Cyclic AMP-dependent and Epac-mediated Activation of R-Ras by G Protein-coupled Receptors Leads to Phospholipase D Stimulation*

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The activation of the Ras-related GTPase R-Ras, which has been implicated in the regulation of various cellular functions, by G protein-coupled receptors (GPCRs) was studied in HEK-293 cells stably expressing the M3 muscarinic acetylcholine receptor (mACHR), which can couple to several types of heterotrimeric G proteins. Activation of the receptor induced a very rapid and transient activation of R-Ras. Studies with inhibitors and activators of various signaling pathways indicated that R-Ras activation by the M3 mACHR is dependent on cyclic AMP formation but is independent of protein kinase A. Similar to the rather promiscuous M3 mACHR, two typical Gαi-coupled receptors also induced R-Ras activation. The receptor actions were mimicked by an Epac-specific cyclic AMP analog and suppressed by deletion of endogenous Epac1 by small interfering RNAs, as well as expression of a cyclic AMP binding-deficient Epac1 mutant, but not by expression of dominant negative Rap GTPases. In vitro studies demonstrated that Epac1 directly interacts with R-Ras and catalyzes GDP/GTP exchange at this GTPase. Finally, it is shown that the cyclic AMP- and Epac-activated R-Ras plays a major role in the M3 mACHR-mediated stimulation of phospholipase D but not phospholipase C. Collectively, our data indicate that GPCRs rapidly activate R-Ras, that R-Ras activation by the GPCRs is apparently directly induced by cyclic AMP-regulated Epac proteins, and that activated R-Ras specifically controls GPCR-mediated phospholipase D stimulation.

R-Ras, a member of the Ras superfamily of small GTPases, originally cloned through its homology to prototypic H-Ras, has been shown to interact in vitro with the three H-Ras downstream effectors, Raf-1, phosphatidinositol (PI)3-kinase, and RapGDS (1–4). In addition, in vitro interaction of R-Ras with several Ras regulatory proteins, including various guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), has been reported (5–8). Despite these similarities, studies in different cell types demonstrated that R-Ras has biological functions distinct from classic H-Ras. Notably, although H-Ras inhibits cell adhesion in fibroblasts by reducing the affinity of integrins for their ligands, R-Ras has been shown to promote integrin activation (7, 9–11). Recently, it has been proposed that R-Ras might enhance integrin-mediated cell attachment and spreading through alterations in the cellular Ca2+ handling, by decreasing the Ca2+ content of the endoplasmic reticulum (12). The capacity of R-Ras to modulate cell adhesion by maintaining integrin activity can be regulated by phosphorylation of a tyrosine residue in its effector domain by an Eph receptor kinase (13). Meanwhile, it has been reported that Eph receptor signaling to R-Ras is controlled by tyrosine phosphorylation of R-Ras by the Src kinase on tyrosine 66, and by binding of R-Ras to the Src homology 2 domain-containing Eph receptor-binding protein SHERP1, which binds in addition to the Ras-related GTPase Rap1A (14, 15). Notably, Rap1 has been shown to play a key role in the regulation of several aspects of cell adhesion, in particular in integrin-mediated cell adhesion (16), and thus R-Ras might modulate integrin signaling in concert with Rap GTPases. Other studies have shown that the semaphorin 4D receptor, plexin-B1, can down-regulate R-Ras activity by acting as an R-Ras-specific GAP in the presence of the Rho family member Rnd1, and thereby suppresses integrin activation and cell adhesion (17). Furthermore, recent studies in different cell types demonstrated that R-Ras is involved in

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‡ The abbreviations used are: PI, phosphatidinositol; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GPCR, G protein-coupled receptor; mACHR, muscarinic acetylcholine receptor; PLC, phospholipase C; PLD, phospholipase D; PGE1, prostaglandin E1; 8-Br-cAMP, 8-bromo-cAMP; 8-pCPT-2Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP; Rp-CPT-cAMPS, 8-pCPT-adenosine-3′,5′-cyclic monophosphorothioate; VASP, vasodilator-stimulated phosphoprotein; PtdEIOH, phosphatidylethanol; IP3, inositol 1,4,5-trisphosphate; GST, glutathione S-transferase; siRNA, small interfering RNA; PKA, protein kinase A; GTPγS, guanosine 5′-[γ-thio]triphosphate; ddAdo, 2′, 5′-dideoxyadenosine; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxyethyl)ester; AMP-PNP, adenosine 5′-[(β,y-imido)triphosphate].
control of cell migration, apparently through spatio-temporal regulation of Rho and Rac activities (18–20). In a recently generated R-Ras-null mouse, increased proliferation of vascular smooth muscle cells and enhanced angiogenesis was observed (21).

Despite the increasing evidence that R-Ras is obviously a key regulator of several cellular processes, our knowledge about the mechanisms leading to activation of R-Ras is very limited. In particular, it should be mentioned that in most studies the biological function of R-Ras was analyzed by ectopically expressing constitutively active or dominant negative R-Ras mutants. Activation of R-Ras, i.e. GTP loading, is catalyzed by several distinct GEFs, comprising about 20 distinct proteins (5–7). Of note, there is no report on a GEF that is specific for R-Ras. For example, the Ca\(^{2+}\)/diacylglycerol-regulated GEFs, RasGRP1–3, have been reported to activate R-Ras, H-Rras, and Rap1 (22, 23), although C3G and AND-34 have been demonstrated to promote GTP loading on R-Ras and Rap1 (22, 24, 25). It should be emphasized, however, that none of these studies addressed the question whether activation of R-Ras by these GEFs is triggered by specific membrane receptors.

The aim of this report was to study whether and by which mechanisms G protein-coupled receptors (GPCRs) activate R-Ras and to define a biological function of R-Ras in the GPCR actions. For this, we used the M\(_3\) muscarinic acetylcholine receptor (mACHR) stably expressed in HEK-293 cells. Numerous studies have demonstrated that this prototypical GPCR can couple to all major subtypes of heterotrimeric G proteins, not only to G\(_i\) proteins, as initially assumed (26–28), but also to G\(_{12}\), G\(_i\), and G\(_p\) proteins. By this distinct G protein coupling, the M\(_3\) mACHR can lead to the regulation of various effector enzymes, including phospholipase C (PLC), phospholipase D (PLD), and adenylyl cyclase, and activation of small GTPases from distinct families (26, 28, 29). Here we report that the M\(_3\) mACHR strongly activates R-Ras, that this activation requires endogenously expressed cyclic AMP-activated Epac1 proteins as shown by silencing of cellular Epac1 using siRNAs, and that this cellular response is mimicked by typical G\(_i\)-coupled receptors. Evidence is provided that Epac-activated R-Ras controls the M\(_3\) mACHR-mediated stimulation of PLD.

**EXPERIMENTAL PROCEDURES**

**Materials, Expression Plasmids, and Transfection**—Forskolin, prostaglandin E\(_2\), 2’,5’-dideoxyadenosine (dd-Ado), BAPTA/AM, and H-89 were from Calbiochem, Merck Biosciences, and adren- aline and wortmannin were from Sigma. 8-Br-cAMP, LY294002, and tyrphostin 23 were from Biomol, and 8-(4-chlorophenylthio)-2’-O-methyl cyclic AMP (8-pCPT-2Me-cAMP) and 8-pCPT-adenosine-3’,5’-cyclic monophosphorothioate (Rp-CPT-cAMPS) were from BIOLOG Life Science Institute (Bremen, Germany). GTP\(_\gamma\)S was from Roche Applied Science. Antibodies against R-Ras, RhoA, and Epac1 were from Santa Cruz Biotechnology; the anti-phosphovasodilator-stimulated phosphoprotein (VASP, Ser-157) antibody was from Cell Signaling Technology; the anti-VASP antibody was from Calbiochem, Merck Biosciences, and the anticofilin antibody was from tebu-bio. The anti-GAP1 IP4BP antibody was kindly provided by D. Bouyoucef and P. Cullen, and a mouse monoclonal antibody against Epac1 by J. Bos (30). [\(^{3}H\)]Oleic acid (5 Ci/mmol) was from PerkinElmer Life Sciences, and [\(^{3}H\)]GDP (14.2 Ci/mmol) was from GE Healthcare. cDNAs encoding wild-type R-Ras, S43N R-Ras, G38V R-Ras, Epac1 (each subcloned into pMT2-HA), S17N Rap2B (subcloned in pRK5), the β\(_2\)-adrenergic receptor (subcloned into pCDNA3), and GAP1 IP4BP (subcloned into pC-I-neo) were kindly provided by Drs. M. Spaargaren, R. Rehmann, J. de Gunzburg, R. Jockers, D. BouyouCEF, and P. Cullen. M\(_3\) mACHR-expressing HEK-293 cells and N1E-115 neuroblastoma cells grown to near confluence on 145-mm culture dishes were transfected with an efficiency of at least 50% as reported before (31, 32), typically with 25 μg of DNA from each of the β\(_2\)-adrenergic receptors and wild-type Epac1, 50 μg each of wild-type R-Ras and G38V R-Ras, 100 μg each of S17N Rap2B, S43N R-Ras, and R279K Epac1, or with the indicated amounts of plasmid DNA. For transfection of HEK-293 cells with siR-NAs, cells on 35-mm dishes were transfected with 20 μl of Lipofectamine 2000 in 2 ml of Opti-MEM and 200 pmol of siRNA according to the manufacturer’s instructions (Invitrogen). If vector DNA was co-transfected with siRNA, 4 μg of the individual vector DNA was used in a total amount of 12 μg. The specific siRNA (Eurogentec) was si-Epac1: 5’-CCAUCAUCC-UGCCGAGAGAA99-3’ (effective against human Epac1). To evaluate transfection efficiency, fluorescence-labeled siRNA (Alexa Fluor 488, Qiagen) was used, which also served as unspecific control siRNA. Expression of the encoded proteins was verified by immunoblotting of cell lysates with specific antibodies. Assays were performed 48 h after transfection.

**Measurement of PLD and PLC Activities**—PLD activity was measured in HEK-293 cells prelabeled with [\(^{3}H\)]oleic acid as formation of the specific PLD product, [\(^{3}H\)]phosphatidylethanol (\([\(^{3}H\)]PtdEtOH\)), for 30 min at 37 °C in the presence of ethanol as reported previously (28). Formation of [\(^{3}H\)]PtdEtOH is expressed as percentage of total labeled phospholipids. PLC activity was measured for 1 min at 37 °C as accumulation of inositol 1,4,5-trisphosphate (IP\(_3\)) as described before (29).

**Activation of R-Ras and VASP Phosphorylation**—Serum-starved HEK-293 cells and N1E-115 neuroblastoma cells transfected with wild-type R-Ras were incubated for the indicated periods of time at 37 °C with the indicated agents. After cell lysis, activated R-Ras was extracted with glutathione S-transferase (GST)-tagged Raf1-RBD (Ras-binding domain of Raf-1) bound to glutathione-Sepharose beads and immunoblotting with the anti-R-Ras antibody as described before (29, 33, 34). Densitometric analysis of the bands was performed with ImageQuant software (Amersham Biosciences). For measurement of VASP phosphorylation, serum-starved HEK-293 cells were incubated for 5 min at 37 °C with 30 μM forskolin, followed by cell lysis in a buffer containing 1% SDS and 10 mM Tris/HCl, pH 7.4, and five passages through a 25-gauge needle. Thereafter, the lysates were clarified by centrifugation, followed by determination of protein concentration and incubation in Laemmli buffer for 10 min at 95 °C. After SDS-PAGE and transfer to nitrocellulose membranes, phosphorylated VASP (Ser-157) was detected with the anti-phospho-VASP antibody.

**Purification of Proteins**—GST-tagged R-Ras, Rap2B, and ΔDEP Epac1 (Epac1-(149–881)) and His\(_6\)-tagged C3G-(830–1078) (cDNAs kindly provided by Drs. M. Spaargaren, J. de
Gunzburg, and H. Rehmann) were expressed in *Escherichia coli* and purified with glutathione-Sepharose or nickel-nitrotriacetic acid superfrown agarose beads as described before (31, 33, 35). To obtain the soluble and untagged proteins, the immobilized proteins were proteolytically digested by thrombin (Rap2B, ΔDEP Epac1), factor Xa (R-Ras) or eluted with 400 mM imidazole (C3G). For protease digestion, the washed beads were resuspended in thrombin buffer (50 mM Tris/HCl, pH 8.6, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% 2-mercaptoethanol) or factor Xa buffer (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 8.8 mM CaCl₂, pH 7.2), respectively, and incubated for 2 h at room temperature with thrombin or factor Xa (followed by overnight incubation at 4 °C). Purification of the proteins was analyzed by SDS-PAGE. Expression and purification of GST-tagged ARF1 in *Spodoptera frugiperda* cells were performed as described before (36), except that a different buffer (50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 10 μM phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin) was used.

**Measurement of the GEF Activities of Epac1 and C3G**—Binding of GDP to R-Ras, Rap2B, and ARF1 was determined at room temperature by the filter binding method (37). In brief, for GDP binding the recombinant GTPases were first made nucleotide-free by incubation for 5 min in an EDTA-containing loading buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM AMP-PNP, and 1 mM dithiothreitol), supplemented with 3 μM [³H]GDP. Thereafter, MgCl₂ was added to a final concentration of 5 mM, and the incubation was continued for a further 20 min. Finally, purified ΔDEP Epac1 or C3G-(830–1078) equilibrated for 15 min in exchange buffer, containing 20 mM Tris/HCl, pH 7.5, 80 mM NaCl, 5 mM MgCl₂, 0.4 mg/ml bovine serum albumin, 1 mM dithiothreitol, 10 mM AMP-PNP, and 10 mM GTP, was added in the absence (C3G-(830–1078)) or presence of 1 mM 8-Br-cAMP (ΔDEP Epac1), and the reaction was continued for the indicated periods of time. Bound and free [³H]GDP were separated by adding 1.5 ml of washing buffer (20 mM Tris/HCl, pH 7.5, 8 mM NaCl, 10 mM MgCl₂, 0.05% 2-mercaptoethanol) and filtration through nitrocellulose filters. The ratio of GEF protein to the GTPases in the exchange assays typically was 1:1, as assessed from the protein bands of Coomassie-stained gels.

**Protein Binding Assays**—Serum-starved HEK-293 cells transfected with wild-type Epac1 were lysed in a buffer containing 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, 1% phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Nucleotide-free, GDP-bound, and GTPyS-bound forms of recombinant GST-tagged R-Ras, Rap2B, and ARF1, immobilized on glutathione-Sepharose beads, were prepared exactly as described (38). Thereafter, the beads (5–10 or 50 μg of protein) were incubated with lysates of the transfected cells (3–4 mg of protein) or purified ΔDEP Epac1 (50 μg of protein) overnight at 4 °C. Finally, after three washes, the beads were resolved in Laemmli buffer, subjected to SDS-PAGE, and transferred to nitrocellulose for Western blotting, using anti-Epac1 antibodies as indicated.

**Immunoblot Analysis**—For detection of R-Ras, phospho-VASP, VASP (each at a dilution of 1:500), and Epac1 (dilution of 1:1000–1:10,000), equal amounts of protein were separated by SDS-PAGE on 10% acrylamide gels. After transfer to nitrocellulose membranes and a 1-h incubation with the antibodies at the above given dilution factors, the proteins were visualized by enhanced chemiluminescence.

**Generation of HEK-293 Cells Stably Expressing Retroviral Encoded siRNA Epac1—siRNA molecules targeted against Epac1 were delivered to HEK-293 cells by retroviral transfer as described (39) with the following modifications.** First, the human H1 promoter, suitable for expression of short RNA molecules, was cloned by PCR from genomic DNA using the primers 5’-CGCGTTCACATGAAATCGAAGCTGACGTCTACAAACCGCTC-3’ (forward) and 5’-CGCGTTCGACTCCTCGAGGCCCTTCGAAAGCTGTCCTCTCATACAGAATTATAGCTC-3’ (reverse). This construct contains two artificial restriction sites for XhoI and Bsp191I for site-directed insertion of the respective siRNA constructs, including suitable transcription stop signals. The entire construct was sequenced and cloned into the Sall site within the 3′-long terminal repeat of the pQCGXH vector (Clontech). This vector contains a hygromycin resistance gene under the control of a cytomegalovirus promoter followed by an internal ribosome entry site as well as enhanced green fluorescent protein sequence. siRNA sequences targeted against human Epac1 were designed according to current algorithms and consisted of the cDNA of the target transcript followed by nine nucleotides hairpin sequences and the reverse sequence. For human Epac1 (GenBank™ accession number NM_006105), the entire sequences were 5’-CGAACCAGCGCCTTCGTTGATTACATTACCAAGAGAATGTCAGCGTCTTTTGGGAC-3’ (forward) and 5’-TCGAGTTCGAAAAAGGCACTCGTGTGATTTCTTGGGAAATGTCAGCGTCTTTTGGGAC-3’ (reverse). Two oligonucleotides (0.06 μg/μl, final concentration) encoding these sequences were annealed in a stepwise process for 4 min at 90 °C, 10 min at 70 °C, 5 min at 62 °C, 5 min at 37 °C, 5 min at 25 °C, and 5 min at 10 °C in 100 mM NaCl, 50 μM Hesper buffer, pH 7.4. After hybridization the construct was ligated into the Xhol/Bsp191I site of the modified pQCGXH vector. All vector constructs used were sequence-verified. Packaging GP2–293 cells (Clontech) were co-transfected with the respective pQCGXH-siRNA vector and the pVSV-G plasmid (encoding the envelope protein of the vesicular stomatitis virus) by calcium-phosphate transfection. Supernatants were harvested and ultracentrifuged for enrichment of recombinant retroviruses. M13 mACHR-expressing HEK-293 cells were infected with the recombinant retroviruses for 24 h at 37 °C, and infection was monitored by fluorescent microscopy detection of enhanced green fluorescent protein. Stably infected cells were further selected by incubation with 200 μg/ml hygromycin. Epac1 transcripts were measured by quantitative real time PCR using the fluorescent dye SYBR Green. Total RNA samples were obtained using the NucleoSpin RNA II kit, including a DNA digestion step (Macherey-Nagel) according to the manufacturer’s instructions. As control, cells infected with retroviruses lacking a siRNA insert were used. Reverse transcription was done with an oligo(dT)₁₅ primer and 1 μg of RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed by monitoring the fluo-
rescence of the SYBR Green dye (Platinum SYBR Green qPCR SuperMix UDG; Invitrogen) on a 7500 real time PCR system (Applied Biosystems). Applied primer pairs were specific for the sequences of Epac1 (5′-AGTTTCCACCCTCAGAGGCAGAGC-3′; 5′-ACATAGGGCCAGGGTCTGCTG-3′) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (5′-GGCGATGCTGGCTGAGT-3′; 5′-CATGCTTCACACCATTAGCA-3′), which was used for normalization. The relative transcription level of the EPAC1 gene in siRNA expressing cells compared with vector controls is expressed as ΔCt values (40).

**Data Presentation**—Data shown in the figures are either representative experiments or the mean ± S.E. of n independent experiments, each performed in triplicate. Comparisons between means were either with the Student’s paired t test or one-way analysis of variance test, and a difference was regarded significant at p < 0.05.

**RESULTS**

**Activation of R-Ras by the M₃ mACHr**—The M₃ mACHr stably expressed in HEK-293 cells can couple to several types of heterotrimeric G proteins, thereby leading to stimulation of various effector enzymes and activation of small GTPases from different families (26–29). Thus, we used the M₃ mACHR-expressing HEK-293 cells to study whether and by which mechanisms GPCRs activate R-Ras. For this, wild-type R-Ras was overexpressed in the cells, and upon stimulation GTP-loaded R-Ras was extracted from cell lysates with the immobilized Ras-binding domain of Raf-1. As shown in Fig. 1, agonist (carbachol) activation of the receptor induced a very rapid but transient activation of R-Ras. The stimulatory effect of carbachol reached its maximum at ~1 min and declined thereafter (Fig. 1A). Half-maximal and maximal activation was observed at about 5 and 100 μM carbachol, respectively (Fig. 1B). To elucidate the mechanisms of R-Ras activation, we interfered with the known signaling pathways of this receptor. The M₁ mACHR potently stimulates PLC and increases [Ca²⁺], in HEK-293 cells, a process mainly mediated by Gq-activated PLC-β1 (29). On the other hand, PLD stimulation by the M₃ mACHR is mediated by G₁₂ types of G proteins and the small GTPases, RhoA and ARF1, which are activated by a tyrosine kinase- and a PI 3-kinase-dependent mechanism, respectively (28, 36, 41). As shown in Fig. 2A, chelation of intracellular Ca²⁺ with BAPTA/AM (20 μM) had no effect on the carbachol-induced activation of R-Ras. Similarly, treatment of HEK-293 cells with the general tyrosine kinase inhibitor, tyrphostin 23 (100 μM), which prevented M₃ mACHR-mediated RhoA activation and PLD stimulation (41), had no effect on activation of R-Ras (Fig. 2A). Activation of R-Ras was also not affected by the PI 3-kinase inhibitors, LY294002 (10 μM) and wortmannin (200 nM) (Fig. 2B), which on the other hand prevented activation of ARF1 and stimulation of PLD by the M₁ mACHR (36). Furthermore, treatment of HEK-293 cells with pertussis toxin and expression of the C terminus of the β-adrenergic receptor kinase, a Gβγ scavenger, had no effects on the carbachol-induced R-Ras activation (data not shown). These data suggested that signaling of the M₃ mACHR to R-Ras does not require Gq-mediated and PLC-dependent [Ca²⁺] increase, G₁₂-mediated PLD stimulation, including activation of PI 3-kinase and tyrosine kinases, and the small GTPases, RhoA and ARF1, and also not G₁ and Gβγ proteins.

**Cyclic AMP-dependent Epac-mediated Activation of R-Ras by the M₃ mACHR and G₁₂-coupled Receptors**—We have reported recently that the M₃ mACHR activates the Rap GTPase Rap2B via G₁₂-dependent cyclic AMP formation (29). Activation of R-Ras by the M₃ mACHR apparently involves...
cyclic AMP as well. Treatment of HEK-293 cells with the P-site adenylyl cyclase inhibitor, dd-Ado (10 μM), fully suppressed the stimulatory effect of carbachol on activation of R-Ras (Fig. 3A). Furthermore, direct activation of adenylyl cyclase with forskolin (30 μM) and treatment of the cells with the membrane-permeable cyclic AMP analog, 8-Br-cAMP(1 mM), strongly enhanced, by 2–2.5-fold, GTP loading of R-Ras (Fig. 3C). However, the activation of R-Ras by cyclic AMP was apparently not mediated by protein kinase A (PKA), the major cellular cyclic AMP target. Treatment of HEK-293 cells with the PKA inhibitor, H-89 (10 μM), did not alter activation of R-Ras by either carbachol, forskolin, or 8-Br-cAMP (Fig. 3, B and C). Similar to H-89, the PKA inhibitor, Rp-CPT-cAMPS (10 μM), did not affect GTP loading of R-Ras by the stimulatory agents (data not shown). On the other hand, both PKA inhibitors, H-89 and Rp-CPT-cAMPS (not shown), suppressed the forskolin (30 μM)-induced and PKA-mediated phosphorylation of endogenous VASP proteins (Fig. 3D). Thus, the M3 mAChR-mediated activation of R-Ras is apparently cyclic AMP-dependent but independent of PKA.

Activation of Rap2B by the M3 mAChR in HEK-293 cells is mediated by cyclic AMP-regulated Epac proteins (29, 42). Again, the cyclic AMP-dependent and PKA-independent activation of R-Ras is apparently also mediated by Epac proteins, endogenously expressed in HEK-293 cells (34). Treatment of HEK-293 cells with the membrane-permeable Epac-specific cyclic AMP analog, 8-pCPT-2Me-cAMP (100 μM) (43), induced a robust and rather long lasting activation R-Ras, with a maximum at 5–10 min (Fig. 4A). Half-maximal and maximal activation was observed at about 10 and 100 μM 8-pCPT-2Me-cAMP, respectively (Fig. 4B). R-Ras activation by 8-pCPT-2Me-cAMP was not affected by the PKA inhibitor H-89 (10 μM) (Fig. 4C).
R-Ras Activation by Epac

FIGURE 5. Epac-mediated but Rap-independent activation of R-Ras by the M3 mACHr and Gs-coupled receptors. HEK-293 (A and B, upper panel, and C) and N1E-115 neuroblastoma cells (B, lower panel) were transfected with wild-type R-Ras alone (Control) together with the β2-adrenergic receptor (B, upper panel) and with R279K Epac1, S17N Rap2B, unspecific siRNA (Alexa Fluor 488), or Epac1-specific siRNA as indicated. At 48 h after transfection, the cells were incubated for 1 min without (Basal) and with 100 μM carbachol, for 5 min with 100 μM 8-pCPT-2Me-cAMP, for 2 min with 10 μM adrenaline or 1 μM PGE1, as indicated, followed by determination of R-Ras-GTP and total R-Ras (Lysate) levels. Blots are representative for 3–5 experiments. C, left panel, immunoblot detection of Epac1, RhoA, and cofilin in lysates of control cells (siRNA Control) and Epac1-specific siRNA transfected cells (siRNA Epac1).

To substantiate the involvement of Epac proteins in R-Ras activation, HEK-293 cells were transfected with R279K Epac1, an Epac1 mutant deficient in cyclic AMP binding, which interfered with cyclic AMP-dependent Rap2B activation (34). As shown in Fig. 5A, expression of R279K Epac1 suppressed the activation of R-Ras by both the M3 mACHr agonist, carbachol, and the Epac-specific cyclic AMP analog, 8-pCPT-2Me-cAMP. Epac proteins act as GEFs for Rap GTPases (35, 42). Therefore, we studied whether Rap GTPases are involved in the activation of R-Ras. However, co-expression of dominant negative S17N Rap2B and dominant negative S17N Rap1A (not shown), which prevented activation of H-Ras and extracellular signal-regulated kinases by Gαi-coupled receptors in HEK-293 cells (34), did not interfere with activation of R-Ras by either carbachol or 8-pCPT-2Me-cAMP (Fig. 5A). These data suggested that the cyclic AMP-dependent activation of R-Ras by the M3 mACHr is mediated by Epac proteins but apparently independent of Rap GTPases. The data presented thus far suggested that the M3 mACHr, which can couple to several heterotrimeric G proteins, induces R-Ras activation specifically by enhancing cyclic AMP formation. Therefore, it was of interest to know whether typical Gαs- and adenylyl cyclase-coupled receptors may induce activation of R-Ras as well. Stimulation of the β2-adrenergic receptor transiently expressed in HEK-293 cells by adrenaline (10 μM) induced GTP loading of R-Ras, and this R-Ras activation was fully suppressed by co-expression of R279K Epac1 (Fig. 5B). Furthermore, agonist activation of the Gs-coupled receptor for PGE1 (α2) endogenously expressed in N1E-115 neuroblastoma cells induced activation of R-Ras by about 2-fold, similar to treatment of the cells with 8-pCPT-2Me-cAMP (Fig. 5B). Again, expression of R279K Epac1 suppressed activation of R-Ras by PGE1 and the Epac-specific cyclic AMP analog. To assign Epac1 as an essential regulator of R-Ras activation, its endogenous expression in HEK-293 cells was suppressed by specific siRNA. As shown in Fig. 5C, this maneuver abolished cellular expression of Epac1 leaving the expression of RhoA and cofilin unaffected. Most important, silencing of cellular Epac1 severely impaired activation of R-Ras by carbachol and the Epac-specific cyclic AMP analog (Fig. 5C).

Interaction of Epac1 with R-Ras—The activation of R-Ras by Epac proteins observed in intact cells suggested that Epac proteins may bind directly to R-Ras and catalyze GDP/GTP exchange at this GTPase. Binding of Epac proteins to R-Ras was studied in comparison to Rap2B and ARF1. For this, GST-tagged Rap2B, R-Ras, and ARF1, each either in the nucleotide-free, GDP-bound, or GTPγS-bound state, and immobilized on glutathione-Sepharose beads, were examined for binding of full-length Epac1 from lysates of HEK-293 cells overexpressing the protein or purified ΔDEP Epac1, Epac1-(149–881), an Epac1 mutant lacking the N-terminal DEP domain (35). As expected, both full-length Epac1 and ΔDEP Epac1 specifically bound to Rap2B in its nucleotide-free state (Fig. 6A, upper panel). Most strikingly, a similar efficient binding of full-length Epac1 and ΔDEP Epac1 to nucleotide-free R-Ras was observed (Fig. 6A, middle panel), whereas no binding at all was observed to ARF1, even when this GTPase was presented at a 10-fold excess (Fig. 6A, lower panel).

The GEF activity of Epac1 was determined by measuring release of [3H]GDP bound to purified Rap2B, R-Ras, and ARF1, in the absence and presence of 8-Br-cAMP and purified ΔDEP Epac1. As expected, ΔDEP Epac1 stimulated release of [3H]GDP from purified Rap2B, but only in the presence of 8-Br-cAMP (Fig. 6B). In contrast, ΔDEP Epac1
exhibited no GEF activity toward ARF1. Most important, ΔDEP Epac1 induced release of [3H]GDP from R-Ras (Fig. 6B). As observed with Rap2B, significant GEF activity of ΔDEP Epac1 toward R-Ras, although less efficient, was only observed in the presence of 8-Br-cAMP. The release of [3H]GDP from R-Ras induced by 8-Br-cAMP-activated ΔDEP Epac1 was of a similar magnitude as the release induced by purified C3G-(830–1078), containing the catalytic GEF domain of the protein (44).

### Role of R-Ras in M₃ mAChR Signaling

To study whether R-Ras plays a role in M₃ mAChR signaling, we first studied the effects of the expression of the dominant negative and constitutively active R-Ras mutants, S43N R-Ras and G38V R-Ras, respectively (2, 3), on M₃ mAChR-mediated PLC and PLD stimulation. As shown in Fig. 7A, neither expression of S43N R-Ras nor that of G38V R-Ras had any effect on M₃ mAChR-mediated IP₃ formation. Expression of these R-Ras mutants had also no effect on the cellular PLC substrate (phosphatidylinositol 4,5-bisphosphate) levels (data not shown). In contrast, expression of S43N R-Ras strongly decreased (by up to 70%) and expression of G38V R-Ras strongly increased (by up to 2-fold) the M₃ mAChR agonist-induced IP₃ stimulation (Fig. 7B). Inhibition of R-Ras signaling by expression of the GTPase-activating protein GAP1 IP4BP, known to stimulate GTP hydrolysis on R-Ras in vitro (45), mimicked the inhibitory effect of dominant negative R-Ras on PLD stimulation by the M₃ mAChR (Fig. 7C). These data suggested that R-Ras plays a major and specific role in the M₃ mAChR-mediated PLD stimulation.

R-Ras activation by the M₃ mAChR was dependent on cyclic AMP formation (see Fig. 3A). In agreement with these data, we observed that treatment of HEK-293 cells with the adenylyl cyclase inhibitor, dd-Ado (10⁻⁶ M), strongly reduced, by about 50%, the M₃ mAChR-mediated PLD stimulation (Fig. 8A). Furthermore, in agreement with the involvement of Epac proteins, but not PKA, in the M₃ mAChR-mediated R-Ras activation, it was found that the PKA inhibitor, H-89 (10 μM), had no effect (Fig. 8A), whereas expression of R279K Epac1 reduced the M₃ mAChR-mediated PLD stimulation (Fig. 8B). Conversely, direct stimulation of Epac proteins with 8-pCPT-2Me-cAMP induced PLD stimulation (Fig. 8C). Compared with the M₃ mAChR action, PLD stimulation induced by 8-pCPT-2Me-cAMP was much less efficient, only about 30% (see ordinates in Fig. 8, A and C), but on the other hand was more sensitive to inhibition by R279K Epac1. Depletion of cellular Epac1 by transient transfection with Epac1-spe-
cific siRNA diminished activation of R-Ras by the M₃ mAChR and the Epac-specific cyclic AMP analog (see Fig. 5E). As shown in Fig. 9A, silencing of cellular Epac1 also reduced the PLD response by the M₃ mAChR. To corroborate these data, HEK-293 cells stably expressing retroviruses encoding siRNA targeted against human Epac1 were generated. Expression of endogenous Epac1 was clearly reduced in these cells both on the mRNA and protein level, whereas expression of R-Ras was not altered (Fig. 9B, upper panel). The specific abrogation of Epac1 expression resulted in a reduced activation of PLD by the M₃ mAChR.

**DISCUSSION**

Despite its similarities to H-Ras, it is now generally accepted that R-Ras exhibits unique biological functions. In particular, R-Ras promotes integrin-mediated cell adhesion, in part through Rap-dependent signaling, and cell migration by modulating the spatio-temporal regulation of Rho and Rac activities (9–11, 18–20). Activation of R-Ras, i.e. GTP loading, is catalyzed by several distinct GEFs, e.g. RasGRP1–3, C3G, and AND-34 (5–7, 22–25). However, none of these GEFs appears to be specific for R-Ras, and even more important, activation of R-Ras by membrane receptors has not been studied at all. Therefore, we examined whether and by which mechanisms GPCRs activate R-Ras. Here we show for three GPCRs, the M₃ mAChR and the β₂-adrenergic receptor stably and transiently expressed in HEK-293 cells, respectively, and the PGE₁ receptor endogenously expressed in N1E-115 neuroblastoma cells, strong and rapid activation of R-Ras. The activation of R-Ras by these three GPCRs is apparently because of their capacity to couple to the cyclic AMP-regulated Epac exchange factors.

For screening of GPCR-mediated R-Ras activation, we used HEK-293 cells stably expressing the M₃ mAChR, which can couple to all major subtypes of heterotrimeric G proteins, most prominently to Gq proteins (26–28). Surprisingly, we found that GTP loading of R-Ras by this rather promiscuous GPCR is specifically dependent on cyclic AMP formation by adenylyl cyclase, which is not considered a primary function of the M₃ mAChR. Subsequent studies with forskolin, a direct adenylyl cyclase activator, cyclic 8-Br-AMP, a membrane-permeable cyclic AMP analog, and particularly the β₂-adrenergic receptor and the PGE₁ receptor, two typical Gi-coupled receptors, confirmed that R-Ras activation by GPCRs is indeed dependent on cyclic AMP formation. However, the
activation of R-Ras was apparently not mediated by PKA, the major cellular cyclic AMP effector. Instead, Epac1 proteins, endogenously expressed in HEK-293 and N1E-115 neuroblastoma cells (34), apparently served as mediators of cyclic AMP-dependent R-Ras activation. First, specific activation of Epac proteins with the cyclic AMP analog, 8-pCPT-2Me-cAMP (43), induced a PKA-independent R-Ras activation. Second, expression of R279K Epac1, an Epac1 mutant deficient in cyclic AMP binding (34), suppressed activation of R-Ras by both 8-pCPT-2Me-cAMP and the GPCR agonists in HEK-293 and N1E-115 neuroblastoma cells. Third, silencing of cellular Epac1 by siRNAs either by transient transfection of Epac1-specific siRNA or stable expression of retroviral encoded siRNA Epac1 in HEK-293 cells (data not shown) impaired activation of R-Ras by the M₃ mAChR and by the Epac-specific cyclic AMP analog. Epac1 directly binds to R-Ras and, when activated by cyclic AMP, catalyzes the GDP/GTP exchange at this GTPase, probably leading to activation of R-Ras under cellular settings.

We have recently reported that the same GPCRs shown here to activate R-Ras in HEK-293 and N1E-115 neuroblastoma cells induce activation of the Rap GTPase Rap2B and that this process is mediated by cyclic AMP-activated Epac proteins (31), similar as shown here for activation of R-Ras. As Epac proteins have been characterized as GEFs specific for Rap GTPases (35, 42), we studied whether Rap GTPases may mediate activation of R-Ras. However, co-expression of dominant negative Rap2B, which on the other hand prevented activation of H-Ras and extracellular signal-regulated kinases by Gₛ-coupled receptor in HEK-293 cells (34), or Rap1A did not prevent R-Ras activation by 8-pCPT-2Me-cAMP and the M₃ mAChR. Thus, in line with the direct interaction of Epac1 with R-Ras observed in vitro with purified proteins, these data suggest that cyclic AMP-activated Epac proteins can induce activation of R-Ras in intact cells largely independent of Rap GTPases. The ineffectiveness of the dominant negative Rap mutants to prevent Epac-mediated R-Ras activation in intact cells may be due to discrete functional and physical Epac signaling units. However, although Epac1 bound to R-Ras and Rap2B with similar efficiency, the GEF activity of ΔDEP Epac1 toward R-Ras was less than toward Rap2B but similar to the GEF activity of the R-Ras GEF C3G. Thus, despite previous studies (46–48) we have obtained convincing evidence that the cyclic AMP-dependent activation of R-Ras is directly mediated by Epac proteins both in vivo and in vitro. Several factors may contribute to explain our observation of Epac-mediated R-Ras activation in intact cells may be due to discrete functional and physical Epac signaling units. However, although Epac1 bound to R-Ras and Rap2B with similar efficiency, the GEF activity of ΔDEP Epac1 toward R-Ras was less than toward Rap2B but similar to the GEF activity of the R-Ras GEF C3G. Thus, despite previous studies (46–48) we have obtained convincing evidence that the cyclic AMP-dependent activation of R-Ras is directly mediated by Epac proteins both in vivo and in vitro. Several factors may contribute to explain our observation of Epac-mediated R-Ras activation, including the need for specific cofactors that direct the catalytic activity and/or substrate selectivity of Epac, a divergence in the pattern of proteins expressed by different cell lines, and the subcellular partitioning of signaling factors. Similarly, activation of R-Ras by C3G was also disputed, and inconsistent observations to some extent were explained by the notification that R-Ras may act as a preferable substrate for C3G in specific subcellular domains (22, 24, 25).

FIGURE 8. Involvement of cyclic AMP and Epac proteins in M₃ mAChR-mediated PLD stimulation. A, HEK-293 cells were first treated for 15 min without (Control) and with 10 μM H-89 or 10 μM dd-Ado. Thereafter, PLD activity was determined in the absence (Basal) and presence of 100 μM carbachol. B and C, HEK-293 cells were transfected without (Control) and with R279K Epac1. After 48 h, PLD activities were measured in the absence (Basal) and presence of 100 μM carbachol (B) or at the indicated concentrations of 8-pCPT-2Me-cAMP (C). Data shown are means ± S.E. (n = 3–5).
R-Ras Activation by Epac

**FIGURE 9. Effect of Epac1 depletion on M3 mAChR-mediated PLD stimulation.** HEK-293 cells either transfected with unspecific siRNA (Alexa Fluor 488) or Epac1-specific siRNA (siRNA Epac1-expressing cells) (B), were processed to determine PLD activity in the absence (Basal) and presence of 5 μM carbachol. Data shown are means ± S.E. (n = 3). B, upper panel, mRNA expression of Epac1 in vector controls and siRNA Epac1-expressing cells (n = 5). Higher ΔCt values represent lower mRNA levels. Immunoblot detection of Epac1 and R-Ras in lysates of control cells (Vector Control) and siRNA Epac1-expressing cells.

It may be considered that Epac proteins not only act as GEFs for these GTPases but additionally function as effector of the activated GTPase or may translocate the active GTPase to potential effector target sites in the cells. Indeed, Quilliam and co-workers (50) recently reported that Epac2 is able to couple H-Ras and cyclic AMP signals at the plasma membrane.

In search for a biological function of R-Ras in the GPCR actions, a possible role of R-Ras in the M3 mAChR-mediated stimulation of PLC and PLD was analyzed, using dominant negative and constitutively active R-Ras mutants. In line with findings on the cholecystokinin A receptor expressed in Chinese hamster ovary cells (12), no effects of these R-Ras mutants on PLC stimulation by the M3 mAChR expressed in HEK-293 cells were observed. In contrast, dominant negative R-Ras largely inhibited and constitutively active R-Ras enhanced PLD stimulation by the M3 mAChR. The GTPase-activating protein GAP1 IP4BP, known to stimulate GTP hydrolysis on R-Ras in vitro (45), mimicked the inhibitory effect of dominant negative R-Ras on PLD stimulation by the M3 mAChR. Furthermore, in line with the findings observed on R-Ras activation, PLD stimulation by the M3 mAChR was reduced by inhibition of adenyl cyclase, but not PKA, and by expression of R279K Epac1, and direct activation of Epac proteins increased PLD activity. Most important, silencing of cellular Epac1 decreased M3 mAChR signaling to PLD as well. As dominant negative Rap GTPases did not alter the PLD response (data not shown), and as we have reported previously that M3 mAChR-mediated PLD stimulation is Ras- and Ral-independent (51), such mechanisms probably do not contribute to PLD regulation by R-Ras. However, as shown here Epac-activated R-Ras apparently plays a prominent and specific role in PLD stimulation by GPCRs. Studies are in progress to define the mechanisms by which R-Ras, in combination with Rho and ARF GTPases (28, 36, 41), controls PLD activity. As Epac-activated Rap2B has been shown to control stimulation of PLC-ε by GPCRs (29, 31, 32), it appears that Epac proteins by activating the GTPases, Rap2B and R-Ras, act as multifunctional proteins in GPCR signaling systems. Interestingly, R-Ras and PLC-ε have been shown recently to act together in sustaining protrusive motility in MCF10A cells (52).

In conclusion, our findings obtained with three different GPCRs in two cell types indicate that activation of R-Ras is a rapid and prominent response to GPCR activation and that these receptors activate R-Ras by enhancing cyclic AMP formation with subsequent activation of Epac proteins, apparently directly controlling the GDP/GTP exchange reaction at R-Ras independent of Rap GTPases. As studied with the M3 mAChR, Epac-activated R-Ras is apparently not involved in PLD stimulation but plays a major role in stimulation of PLD by this GPCR.

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