized by autoradiography and quantified with a phosphorimager.

Detection of EGFR tyrosine phosphorylation. Lysates from treated and untreated COS-7 cells transfected with pcDNA3- B_2R were immunoprecipitated as described for the MAPK assay. Immunoprecipitation was performed with 1 μ l of EGFR MAb (sc-101; Santa Cruz Biotechnology, Santa Cruz, Calif.). Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and blotted onto polyvinylidene difluoride PVDF membranes. Tyrosine phosphorylation of EGFR was detected with an antiphosphotyrosine MAb, 4G10 (Upstate Biotechnology, Lake Placid, N.Y.). For reblotting, a polyclonal anti-EGFR antibody (sc-03) was used.

Identification of BK-induced G_i loading with [α - ^{32}P]GTP azidoanilide. Synthesis and purification of the photoaffinity label [α - ^{32}P]GTP azidoanilide as well as determination of receptor-activated $G_{i\alpha}$ -subunits in membranes were performed according to the method of Offermanns et al. (26). 4-Azidoanilide hydrochloride was from Fluka (Buchs, Switzerland), and [α - ^{32}P]GTP was purchased from NEN Life Science Products (Boston, Mass.). Briefly, for membrane preparation, COS-7 cells containing genes encoding B_2 receptors were homogenized in 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA and 5% (wt/vol) sucrose with a Dounce homogenizer. For some experiments, cells were treated with pertussis toxin (PTX; 400 ng/ml) for 24 h. Intact cells and nuclei were removed by centrifugation at $500 \times g$ for 5 min. Crude membranes were obtained by centrifuging the resulting supernatant at $140,000 \times g$ for 30 min. Membranes

FIG. 1. Costimulation of β -ARs and B_2R s in COS-7 cells: effects on cAMP accumulation. COS-7 cells were transiently transfected with pcDNA3- B_2R (1 μ g per well) in 24-well plates. (A) Time-dependent accumulation of cAMP in response to either 10 μ M isoproterenol or 1 μ M BK. For costimulation, COS-7 cells were pretreated with 10 μ M isoproterenol for the times indicated followed by stimulation with 1 μ M BK for 5 min. The cAMP content was measured with a [3H]cAMP binding assay from Amersham. The results expressed as picomoles of cAMP per well represent means $\pm$ standard error from three independent experiments in duplicate determinations. (B) Accumulation of intracellular cAMP in response to simultaneous treatment (5 min) of COS-7 cells with isoproterenol (Iso [10 μ M]) and BK (1 μ M). Data are means of three separate experiments in duplicate. *, significantly different from control; **, significantly different from stimulation with isoproterenol alone ($P < 0.05$; Student's t test). (C) Concentration-dependent cAMP accumulation in response to BK. COS-7 cells were incubated with increasing concentrations of BK together with 10 μ M isoproterenol for 5 min. The results represent means $\pm$ standard errors of three independent experiments in triplicate determinations. (D) COS-7 cells were pretreated with 10 nM CTX for 60 min followed by stimulation with 1 μ M BK for 5 min. Then the cAMP content was measured. The results represent the means $\pm$ standard errors from three separate experiments performed in duplicate determinations. *, significantly different from the basal value; **, significantly different from CTX alone ($P < 0.05$; Student's t test).

were aliquoted and stored at -80°C in Tris buffer with 1 mM EDTA. Protein content was determined according to the method of Lowry et al. (21). For G_i loading, membrane proteins (150 μ g/assay) were preincubated in 30 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM $MgCl_2$, 20 mM NaCl, 10 mM GDP, and the agonists as indicated for 3 min at 30°C . Then, [α - ^{32}P]GTP azidoanilide (2 μ Ci/sample) was added for another 3 min. After incubation, the samples were centrifuged at $14,000 \times g$ for 5 min at 4°C . The membrane pellets were resuspended in 60 μ l of GDP-free incubation buffer supplemented with 2 mM dithiothreitol. The samples were irradiated (as drops) for 15 s at 4°C with a UV lamp (254 nm; 150 W) from a distance of 3 cm. The samples were centrifuged again. The pellets were solubilized in Laemmli buffer, subjected to SDS-

FIG. 2. (A) Involvement of $\beta\gamma$ -complexes from a G_i protein in cAMP accumulation in response to BK. COS-7 cells transiently transfected with pcDNA-B₂R in 24-well plates were preincubated overnight with PTX (400 ng/ml) for 24 h or with the PKC inhibitor Bis (5 μ M) for 30 min. In parallel experiments, pcDNA-B₂R was cotransfected with plasmids containing CD8- β ARK chimera. Serum-starved cells were stimulated with isoproterenol (Iso) alone (10 μ M) or isoproterenol together with BK (1 μ M) for 5 min. Then the cAMP content was measured. The results are

PAGE (10% polyacrylamide gels), transferred to polyvinylidene difluoride membranes, and subjected to autoradiography. After autoradiography, G proteins were immunologically identified with anti-G₁₂ antibody and, for control, anti-G_{q/11} and anti-G_s antibody (Santa Cruz, Calif.).

Determination of intracellular cAMP. COS-7 cells transiently transfected with pcDNA3-B₂R were treated with serum-free DMEM for 2 h. Then the cells were exposed to the agents tested for 20 min in 500 μ l of serum-free HEPES-buffered DMEM (pH 7.2), supplemented with 100 μ M 3-isobutyl-methylxanthine (IBMX) and 10 μ M captopril. The reaction was stopped by the addition of 1 ml of ice-cold ethanol (96% [vol/vol]), giving a final concentration of 65% (vol/vol). The ethanol cell extracts were centrifuged at 14,000 $\times$ g for 5 min at room temperature. The supernatants containing the extracted cAMP were removed, and the pellets were washed with 500 μ l of ethanol (65% [vol/vol]) and centrifuged as described above. Supernatants were pooled, evaporated to dryness, and resolved in 150 μ l of 50 mM Tris buffer (pH 7.5) containing 4 mM EDTA. The samples were taken for estimation of cAMP by a [³H]cAMP protein binding assay from Amersham. For some experiments, COS-7 cells were preincubated with PTX (400 ng/ml) for 24 h and then assayed as described previously.

Immunocytochemical detection of AC II. Lysates from COS-7 cells were immunoprecipitated and reblotted with polyclonal anti-AC II or anti-AC IV antibodies (sc-587 and sc-589; Santa Cruz Biotechnology). Immunoprecipitation and Western blotting were performed as described above with 10% polyacrylamide gels.

Phosphatidylinositol turnover. COS-7 cells (5×10^5 cells per well) grown in 24-well plates and transiently transfected with the pcDNA3-B₂R were prelabeled with 4 μ Ci of [³H]myo-inositol (NEN Life Science Products, Boston, Mass.) per well for 24 h. At 2 h prior to stimulation, the cells were incubated in serum-free medium containing 20 mM HEPES (pH 7.4). The cells were stimulated in presence of LiCl as indicated in the figure legend. PLC activity was determined by analyzing total inositol phosphate formation as recently described (1).

Measurement of DNA synthesis. Subconfluent cells were deprived of serum for 24 h and then treated with BK, isoproterenol, and EGF as indicated. The cells were incubated for another 24 h, followed by the addition of [³H]thymidine (1 μ Ci/ml; Amersham Pharmacia Biotechnology) for 2 h. Incorporation of [³H]thymidine was measured by washing cells sequentially twice with ice-cold PBS, 5% trichloroacetic acid, and 95% ethanol. Then, the DNA was extracted with 1 N NaOH, and the [³H]thymidine incorporation into the trichloroacetic acid precipitate was assayed by liquid scintillation counting.

RESULTS

Costimulation of COS-7 cells with isoproterenol and BK leads to augmentation of β -AR-mediated cAMP accumulation.

G_{s α} -mediated activation of the cAMP-PKA system is the well-known main signaling pathway of β -ARs. Thus, in COS-7 cells, isoproterenol induced an increase in intracellular cAMP concentration, whereas BK did not change the cAMP level (Fig. 1A and B). Surprisingly, pretreatment of COS-7 cells with isoproterenol for 5 to 10 min followed by stimulation with BK as well as simultaneous application of isoproterenol and BK resulted in a significant amplification of the isoproterenol-induced cAMP response by BK (Fig. 1A and B). The effect of BK on cAMP accumulation in presence of isoproterenol was concentration dependent and reveals a 50% effective concentration of approximately 2 nM (Fig. 1C). Costimulation of COS-7 cells with cholera toxin (CTX) and BK also results in a

significant increase in cAMP accumulation compared with the single effect of CTX (Fig. 1D).

BK-induced superactivation of AC activity depends on $\beta\gamma$ -complexes of G_i proteins and is independent of PKC or PKA.

The increase in isoproterenol-induced cAMP formation by BK was reduced to the level of the isoproterenol effect by PTX as well as by coexpression of the $\beta\gamma$ -scavenger CD8- β ARK (Fig. 2A). These findings indicate the involvement of $\beta\gamma$ -subunits of a G_i protein in the effect of BK on AC activity. The increase in cAMP accumulation elicited by BK was not affected by the PKC inhibitor bisindolylmaleimide I (Bis) (Fig. 2A). For comparison, in the concentration used, Bis has been demonstrated to block completely the BK-induced activation of MAPK in COS-7 cells (1). An increase in intracellular cAMP may be due to activation of different AC isoforms with different patterns of activation and/or by inhibition of phosphodiesterase (PDE) activity. An inhibitory effect of BK on PDE may be excluded, since in our cAMP assay, PDE was blocked by IBMX. Among the AC isoforms, AC II or IV is known to be activated via $\beta\gamma$ -complexes of G_i in presence of free α_s -subunits (22). As demonstrated in Fig. 2B, COS-7 cells endogenously express AC II. This finding confirms previous results from functional studies indicating the occurrence of AC II in COS-7 cells (14).

Next we investigated whether the ability of B₂R to activate a G_i protein might be due to heterologous receptor phosphorylation of B₂R by isoproterenol-induced activation of PKA, as has been shown for the switch of β -AR from G_s to G_i protein (9). Treatment of COS-7 cells with H-89, an inhibitor of PKA, failed to prevent the effect of BK on isoproterenol-induced cAMP accumulation (Fig. 2C). In contrast, the increase in the cAMP level in response to BK was enhanced in presence of H-89. This rather unexpected finding might be explained by the inability of β -AR to couple to G_i when PKA is blocked (9). Thereby, both the activation of G_{s α} becomes stabilized and the amount of G_i, which is now susceptible to B₂R, becomes enhanced. It may be concluded that the coupling of B₂R to G_i is independent of prior receptor phosphorylation via the cAMP-PKA pathway.

To demonstrate that stimulation of B₂R directly results in G_i activation, COS-7 cell membranes were costimulated by BK and isoproterenol in presence of the photoreactive GTP analog [α -³²P]GTP azidoanilide. We used assay conditions (short incubation time, presence of GDP) that have been optimized for photolabeling of G_i proteins (17). Figure 3 shows that the basal [α -³²P]GTP azidoanilide accumulation of α_i was clearly enhanced by BK in the presence of isoproterenol compared with the single effects of both agonists. The specificity of G_i loading via the B₂R is verified by three lines of evidence: (i) the

the means $\pm$ standard error from three (Bis) or six (PTX, CD8- β ARK) independent experiments performed in duplicate. *, significantly different from control; **, significantly different from isoproterenol alone. +, significantly different from costimulation with isoproterenol and BK ($P < 0.05$; Student's *t* test). (B) Detection of AC II. COS-7 cells were lysed and subjected to immunoprecipitation (IP) with anti-AC II antibody (sc-587; lane 1), with anti-AC II antibody in the presence of an excess of blocking peptide (sc-587P; lane 2), and with nonimmune serum (NIS; lane 3). Reblotting (Western blot [WB]) of immunoprecipitates was performed with anti-AC II antibody. The blot shown is representative of three similar experiments. (C) PKA-mediated receptor phosphorylation is not required for the coupling of B₂R to G_i. COS-7 cells expressing human B₂R were incubated for 2 h in serum-free media. Cells were pretreated with the PKA inhibitor H-89 (10 μ M) for 30 min followed by stimulation with isoproterenol (10 μ M) alone or together with 1 μ M BK. The cAMP concentration was measured as described previously. The values shown represent means $\pm$ standard error from six separate experiments in triplicate determinations. *, significantly different from isoproterenol alone; **, significantly different from isoproterenol and BK.

FIG. 3. Photolabeling of $G_{\alpha i}$ proteins in COS-7 cell membranes. Crude membranes (150 μ g/tube) were incubated with [α - 32 P]GTP azidoanilide in the absence (–) or presence (+) of BK (100 nM) and/or isoproterenol (Iso [10 μ M]). PTX refers to membranes prepared from COS-7 cells pretreated with PTX (400 ng/ml) for 24 h. The PKA inhibitor H-89 (10 μ M) was added 20 min prior to the addition of agonists. Incubation with the photolabel was performed for 3 min. Solubilized membranes were subjected to SDS-PAGE, blotted, and autoradiographed. After autoradiography, the G proteins were identified by Western blotting (WB). The numbers at the right margin indicate the molecular mass marker (kilodaltons). (A) Shown in an autoradiogram representative for three separate experiments. The positions of G protein α -subunits comigrating with the photolabeled proteins are indicated in the left margin. (B) Western blot with anti- α_{i2} antibody corresponding to the autoradiogram shown in panel A. The immunologically identified G_i subunits closely comigrate with the photolabeled proteins.

comigration of [α - 32 P]GTP azidoanilide accumulation and $G_{\alpha i2}$, (ii) the failure of G_i loading in membranes pretreated with PTX, and (iii) the finding that the PKA inhibitor, H-89, does not block the additional increase in [α - 32 P]GTP azidoanilide accumulation after costimulation of isoproterenol with BK. For control, the G_i loading in response to isoproterenol alone was inhibited by H-89 (Fig. 3) (9).

Costimulation of B_2 R and β -AR leads to prevention of BK-induced PLC activation. In most cells and tissues investigated, activation of the phosphatidylinositol metabolism by G_q -mediated stimulation of PLC activity represents the main signaling pathway of B_2 R. Treatment of COS-7 cells with BK results in a concentration-dependent increase in inositol phosphate formation (1, 25). Stimulation of COS-7 cells with isoproterenol has been shown to be without influence on phosphatidylinositol metabolism (8). As demonstrated in Fig. 4A, simultaneous treatment of COS-7 cells with BK and isoproterenol abolished the stimulatory effect of BK on phosphatidylinositol metabolism. The inability of BK to increase inositol phosphate formation in the presence of isoproterenol was restored by addition of the PKA inhibitor H-89 (Fig. 4B). This finding is in accordance with previous results demonstrating the attenuation of PLC by cAMP and PKA (6, 7). Surprisingly, preincubation of COS-7 cells with PTX did not reverse the effect of isoproter-

FIG. 4. Costimulation with isoproterenol inhibits BK-induced inositol phosphate formation in COS-7 cells. (A) COS-7 cells expressing the human B_2 R were prelabeled with 4 μ Ci of myo -[3 H]inositol (20.5 Ci/mmol; NEN Life Science Products) per ml for 24 h. Serum-starved cells were stimulated with increasing concentrations of BK in the absence and presence of 10 μ M isoproterenol. In parallel experiments, COS-7 cells were preincubated with PTX (400 ng/ml) for 24 h followed by costimulation with 10 μ M isoproterenol and 1 μ M BK. Then, inositol phosphate accumulation was determined. PTX alone was without influence on inositol phosphate formation (not shown). The results presented are the means $\pm$ standard errors of two or three (with PTX) independent experiments in quadruplicate. (B) COS-7 cells were pretreated with 10 μ M PKA inhibitor H-89 for 30 min and then stimulated as indicated. The data shown are means $\pm$ standard errors for three independent experiments in quadruplicate determinations. *, significantly different from basal value; **, significantly different from the BK effect ($P < 0.05$; Student's t test).

enol (Fig. 4A), suggesting that the rise in cAMP and PKA activity in response to isoproterenol alone is sufficient to inhibit PLC activation by BK.

EGFR transactivation by isoproterenol depends on but EGFR transactivation by BK is independent of the cAMP-PKA pathway. Both isoproterenol (24) and BK (1) have been shown to induce transactivation of EGFR in COS-7 cells. Simultaneous stimulation of COS-7 cells by BK and isoproterenol leads to additive EGFR tyrosine phosphorylation compared with the individual effects (Fig. 5A). Pretreatment of COS-7 cells with Rp-8-Br-cAMPS, another specific and cell permeable inhibitor of PKA, reduced EGFR transactivation by isoproterenol, but did not significantly change EGFR transactivation in response to BK. These findings confirm the key role of PKA in the activation of G_i proteins by β -AR as the essential step for both EGFR transactivation and activation of MAPK by isoproterenol (8, 9, 24). They suggest, furthermore, that the increased activation of the cAMP-PKA pathway after costimulation of COS-7 cells with isoproterenol and BK does not affect the EGFR transactivation part of the bifurcated mitogenic pathway from B_2 R to MAPK. The reduced additive EGFR transactivation after costimulation of B_2 R and β -AR in the presence of Rp-8-Br-cAMPS may be explained by the selective inhibition of the PKA-mediated EGFR transactivation in response to isoproterenol. In a control experiment, it was demonstrated that Rp-8-Br-cAMPS influenced neither the basal nor the EGF-stimulated tyrosine phosphorylation of EGFR (Fig. 5B).

Simultaneous activation of β -AR selectively prevents activation of MAPK by BK. MAPK represents the final convergence point of the mitogenic pathways of both B_2 R and β -AR.

FIG. 5. Effects of costimulation on BK- and isoproterenol-induced tyrosine phosphorylation of EGFR. (A) COS-7 cells transfected with pcDNA-B₂R were serum starved overnight, preincubated with the PKA inhibitor Rp-8-Br-cAMPS (50 μ M) for 30 min, and stimulated with BK (100 nM) or isoproterenol (10 μ M) alone or together as indicated. EGFR was immunoprecipitated (IP), subjected to SDS-PAGE, and blotted and analyzed by Western blotting (WB). (B) For control, COS-7 cells were stimulated with isoproterenol and/or EGF (100 ng/ml) as indicated and EGFR tyrosine phosphorylation was assayed. The results shown are representative of two (B) and four (A) experiments.

Selective treatment of COS-7 cells with either isoproterenol or BK leads to an increase in MAPK activity. In our assay system, the effect of isoproterenol on MAPK was approximately 29.8% compared with that of BK and approximately 19.6% compared with that of EGF. For comparison, in binding studies with [³H]BK, the mean expression level of the human B₂R in COS-7 cells was determined with approximately 2×10^5 sites per cell (1, 25). Costimulation of COS-7 cells with both mitogenic agonists BK and isoproterenol resulted in a decrease in BK-induced activation of MAPK to the level of that induced by isoproterenol alone (Fig. 6A). For comparison, stimulation of MAPK activity by EGF was not affected by cotreatment with isoproterenol (Fig. 6B).

Prevention of MAPK activation by BK due to costimulation of β -AR is independent of the BK-induced and G_i-mediated amplification of cAMP accumulation. As shown in Fig. 7, pretreatment of COS-7 cells with PTX did not reverse the inhibitory effect of isoproterenol on BK-induced MAPK activation. In addition, when G_i is blocked, the MAPK activation in response to isoproterenol is also reduced to the level of basal activity. These findings reflect the inhibition of BK-induced activation of MAPK via the intact PKA pathway and the inability of β -AR to activate MAPK when G_i is blocked. They support the results shown in Fig. 4.

FIG. 6. Activation of MAPK in COS-7 cells by BK and isoproterenol (Iso): effect of costimulation. (A) Two days after transfection with pcDNA-B₂R and pcDNA-HA-MAPK, cells were exposed to serum-free medium overnight and then treated for 5 min with 100 nM BK and 10 μ M isoproterenol separately or simultaneously as indicated. Cells were lysed and immunoprecipitated, and MAPK activity was determined. (B) For comparison, COS-7 cells expressing HA-tagged MAPK were stimulated with EGF (100 ng/ml) and/or isoproterenol as indicated. Then cells were lysed, and MAPK activity was determined. The blots shown are representative of three separate experiments.

Costimulation with isoproterenol inhibits the BK-induced increase in DNA synthesis. In COS-7 cells, both BK and isoproterenol are capable of increasing DNA synthesis as measured by [³H]thymidine incorporation. The rise in DNA synthesis in response to BK or isoproterenol compared with that after EGF treatment corresponds to the effects of these mitogenic stimuli on MAPK activity and is abolished in the presence of the MEK inhibitor PD 098059, suggesting the involvement of MAPK (not shown). When COS-7 cells are simultaneously stimulated with BK and isoproterenol, the ef-

FIG. 7. Costimulation of MAPK activity by BK and isoproterenol: effect of PTX. COS-7 cells transiently expressing B₂R and HA-MAPK were preincubated with PTX (400 ng/ml) for 24 h and then stimulated with BK (100 nM), isoproterenol (10 μ M), or both together for 5 min in serum-free medium. MAPK activity was assayed as described previously. WB, Western blotting. The results shown are representative of two independent experiments performed in duplicate.

FIG. 8. Effects of BK, isoproterenol (Iso), and EGF on the rate of DNA synthesis in COS-7 cells. Serum-starved cells transfected with pcDNA-B₂R were stimulated in 24-well plates with 1 μ M BK or 10 μ M isoproterenol, costimulated with BK and isoproterenol, or treated with 100 ng of EGF per ml for comparison. Then, cells were assayed for [³H]thymidine incorporation as described under Materials and Methods. The results are expressed as the means $\pm$ standard errors from three separate experiments in quintuplicate determinations. *, significantly different compared with the basal value; **, significantly different compared with the singular effect of BK ($P < 0.05$; Student's *t* test).

fect of BK on DNA synthesis is clearly reduced (Fig. 8). These results indicate that the cooperation of β -AR and B₂R might represent a cellular response to avoid hyperproliferation due to multiple mitogenic stimuli.

DISCUSSION

When β -AR and B₂R are costimulated, cross talk between their signaling pathways predominantly changes the signal transduction of B₂R. First, the receptor additionally activates a G protein of the G_i family and amplifies the β -AR-mediated cAMP accumulation via activation of AC II. Second, PKA activated via the β -AR leads to a selective switch off at the level of PLC activation in response to BK. Thereby the second messenger diacylglycerol cannot be generated and fails to activate PKC as an essential step in MAPK activation by BK (1).

BK alone does not significantly change the cAMP level in COS-7 cells. Surprisingly, when β -ARs are costimulated by isoproterenol, BK increases the isoproterenol-induced cAMP accumulation. This effect of BK is inhibited by PTX as well as by coexpression of a $\beta\gamma$ -scavenger, suggesting the involvement of a G_i protein as well as AC II or IV (AC II here). Indeed, a significant increase in G_i loading after costimulation with BK and isoproterenol was directly demonstrated by using [α -³²P]GTP azidoanilide as a photoaffinity label. Under our assay conditions, both BK and isoproterenol induced a weak increase in G_i loading compared with the basal activity. However, the higher increase in [α -³²P]GTP azidoanilide accumu-

lation after costimulation does not simply reflect an additive effect, because it was not significantly reduced by the PKA inhibitor H-89. In contrast, the β -AR-induced G_i activation, which is mediated via PKA (9), was completely prevented by H-89.

Among the different AC isoforms, only AC II is able to integrate stimulatory inputs from G_s-, G_i-, and G_q-coupled receptors. The activation may be mediated by G_s α , $\beta\gamma$ -complexes, or PKC (22). Furthermore, the presence of activated α_s -subunits represents a prerequisite for the activation of AC II by $\beta\gamma$ -complexes (14). In COS-7 cells, interestingly, permanent activation of G_s α by CTX is also sufficient to induce the cAMP-amplifying activity by BK. Therefore, these results and the detection of endogenously expressed AC II in COS-7 cells suggest that AC II may be a downstream target of B₂R when simultaneously a G_s-coupled signaling pathway is activated. Alternatively, AC II activity might be stimulated as well in response to PKC. This was demonstrated, for example, for AC activation by BK in guinea pig airway smooth muscle cells (28). In COS-7 cells, the BK-induced activation of AC II via the G_q-PLC-PKC pathway may be excluded from several reasons: (i) BK alone fails to stimulate cAMP formation, (ii) costimulation of β -AR and B₂R turns off PKC activation by BK, and (iii) PKC inhibitors are without influence on BK-induced increase in cAMP accumulation.

The molecular mechanism of G_i activation via the B₂R, which is, at least in COS-7 cells, predominantly G_q coupled, is not yet fully understood. In fact, the switch of B₂R from G_q to G_i protein is different from that from G_s to G_i protein described for the β -AR (9), because no PKA-mediated heterologous receptor desensitization is involved. Furthermore, only a part of B₂R appears to switch to G_i proteins. In fact, the coupling of B₂R to G_q protein remains intact under conditions of costimulation, because the B₂R retains its ability to induce tyrosine phosphorylation of EGFR, which is mediated via G_q α subunits (1). Another possibility might be that the overexpression of B₂R is responsible for the coupling to G_i protein, as was recently demonstrated for the β -AR in HEK293 cells (29). However, overexpression of B₂R cannot be the reason for its G_i coupling, because stimulation with BK alone does not increase the cAMP level in COS-7 cells. Very recently it was shown that in HEK-293 T cells the B₂R is dually coupled to G_q and G_i (4). In these cells, the BK-induced stimulation of ERK2 requires the cooperation of both the G_q-coupled PKC pathway and a G_i-mediated but EGFR-independent activation of Ras (4). Furthermore, we have previously shown that in smooth muscle or tumor cells, BK may activate G_i proteins, too (13, 19). We assume, therefore, that in COS-7 cells, a latent coupling exists between B₂R and G_i proteins and that due to costimulation of β -AR, this latent coupling comes into effect. This assumption is supported by our finding that even in the presence of BK alone, a small increase in G_i loading was detectable compared with the basal value (Fig. 3). The molecular mechanism of G_i activation in response to BK is not yet clear. It might be speculated, for example, that the activation of ACII via latently G_i-coupled B₂R is masked by PKC α . Indeed, PKC α has been demonstrated to eliminate the responsiveness of ACII to $\beta\gamma$ -regulation (32). We have recently shown that stimulation of COS-7 cells by BK leads to activation of PKC α (1). Costimulation of β -AR, in contrast, prevents the activation

FIG. 9. Cooperation between β -AR and B_2 R signaling leads to augmentation of isoproterenol-induced cAMP accumulation and prevention of BK-induced activation of MAPK. The model depicts molecular events following costimulation of β -AR and B_2 R in COS-7 cells. (i) Activation of the cAMP pathway by the β -AR leads to inhibition of the G_q -mediated activation of the PLC_β -PKC pathway by the B_2 R and thereby prevents BK-induced stimulation of MAPK activity. The transactivation of EGFR via the B_2 R remains intact. (ii) Activated G_s protein is the first requirement of B_2 R to activate G_i protein. $G_{\beta\gamma}$ subunits released from G_i stimulate AC II leading to augmentation of β -AR-triggered cAMP accumulation.

PKC by BK, whereby AC II could become susceptible to $\beta\gamma$ -complexes from activated G_i proteins. The biological importance of the superactivation of AC in response to BK is also not yet understood and needs additional investigation. However, it is evident that the additional rise in isoproterenol-induced cAMP accumulation by BK is a prerequisite neither for the blockade of PLC toward BK nor for the inhibition of BK-induced activation of MAPK. The amplification of cAMP accumulation by BK could play a role in metabolic signaling of β -AR. Nevertheless, an additional regulatory importance of the BK-induced superactivation of the cAMP-PKA system downstream of MAPK activation (e.g., for nuclear translocation of MAPK [3] or for cell cycle arrest [18]) cannot be excluded.

Our results confirm the key role of AC II as coincidence detector in the network of GPCR signaling pathways as well as the ability of PKA to block PLC activation. They also confirm the previously published results that the β -AR-mediated activation of MAPK involves both G_i protein (9) and transactivation of EGFR (24). Novel aspects of our work are (i) that due to costimulation of β -AR the G_q -coupled B_2 R activates a G_i protein and, subsequently, ACII; and (ii) that the switch of B_2 R from G_q to G_i is different from the switch of β -AR from G_s to G_i (9). The most important novelty in the present study is the finding that two mitogenic pathways when simultaneously activated may cooperate to avoid additive or multiple stimulation of MAPK activity and do not significantly increase DNA synthesis. The cAMP-PKA pathway activated by the β -AR exerts two opposite functions: (i) the β -AR becomes phosphorylated and is thereby enabled to couple to G_i and to induce a mitogenic response, and (ii) simultaneously PLC, a

key element of BK signaling, also becomes phosphorylated, whereby the mitogenic pathway of B_2 R is switched off.

Taken together, we provide evidence that cross talk between β -AR and B_2 R when costimulated in COS-7 cells results in both synergistic and antagonistic effects (Fig. 9). On the one hand, the B_2 R generates a novel property and becomes able to activate G_i proteins additionally to G_q protein. That leads to stimulation of ACII and augmentation of isoproterenol-induced cAMP accumulation. On the other hand, due to β -AR-induced and PKA-mediated blockade of PLC, the B_2 R becomes unable to activate the PKC pathway and loses its mitogenic potency. Future investigations will show whether a "meaningful" cooperation of mitogenic pathways within the cellular networks might represent a principal molecular mechanism of cells to respond to multiple mitogenic stimuli.

ACKNOWLEDGMENTS

We thank C. Mertens and B. Haarseim for excellent technical support and H. Traber and H. Sack for help in preparation of the figures. We thank Günter Schultz for generous support.

This work was supported by grants from Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie to C.L. and B.N.

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