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Dual Effect of β-Adrenergic Receptors on Mitogen-activated Protein Kinase

EVIDENCE FOR A βγ-DEPENDENT ACTIVATION AND A $G\alpha_s$-cAMP-MEDIATED INHIBITION*

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The enzymatic activity of mitogen-activated protein kinases (MAP kinases) increases in response to agents acting on a variety of cell surface receptors, including receptors linked to heterotrimeric G proteins of the G_i and G_q family. Recently, it has been shown that stimulation of β-adrenergic receptors, which are typical of those that act through G_s to activate adenylyl cyclases, potently activates MAP kinases in the heart, resulting in the hypertrophy of the cardiac muscle (Lazou, A., Bogoyevitch, M. A., Clerk, A., Fuller, S. J., Marshall, C. J., and Sudgen, P. H. (1994) *Circ. Res.* 75, 938–941). We have observed that exposure of COS-7 cells to a β-adrenergic agonist, isoproterenol, raises intracellular levels of cAMP and effectively activates protein kinase A (PKA) and an epitope-tagged MAP kinase. However, MAP kinase stimulation by isoproterenol was neither mimicked by expression of an activated mutant of $G\alpha_s$, nor by treatment with PKA-stimulating agents. Moreover, pretreatment of COS-7 with a permeable cAMP analog, 8-Br-cAMP, markedly decreased MAP kinase activation by either isoproterenol or epidermal growth factor. Thus, in COS-7 cells cAMP and PKA do not appear to mediate MAP kinase activation by β-adrenergic receptors. Signaling from β-adrenergic receptors to MAP kinase was inhibited by transfection of a chimeric molecule consisting of the CD8 receptor and the carboxyl terminus of the β-adrenergic receptor kinase, which includes the βγ-binding domain. MAP kinase activation by isoproterenol was not affected by depletion of protein kinase C, but it was completely abolished by expression of Ras-inhibiting molecules. We conclude that signaling from β-adrenergic receptors to MAP kinase involves an activating signal mediated by βγ subunits acting on a Ras-dependent pathway and a $G\alpha_s$-induced inhibitory signal mediated by cAMP and PKA. The balance between these two opposing mechanisms of regulation would be expected to control the MAP kinase response to β-adrenergic agonists as well as to other biologically active agents known to act on G_s coupled receptors, including a number of hormones, neurotransmitters, and lipid mediators.

Mitogen-activated protein kinases (MAP kinases)[1] appear to play a central role in mitogenic signaling pathways stimulated by growth-promoting factors acting on a variety of cell surface receptors (1, 2). These kinases actively participate in converting extracellular stimuli to intracellular signals affecting the expression of genes necessary for a number of biological functions, including cell growth and differentiation (2). The pathway linking cell surface receptors to MAP kinases has just begun to be elucidated. The tyrosine kinase class of receptors signals to MAP kinase by a multistep process. In the case of ligand-activated EGF receptors, it involves binding to the adaptor protein GRB2 which causes the recruitment to the membrane of SOS, a guanine nucleotide exchange factor for $p21^{ras}$ (3, 4) and the consequent exchange of GDP for GTP bound to $p21^{ras}$. This initiates the activation of a linear cascade of protein kinases including c-Raf (5) and MEKK (6), and MEK1 and MEK2 (7), which ultimately phosphorylate MAP kinases on both threonine and tyrosine residues, resulting in a dramatic increase in their enzymatic activity (7). In turn, MAP kinases phosphorylate and modulate the function of key enzymes and nuclear transcription factors (8).

The pathway linking G protein-coupled receptors to MAP kinases is still poorly understood. Recent reports indicate that MAP kinases can be activated by a number of receptors linked to G_i. For example, triggering α_2-adrenergic (9), m2 muscarinic (10, 11), and D2 dopaminergic (11) receptors, as well as receptors for lysophosphatidic acid (12) can all potently activate MAP kinase in a pertussis toxin-sensitive manner. The enzymatic activity of MAP kinases can also be induced upon stimulation of receptors coupled to G_q, such as m1 muscarinic (10, 13) and bombesin (11) receptors, in this case through a pathway only partially dependent on protein kinase C (10, 13). Activation of MAP kinase appears not to be exclusively linked to cell proliferation, as only a few of these receptors can signal cell proliferation. In this regard, accumulating evidence suggests that persistent activation of the MAP kinase might lead to differentiation or hypertrophy in a cell type-specific manner (14). For example, stimulation of nerve growth factor receptors in PC12 rat pheochromocytoma cells or insulin receptors in L1 murine preadipocytic cell line provokes a marked and prolonged activation of MAP kinases and induces cells to acquire a fully differentiated phenotype (15, 16). In addition, hormonal stimulation of a number of G protein-coupled receptors naturally expressed in the heart elevates the enzymatic activity of MAP kinases and causes the hypertrophy of muscle cells (17). Interestingly, MAP kinase activation and the hypertrophic response was shown to be elicited by agonist acting on receptors coupled to either G_q or G_s, such as α_1 and β-adrenergic receptors, respectively (17). The latter represents one of the few examples of receptors coupled to G_s activating MAP kinase so

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[1] The abbreviations used are: MAP kinases, mitogen-activated protein kinases; EGF, epidermal growth factor; PKA, protein kinase a; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MOPS, 4-morpholinepropanesulfonic acid; MBP, myelin basic protein; βARK, β-adrenergic receptor kinase.

far reported (17). This is particularly interesting because β-adrenergic receptors couple to adenylyl cyclases to raise intracellular levels of cAMP (18), and recently available data indicate that elevated cAMP can block MAP kinase activation by oncogenic Ras proteins (19), tyrosine kinase receptors (20–22), and G protein-coupled receptors (23, 24). Thus, receptors coupled to G_s would be expected to diminish rather than to stimulate MAP kinase activity.

In this study, we have used the expression of an epitope-tagged MAP kinase in COS-7 cells as an experimental model to study the signaling pathway connecting endogenously expressed β-adrenergic receptors to MAP kinase. We have found that signaling from these G_s-coupled receptors to MAP kinase has two distinctive components: an activating pathway mediated by βγ subunits acting through Ras, and a $G\alpha_s$-induced inhibitory signal mediated by cAMP and PKA.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP (3000 Ci/mM) was from DuPont NEN. Tissue culture products used were Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and fetal calf serum (Advanced Biotechnologies Inc.). Isoproterenol, propranolol, H-8, and 12-*0*-tetradecanoyl-phorbol-13 acetate (TPA) were purchased from Calbiochem, and EGF from Upstate Biotechnology. All other chemicals were purchased from Sigma.

Expression Plasmids

A 700-base pair DNA fragment encoding the extracellular and transmembrane domains of the CD8 lymphocyte-specific receptor (from codon 1 to codon 209) was amplified by polymerase chain reaction using human CD8 cDNA (25) as a template and the oligonucleotides 5′-ATAAGCTTCTCgagcttcgagccaagc-3′ and 5′-AAGGATCCcctgtggttgcagtaa-3′ (added nucleotides are depicted in uppercase). The resulting DNA was subcloned as a *Hin*dIII-*Bam*HI fragment in the pcDNA expression vector (Invitrogen).

A DNA fragment encoding the carboxyl-terminal 222 amino acids of human bARK1 (26), a region that includes the βγ-binding domain (27), was amplified with the oligonucleotides 5′-CCGGATCCACCATGgaaatcaagttactggac-3′ and 5′-CCGAATTCgaggccgttggcactgcc-3′, and subcloned as a *Bam*HI-*Eco*RI fragment in a modified pGEX4T3 (Pharmacia Biotech Inc.) bacterial expression plasmid containing a short oligonucleotide encoding a COOH-terminal Myc epitope,[2] and then transferred as a *Bam*HI/*Not*I fragment to pcDNA-CD8. The resulting DNA construct, designated pcDNA-CD8-βARK-C, was expected to express the extracellular and transmembrane domains of CD8 fused to an intracellular domain containing the βγ binding portion of human βARK and a COOH-terminal Myc epitope.

Transient Expression in COS-7 Cells—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Plasmid DNA transfection of COS-7 cells was performed by the calcium phosphate precipitation technique (28).

MAP Kinase Assay—COS-7 cells were cotransfected with the different DNA constructs and an expression plasmid containing an amino-terminal hemagglutinin-tagged murine ERK2 cDNA (pcDNA-HA-MAPK) (29), the protein product of which can be efficiently recognized by a murine monoclonal antibody 12CA5 (29). Expression of the tagged MAP kinase was verified by Western blot (not shown). Forty-eight h after transfection, cells were serum-starved overnight and then stimulated with the different agents. After the indicated periods of time, cells were washed with cold phosphate-buffered saline solution, and lysed in a buffer containing 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Following centrifugation, clarified supernatants were immunoprecipitated with an anti-hemagglutinin monoclonal antibody 12 CA5 (Babco) for 1 h at 4 °C, and immunocomplexes were recovered with the aid of protein G-Sepharose. Pellets were then washed three times with phosphate-buffered saline solution supplemented with 1% Nonidet P-40 and 2 mM sodium vanadate, once with 0.5 M LiCl in 100 mM Tris, pH 7.5, and once with kinase reaction 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, 0.5 mM vanadate). Reactions were performed in 30 μl volume of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP/reaction, 20 μM unlabeled ATP, and 1.5 mg/ml myelin basic protein (MBP) (Sigma) at 30 °C for 30 min. Reactions were terminated by addition of 5 × Laemmli buffer, boiled, and electrophoresed in 12.5% polyacrylamide gel electrophoresis. Phosphorylated MBP was visualized by autoradiography. Under these experimental conditions we did not detect any MBP phosphorylating activity in parallel immunoprecipitates from control, vector transfected cells, nor we observed any direct effect on the immunoprecipitated MAP kinase by any of the PKA stimulating or blocking agents described below (data not shown).

cAMP Assays—COS-7 cells were grown to 90% confluence in 24-well plates. Medium was replaced by Eagle's medium containing 1 mM 3-isobutyl-methylxanthine, and the experimental compounds were added for 5 min. The incubation was stopped by replacing medium with ice-cold 0.1 N HCl. Cyclic AMP levels were determined in supernatants of neutralized samples using a commercial [^{3}H]cAMP assay kit (Amersham Corp.), as described (30).

Protein Kinase A Assays—COS-7 cells were grown to confluence in 6-well plates and kept overnight in serum-free conditions after which agents were added for 5 min. Cells were then lysed in a buffer identical to that used for MAP kinase assays, and the activity of protein kinase A was determined using a commercial non-radioactive PKA assay kit (SpinZyme, Pierce) following the manufacturer's instructions.

Phosphatidylinositol Hydrolysis—COS-7 cells were incubated in 24-well plates with 1 μCi/ml [^{3}H]*myo*-inositol for 24 h. Cells were incubated for an additional 4 h in serum-free medium and stimulated with experimental agents for 30 min in the presence of 10 mM LiCl. Inositol phosphates were extracted and analyzed by ion-exchange chromatography, as described (31).

Immunofluorescent Staining—COS-7 cells transfected with the different plasmids were immunostained with anti-CD8 monoclonal antibody (Dako T-8) (1:100) followed by a secondary goat anti-mouse antibody conjugated with fluorescein isothiocyanate, as described previously (32). Cells were viewed under an Olympus-AHTB fluorescence microscope and photographed at a magnification of ×250.

RESULTS

To determine whether stimulation of endogenously expressed β-adrenergic receptors induces MAP kinase activation, COS-7 cells were transfected with an expression plasmid carrying the cDNA for an epitope-tagged MAP kinase, serum starved, and then treated with increasing concentrations of isoproterenol for 5 min. As seen in Fig. 1*A*, the β-adrenergic agonist effectively induced MAP kinase activation in a dose-dependent fashion, reaching a maximum at concentrations of isoproterenol above 10 μM. MAP kinase activation was also time dependent, reaching its maximum between 3–5 min (Fig. 1*B*). Pretreatment of cells with the β-adrenergic antagonist propranolol (4 μM) completely abolished MAP kinase activation by isoproterenol but not by EGF (Fig. 1*B*), thus confirming that stimulation of MAP kinase in response to isoproterenol is mediated by β-adrenergic receptors.

β-Adrenergic receptors are typical of those coupled through G_s to the stimulation of adenylyl cyclase (18). Thus, addition of isoproterenol would be expected to promote an increase in cAMP levels and consequently to activate PKA (33). To study whether this second messenger-generating system was responsible for activating MAP kinase, we treated cells with a number of agents known to raise cAMP levels and/or to activate PKA. As shown in Fig. 2, treatment for 5 min with 100 μM isoproterenol, 10 μM forskolin, or expression of a constitutively activated mutant of $G\alpha_s$, $G\alpha_s$ QL (34) induced a remarkable increase in cAMP levels as compared to control cells. In contrast, neither EGF nor serum elicited any demonstrable effect on intracellular cAMP. Consistent with these results, PKA activity was also greatly enhanced in cells treated with isoproterenol, forskolin, or in cells transfected with a constitutively activated mutant of $G\alpha_s$ (Table I). Furthermore, treatment of COS-7 cells with the cell-permeable analog of cAMP, 8-Br-cAMP (1 mM), elicited a remarkable increase in PKA activity (Table I). However, whereas isoproterenol induced a 10-fold

[2] O. Coso and J. S. Gutkind, unpublished results.

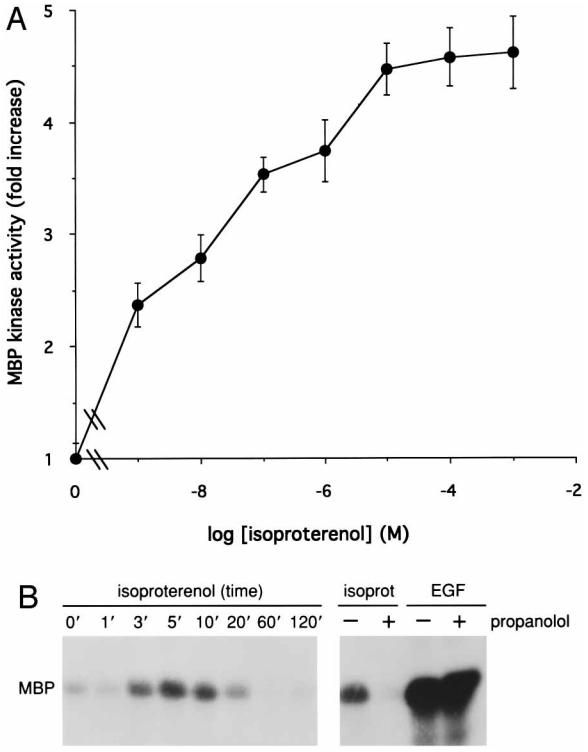

FIG. 1. **MAP kinase activation by isoproterenol.** *A*, dose-response curve. Serum starved COS-7 cells were treated with increasing concentrations of isoproterenol for 5 min. Cell lysates were processed as described under "Experimental Procedures." Data represent the average ± S.E. of triplicate samples and are expressed as fold increase in radioactivity incorporated into MBP with respect to control, untreated cells. Under these experimental conditions, radioactivity incorporated into MBP for control cells was 5377 ± 810 counts/min. *B*, time course activation of MAP kinase by isoproterenol and its blockade by propranolol. Serum-starved cells were treated for the indicated times with 10 mM isoproterenol, and control cells or cells pretreated with 4 μM propranolol for 20 min were stimulated for 5 min with either isoproterenol (10 mM) or EGF (100 ng/ml). MAP kinase activity was determined in immunoprecipitates using MBP as substrate as described under "Experimental Procedures."

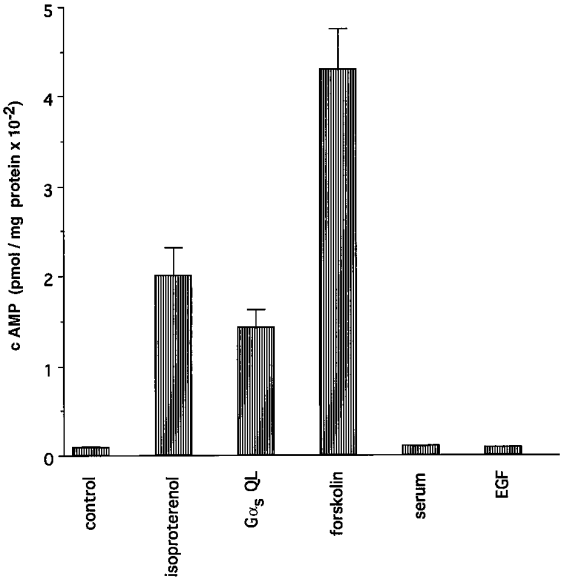

FIG. 2. **Effect of cAMP raising agents on intracellular levels of cAMP.** COS-7 cells transfected with insertless expression vector (control) or transfected with $G\alpha_s$-QL were grown to 90% confluence in 24-well plates. Medium was replaced by Eagle's medium containing 1 mM 3-isobutyl-methylxantine, and cells were stimulated with 10% serum, 10 μM isoproterenol, 10 μM forskolin, or 100 ng/ml EGF for 5 min and processed as described under "Experimental Procedures." Results represent the average ± S.E. of three independent experiments.

TABLE I

Effect of cAMP raising agents on PKA and MAP kinase activities

For PKA activity, COS-7 control cells or cells transfected with Gas-QL were grown to confluence in 6-well plates and kept overnight in serum-free conditions. Cells were then stimulated with 10% serum, 10 μM isoproterenol, 10 μM forskolin, 1 mM 8-Br-cAMP, or 100 ng/ml EGF for 5 min and processed as described under "Experimental Procedures" for protein kinase A assays. One unit of PKA activity is defined as the transfer of 1 nmol of phosphate to histone H III-S/min/mg of protein. Results represent the average ± S.E. of triplicate samples. Similar results were obtained in three independent experiments. For MAP kinase activity, serum-starved COS-7 cells transfected with an epitope-tagged MAP kinase were treated for 5 min with the indicated agents, and MAP kinase activity was determined in anti-HA immunoprecipitates using MBP as substrate. Results are expressed as radioactivity incorporated into MBP under the experimental conditions described under "Experimental Procedures." Data represent average ± S.E. of triplicate samples from a representative experiment, which was repeated three times.

Treatment	PKA activity	MAP kinase activity
	Units	Counts/min
Control	2 ± 1	722 ± 60
Isoproterenol	65 ± 3	70,226 ± 801
$G\alpha_s$-QL	58 ± 8	830 ± 23
Forskolin	132 ± 10	50,906 ± 193
8-Br-cAMP	292 ± 25	1,026 ± 34

increase in MAP kinase activity, neither 8 Br-cAMP nor $G\alpha_s$ QL were able to activate MAP kinase to any significant extent (Table I). Thus, the cAMP and PKA response to isoproterenol might not be sufficient to explain its activating effect on MAP kinase. On the other hand, the fact that forskolin can also elevate MAP kinase activity is more likely to result from some of the pleiotropic effects of this drug (35) rather than as a consequence of stimulating the cAMP/PKA pathway.

Recently, it has been shown that in certain cell types cAMP-raising agents can potently inhibit the activation of MAP kinase in response to a variety of mitogens (19–24). Thus, we examined whether increased cAMP levels and PKA activity would affect β-adrenergic receptor-induced MAP kinase activation. To that end, COS-7 cells were transfected with the activated form of $G\alpha_s$ or were pretreated with 8-Br-cAMP (1 mM) for 20 min prior to stimulation with isoproterenol or EGF. Both cotransfection with $G\alpha_s$ QL (not shown) and pretreatment with 8-Br-cAMP markedly decreased MAP kinase activation by either isoproterenol or EGF, as shown in Fig. 3. This effect of 8-Br-cAMP was blocked when added together with a PKA-specific inhibitor, H-8 (5 μM) (36). As shown in Fig. 4*A*, under these conditions H-8 restored MAP kinase activation by isoproterenol and EGF almost to the levels found in control, unpretreated cells (Fig. 4*A*). Furthermore, treatment of cells with the PKA inhibitor potentiated MAP kinase activation by isoproterenol, up to 2-fold after 5 min of stimulation, when MAP kinase activation is at its peak (Fig. 4B). Taken together, these findings demonstrate that the cAMP-PKA pathway does not mediate activation of MAP kinases in response to the β-adrenergic agonist. On the contrary, this biochemical route negatively regulates the MAP kinase signaling pathway in COS-7 cells.

Agonist-dependent activation of G protein-coupled receptors induces the replacement of GDP by GTP bound to the α subunit and causes the dissociation of α-GTP from βγ subunits (37). Although the GTP-bound α subunit was thought to be alone responsible for activating effector molecules, accumulating evidence supports an active role for the $G_{\beta\gamma}$ dimers in signal transmission (38). To explore whether βγ complexes participate in MAP kinase stimulation by β-adrenergic receptors, we took advantage of the observation that overexpression of the α sub-

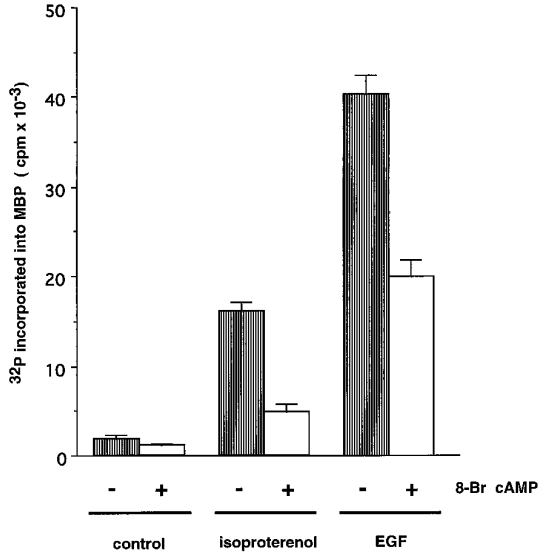

FIG. 3. **Effect of pretreatment with 8-Br-cAMP on MAP kinase activation.** Serum-starved COS-7 cells transfected with pcDNA-HA-MAP kinase were either left untreated or pretreated for 20 min with 1 mM 8-Br-cAMP prior to stimulation with 10 μM isoproterenol or 100 ng/ml EGF for 5 min. MAP kinase activity was determined in anti-HA immunoprecipitates using MBP as substrate. Results represent average ± S.E. of triplicate samples from a representative assay. Similar results were obtained in three independent experiments.

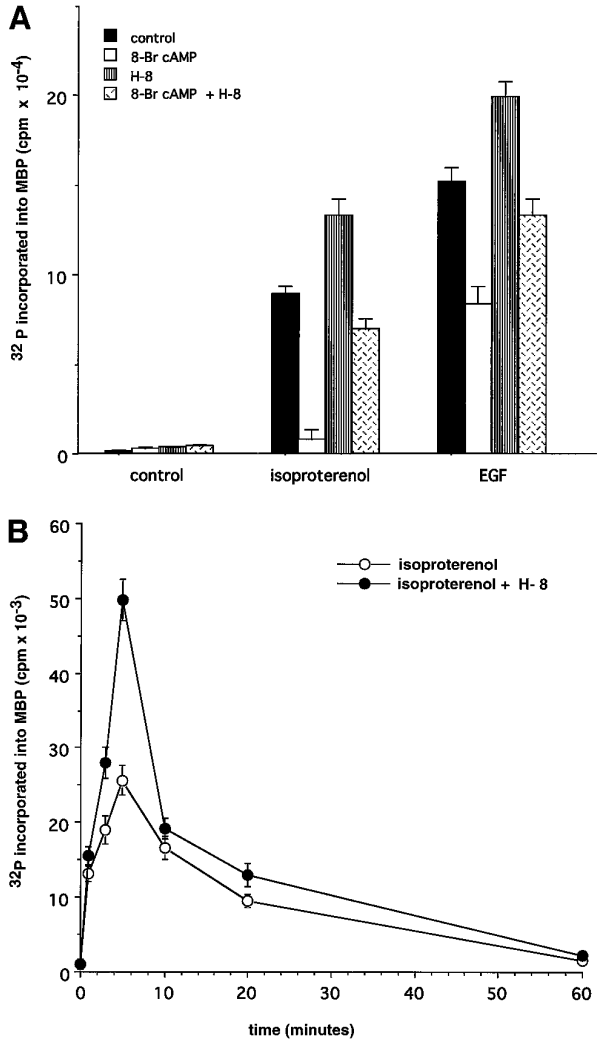

FIG. 4. **Effects of pretreatment with the PKA inhibitor H-8 on MAP kinase activation.** *A*, pretreatment with H-8 reverts the inhibiting effect of 8-Br-cAMP on MAP kinase activation. COS-7 cells transfected with the epitope-tagged MAP kinase were serum starved and pretreated with 5 μM H-8, 1 mM 8-Br-cAMP, or the combination of both for 20 min before stimulation with 10 μM isoproterenol or 100 ng/ml EGF for 5 min. MAP kinase activity was determined in immunoprecipitates using MBP as substrate. Results represent average ± S.E. of three independent experiments. *B*, time course activation of MAP kinase by 10 μM isoproterenol in COS-7 cells transfected with the HA-MAP kinase expression plasmid treated or untreated (control) with 5 μM H-8 for 20 min. MAP kinase activity was determined as above. Results represent average ± S.E. of triplicate samples from a representative experiment.

unit of transducin (G_t) or the carboxyl-terminal domain of β-adrenergic receptor kinase (βARK) can block $\beta\gamma$-dependent pathways, probably by binding and sequestering free $\beta\gamma$ dimers (39). Thus, we engineered a chimeric molecule between the extracellular and transmembrane domain of CD8 (25) fused to the COOH-terminal domain of βARK, which would be expected to express CD8 antigen at the cell surface, and to localize the βARK COOH-terminal domain to the inner face of the plasma membrane. Immunofluorescence analysis of intact cells transfected with this expression construct revealed that both CD8 and CD8-βARK-C chimera were efficiently expressed (Fig. 5). No fluorescence was observed in cells transfected with the vector control or if the primary antibody was omitted (not shown). As shown in Fig. 6, coexpression of G_t (Fig. 6*A*) or CD8-βARK-C (Fig. 6*B*) nearly abolished activation of MAP kinase in response to isoproterenol. In contrast, CD8 alone had no demonstrable effect (Fig. 6*B*). MAP kinase activation by EGF was not affected by any these constructs, thus further demonstrating the specificity of this approach (10). Thus, taken together these data strongly suggest that signaling from β-adrenergic receptors to MAP kinase is mediated by $\beta\gamma$ subunits rather than by the α subunit of G_s.

Addition of isoproterenol to COS-7 cells did not induce any demonstrable hydrolysis of phosphatidylinositol (data not shown). However, triggering β-adrenergic receptors in other cells results in the stimulation of PKC (40). Thus, we asked whether PKC plays a significant role in signaling MAP kinase activation by β-adrenergic receptors by depleting cells of PKC by prolonged treatment with high concentrations of phorbol esters (41). As shown in Fig. 7, this procedure abolished MAP kinase activation by a subsequent challenge with PKC-activating concentrations of TPA. In contrast, PKC depletion did not affect MAP kinase activation by isoproterenol or EGF, demonstrating that signaling from β-adrenergic receptors to MAP kinase involves a PKC-independent pathway.

We next explored a role for Ras in MAP kinase activation by β-adrenergic receptors by transfecting cells with expression plasmids carrying Ras-inhibitory molecules, such as the dominant inhibitory mutant *ras* N17 (42) and Rap-1a (43). As shown in Fig. 8, cotransfection of either *ras*-inhibiting construct nearly abolished MAP kinase activation by isoproterenol. In contrast, expression of Ras-blocking molecules failed to affect MAP kinase stimulation by a constitutively activated form of Raf, Raf BXB (44). Thus, these data strongly suggest that signaling from β-adrenergic receptors to MAP kinase involves a Ras-dependent pathway.

DISCUSSION

In the present study we have set out to investigate the signaling pathway linking β-adrenergic receptors endogenously expressed in COS-7 cells to a transfected, epitope-tagged MAP kinase. We have found that triggering COS-7 cells with the β-adrenergic agonist isoproterenol raises intracellular levels of cAMP, potently stimulates PKA, and induces a time- and dose-dependent activation of MAP kinase. However, we observed that the enhanced MAP kinase activity elicited by

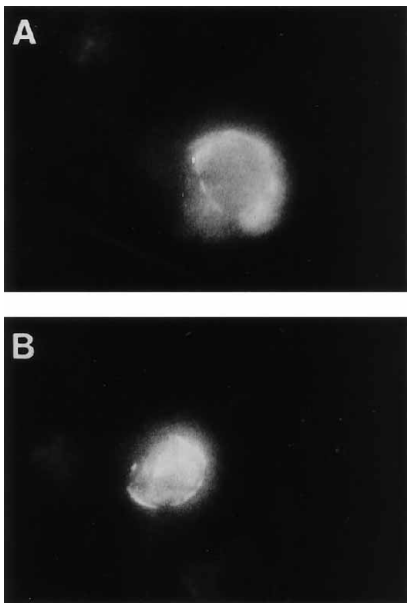

FIG. 5. **Detection of membrane-targeted βARK by immunofluorescence.** COS-7 cells transfected with pcDNA-CD8 (*A*) or pcDNA-CD8-βARK-C (*B*) were immunostained with anti CD8 (1:100) antibody and goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (1:100) as described under "Experimental Procedures."

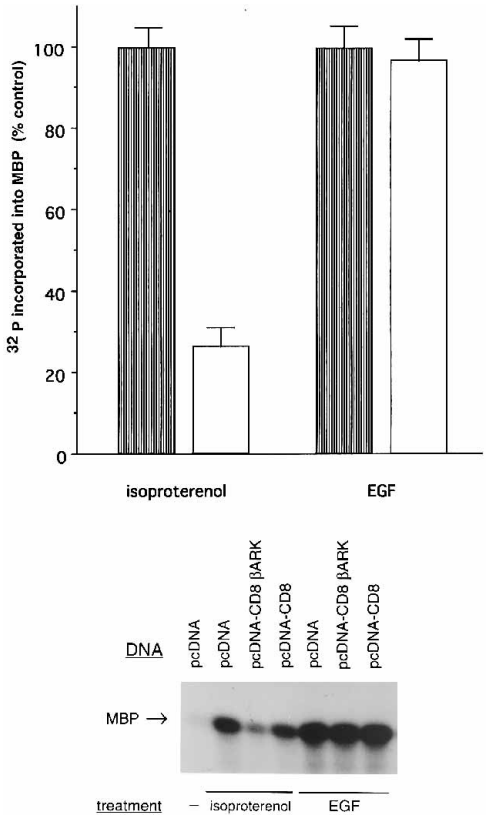

FIG. 6. **Effect of βγ scavenging proteins on MAP kinase activation.** *A*, effect of G_t. Expression plasmids containing G_t or the pcDNA vector (1 μg each) were transfected into COS-7 cells together with pcDNA-HA-MAP kinase. Cells were then serum starved and subsequently stimulated with 10 μM isoproterenol or 100 ng/ml of EGF for 5 min. MAP kinase activity was determined in immunoprecipitates using MBP as substrate. Results represent average ± S.E. of three independent experiments, expressed as percentage of response with respect to vector-transfected cells. Under these experimental conditions, radioactivity incorporated into MBP for vector-transfected cells untreated, or treated with isoproterenol or EGF were 5,377 ± 810, 27,458 ± 2,016, and 111,360 ± 8,945 counts/min, respectively. *B*, membrane-targeted βARK-C blocks MAP kinase activation by isoproterenol. Plasmids containing the CD8-βARK chimera CD8 or the pcDNA vector (1 μg each) were cotransfected into COS-7 cells together with pcDNA-HA-MAP kinase. Cells were then processed as above. MAP kinase activity was determined in immunoprecipitates using MBP as substrate, run in a 12% SDS-polyacrylamide gel electrophoresis gel, and subsequently autoradiographed. Similar results were obtained in three independent experiments. ▥, vector; □, G_t.

isoproterenol was not mimicked by expression of a constitutively activated form of $G\alpha_s$ or by treating cells with a permeable cAMP-analog, 8-Br-cAMP, although both potently stimulated PKA activity. Taken together, these findings strongly suggest that the cAMP-PKA pathway does not mediate MAP kinase elevation in response to isoproterenol. In fact, the only cAMP raising agent capable of eliciting a MAP kinase response was forskolin. Thus, this effect of forskolin, which was previously reported by others (11), is likely to represent a nonspecific effect of this drug rather than being induced by increased PKA activity.

Previous studies have shown that pretreatment with cAMP-raising agents strongly inhibits the MAP kinase activation elicited in response to a variety of mitogens (21–23). In line with these observations, MAP kinase activation in response to isoproterenol or EGF was also markedly decreased by pretreatment with 8-Br-cAMP or by overexpression of an activated form of $G\alpha_s$. It has been proposed that elevation of cAMP blocks signaling to MAP kinase by a mechanism involving the phosphorylation of Raf-1 by PKA (21, 23). Whether this is also the case in COS-7 cells is under current investigation. In this regard, we have observed that pretreatment of COS-7 cells with the PKA inhibitor H-8 (36) prevents the blocking effect of 8-Br-cAMP, thus supporting the existence of a PKA-dependent pathway inhibiting MAP kinase in these cells. Furthermore, exposure of cells to H-8 potentiated MAP kinase activation by isoproterenol, further demonstrating that PKA is not necessary to elevate MAP kinase activity in response to β-adrenergic receptor stimulation. Furthermore, this observation raises the possibility that G_s-coupled receptors might send, simultaneously, both activating and inhibitory signals to MAP kinase, the latter mediated by cAMP acting on PKA.

Recent studies from our laboratory have provided evidence that MAP kinase activation by muscarinic acetylcholine receptors is mediated by the βγ subunits of the heterotrimeric G_q and G_i proteins (10). As discussed above, expression of constitutively activated $G\alpha_s$ failed to mimic the activating effect of isoproterenol on MAP kinase. Thus, we explored whether $G_{s\beta\gamma}$ complexes released upon activation of G_s by β-adrenergic receptors mediate in MAP kinase stimulation. We initially studied the ability of overexpressing G_t to affect the MAP kinase response to isoproterenol. G_t is highly expressed in the retina and participates in linking light-induced changes in rhodopsin to a cGMP phosphodiesterase (45). In COS-7 cells, expression of G_t is expected to associate to free βγ subunits released during G protein stimulation, thus preventing βγ-dependent pathways (45). We observed that coexpression of G_t did not have any demonstrable effect on MAP kinase activation by EGF, but nearly abolished MAP kinase stimulation mediated by β-adrenergic receptors thus suggesting that $G_{s\beta\gamma}$ subunits are involved in linking β-adrenergic receptors to the MAP kinase pathway. As an alternative approach, we took advantage of the recent observation that the carboxyl-terminal domain of the βARK protein binds efficiently to βγ complexes (46). In this case, we anchored the βγ-binding domain of βARK to the plasma membrane by fusing it to the intracellular domain of the CD8 lymphocyte cell surface receptor. Immunofluorescence analysis of intact, transfected COS-7 cells revealed that this construct was effectively expressed and localized to the plasma

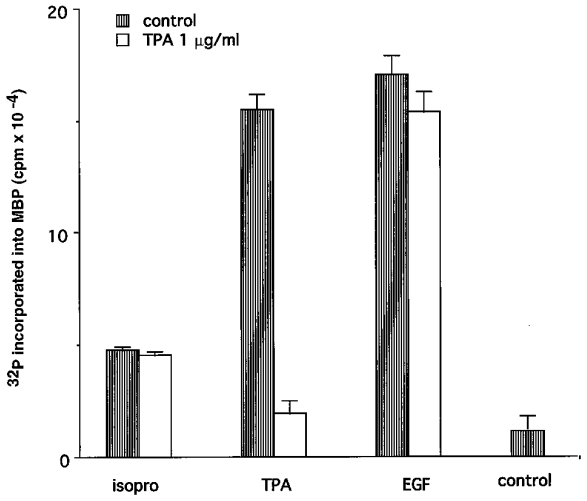

FIG. 7. **Effects of protein kinase C down-regulation on MAP kinase activation by isoproterenol.** Serum-starved cells were treated with either 10 μM isoproterenol, 100 ng/ml EGF, or 100 ng/ml TPA for 5 min with or without a 12-h pretreatment with 1 μg/ml TPA. MAP kinase activity was determined in immunoprecipitates using MBP as substrate as described under "Experimental Procedures." Results represent average ± S.E. of triplicate samples from a representative experiment.

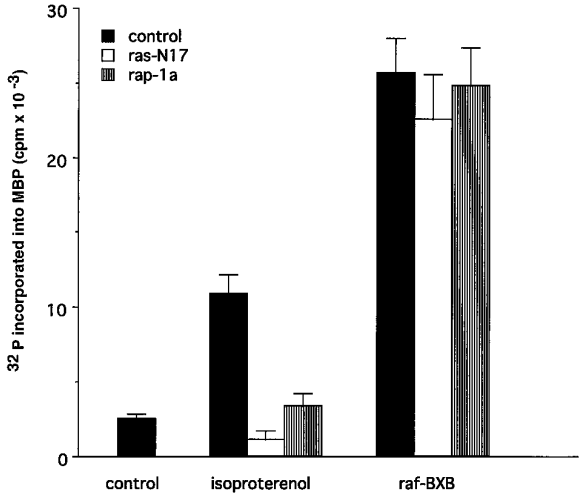

FIG. 8. **Effect of *ras*-inhibitory proteins on MAP kinase activation by isoproterenol.** Plasmids containing *ras*-N17, rap-1a, Raf-BXB, or the pcDNA vector (1 μg each) were transfected into COS-7 cells together with the epitope-tagged MAP kinase cDNA. Serum-starved cells were subsequently stimulated with 10 μM isoproterenol for 5 min. MAP kinase activity was determined in immunoprecipitates using MBP as substrate. Results represent average ± S.E. of triplicate samples from a representative experiment. Similar results were obatined in four independent experiments.

membrane. This CD8-βARK-C chimera abolished signaling from β-adrenergic receptors to MAP kinase, without affecting the EGF-induced response. Thus, we conclude that $G_{s\beta\gamma}$ complexes released upon activation of receptors coupled to G_s are responsible for signaling MAP kinase activation.

Because $\beta\gamma$ dimers have been shown to activate certain subtypes of phospholipase C (47), which might implicate PKC, we next explored a role for PKC in the MAP kinase response to isoproterenol. For that, cells were challenged with this β-adrenergic agonist upon depletion of functional PKC by prolonged treatment with phorbol esters. Under these conditions, the MAP kinase response to a subsequent stimulation of PKCs with phorbol esters was completely abolished, but MAP kinase activation elicited by isoproterenol was identical to that of unpretreated cells. Thus, PKC does not appear to play a significant role in MAP kinase activation by β-adrenergic receptors. On the other hand, it has been recently shown that receptors coupled to G_q and G_i activate MAP kinase in a *ras*-dependent fashion (10, 48–50). In this study, we show that *ras*-blocking proteins such as *ras* N17 (42) and rap-1a (43) completely block isoproterenol-induced MAP kinase activation, thus strongly suggesting that Ras also participates in signaling from G_s-coupled receptors to MAP kinase. Taken together, these findings strongly suggest that $\beta\gamma$ complexes released from G_i, G_q, or G_s can each effectively couple to effector molecules acting on the Ras-MAP kinase pathway. The identity of molecules linking $\beta\gamma$ subunits of these heterotrimeric G proteins to Ras is under current investigation.

Our present findings demonstrate that G_s-coupled receptors such as β-adrenergic receptors signal to MAP kinase in a unique and complex manner, which involves two counteracting pathways: an activating route mediated by $\beta\gamma$ subunits of G proteins acting on a *ras*-dependent pathway and an inhibitory route involving α_s, elevated intracellular cAMP levels, and PKA activation. The balance between these two mechanisms would be expected to determine the outcome of the signal sent to MAP kinases, and a number of cell type-specific factors are likely to regulate this balance. For example, whereas in COS-7 cells isoproterenol triggers a marked activation of MAP kinase, addition of this β-adrenergic agonist to adipocytes not only fails to stimulate MAP kinase, but potently blocks insulin action (22). The identity of those tissue-specific factors involved in balancing these opposing signals acting simultaneously on MAP kinases are still not known and warrant further investigation. On the other hand, a large number of natural agonists such as hormones, neurotransmitters, and lipid mediators are known to stimulate G_s-coupled receptors and, therefore, they would be expected to exert a similar dual effect on MAP kinases. Thus, our study raises the possibility that the $\beta\gamma$-Ras-MAP kinase pathway might play an unexpected role determining the nature of the biological responses elicited *in vivo* by each of these natural agonists.

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