quantities were measured for a given train: (i) calcium currents during the first pulse, (ii) the increase in membrane capacitance during this pulse, and (iii) the average $[Ca^{2+}]$ between the first and second pulses of these trains. These parameters have been compared for trains after augmentation (with slightly enhanced basal calcium between trains) with controls (no previous enhancement of basal calcium). Controls are set to 100%.
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26. To measure step responses (during pulses), linear regressions were performed to three to four data points both before and after a pulse. These fits were extrapolated to the time of the pulse, and their difference was adopted as the step size. The calculation of the derivative of the capacitance trace, as in Fig. 1C, was made by the transferral of short segments of data to the IGOR program (WaveMetrix, Lake Oswego, OR).
27. To simulate responses to pulse trains, the parameters a_1, b_1, and a_3 of the model by Heinemann and co-workers (*7*) have been used unchanged. Peak $[Ca^{2+}]$ at the "release sites" and increments in basal $[Ca^{2+}]_i$ during trains of depolarizing pulses were set to 4.2 μM and 0.42 μM, respectively. A value of 3.45 was adopted as the time constant of $[Ca^{2+}]_i$ decay after pulses, according to experimental results. The constant a_2 for the rate of the depriming of vesicles was increased to 0.012 s^{-1}, and the size of pool A was set to 4000 fF, resulting in a pool B of 230 fF at a $[Ca^{2+}]$ of 100 nM. With these new parameters, we obtained both the correct ratio of capacitance increases between pulses one and five in a train and the experimental result for total secretion during a train of pulses starting from basal $[Ca^{2+}]_i$.
28. All experiments were done in the perforated-patch configuration with the use of nystatin. Amperometric currents were measured as described in (*19*). The voltage at the carbon fiber was continuously held at +800 mV, and the resulting current was measured with an EPC-7 patch-clamp amplifier (List-Electronic, Darmstadt, Germany), filtered at 20 Hz, and sampled by the data acquisition program. Amperometric traces were transferred to the IGOR analysis program for the calculation of current integrals. Before integration, a base line (from a stretch of data without secretory events) was subtracted.
29. We thank M. Pilot for the preparation of chromaffin cells and R. H. Chow, K. D. Gillis, and A. B. Parekh for the reading of and suggestions on the manuscript.

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Inhibition of the EGF-Activated MAP Kinase Signaling Pathway by Adenosine 3′,5′-Monophosphate

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Mitogen-activated protein (MAP) kinases $p42^{mapk}$ and $p44^{mapk}$ are activated in cells stimulated with epidermal growth factor (EGF) and other agents. A principal pathway for MAP kinase (MAPK) activation by EGF consists of sequential activations of the guanine nucleotide exchange factor Sos, the guanosine triphosphate binding protein Ras, and the protein kinases Raf-1, MAPK kinase (MKK), and MAPK. Because adenosine 3′,5′-monophosphate (cAMP) does not activate MAPK and has some opposing physiologic effects, the effect of increasing intracellular concentrations of cAMP with forskolin and 3-isobutyl-1-methylxanthine on the EGF-stimulated MAPK pathway was studied. Increased concentrations of cAMP blocked activation of Raf-1, MKK, and MAPK in Rat1hER fibroblasts, accompanied by a threefold increase in Raf-1 phosphorylation on serine 43 in the regulatory domain. Phosphorylation of Raf-1 in vitro and in vivo reduces the apparent affinity with which it binds to Ras and may contribute to the blockade by cAMP.

Signal transduction pathways used by EGF include activation of $p42^{mapk}$ [or extracellular signal–regulated kinase 2 (ERK2)] and $p44^{mapk}$ (or ERK1), which are closely related protein serine-threonine kinases regulated by dual tyrosine and threonine phosphorylation (*1*). MAPK is likely to mediate at least a subset of cellular responses to EGF, including activation of a number of cytosolic enzymes (phospholipase A_2 and 90-kD ribosomal protein S6 kinase) and nuclear transcription factors (including c-Myc and $p62^{TCF}$) (*1*).

Recent studies have defined several steps in a MAPK activation pathway mediated by Ras. EGF induces autophosphorylation of the EGF receptor (EGFR) on tyrosine residues, creating specific binding sites for Src homology 2 (SH2)–containing proteins, including a multiprotein complex that activates Ras (*2*). The guanine nucleotide–releasing protein Sos, the mammalian homolog of the *Drosophila* protein Son of sevenless, is complexed in the cytoplasm to an SH2-containing adapter protein, growth factor receptor–bound protein 2 (Grb2). After EGFR tyrosine phosphorylation, the Grb2-Sos complex is recruited to the receptor by the SH2 domain of Grb2; localization of Sos with its membrane-bound target Ras catalyzes formation of the active, guanosine triphosphate (GTP)–bound form of Ras. Activated Ras in turn causes activation of Raf-1 by an undefined mechanism that appears to involve direct interaction of Ras and Raf-1 (*3*, *4*). Activated Raf-1 then phosphorylates and activates MKKs, the specific activators of MAPK (*5*).

Cellular control cannot be achieved by collections of linear signal transduction pathways alone; mechanisms for integrating pathways and generating interactions between pathways are required. MAPK is activated by a diverse set of factors, but notably not by agents that increase intracellular concentrations of cyclic adenosine monophosphate (cAMP) (*6*). Because the antagonism between insulin and cAMP-elevating agents is well known and because cAMP counteracts some biologic effects of oncogenes that activate MAPK (*7*), we conducted studies to determine whether cAMP inhibits the MAPK pathway.

Rat1 fibroblasts that overexpress the human EGFR (Rat1hER) were deprived of serum and incubated with or without forskolin or 3-isobutyl-1-methylxanthine (IBMX) or both agents for 15 min before stimulation with EGF (50 or 200 ng/ml). Forskolin directly activates the catalytic subunit of adenylate cyclase (*8*). IBMX inhibits cAMP phosphodiesterase and may also block inhibitory adenosine receptors linked to G_i to increase the intracellular concentrations of cAMP (*9*). Thus, both agents enhance cAMP accumulation but by different mechanisms. Cells treated with forskolin or IBMX contained activity from the catalytic subunit of cAMP-dependent protein kinase (PKA) [assessed by PKA-inhibitor peptide (PKI)–sensitive histone H1 kinase activity] that was 10 and 4 times that in control cells, respectively (*10*). The stimulation of cells with EGF (50 ng/ml for 5 min) without prior incubation with forskolin or IBMX resulted in PKI-insensitive kinase activity in the cell extract supernatant toward myelin basic protein (MBP) that was four times greater than that in untreated cells (*10*). However, the EGF-dependent increase in MBP kinase ac-

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tivity was nearly abolished by prior incubation with forskolin or IBMX or both (*10*).

To confirm that the changes in MBP kinase activity in the supernatants from cell lysates were attributable to MAPK, we performed immune complex kinase assays with a polyclonal antibody (TR2) to $p42^{mapk}$ (anti-$p42^{mapk}$) (*11*) (Fig. 1). MBP kinase activity immunoprecipitated from lysates of cells stimulated with EGF was greater than that from lysates of untreated cells, which indicates that activation of $p42^{mapk}$ had occurred. Treatment of cells with forskolin or IBMX or both blocked stimulation of $p42^{mapk}$ by EGF.

Phosphorylation and activation of MAPK is catalyzed by one or more MKKs (*12–15*). To determine whether forskolin and IBMX also blocked the EGF stimulation of MKKs, we assayed portions of supernatants from cell lysates for MKK activity by ^{32}P incorporation into recombinant kinase–defective $p42^{mapk}$ (KR) (Fig. 2) (*12*). The treatment of cells with EGF increased MKK activity approximately eightfold in cells not treated with forskolin or IBMX (Fig. 2). This response to EGF was not altered in cells treated with the dimethyl sulfoxide (DMSO) vehicle. In contrast, prior treatment with forskolin or IBMX or both blocked the EGF-stimulated activation of MKK by approximately 90% (Fig. 2).

To exclude the possibility that forskolin acted by inhibiting EGF-stimulated receptor autophosphorylation of sites required for Grb2 binding (*16*), we immunoprecipitated EGFR (Fig. 3B) for protein immunoblotting with antibody to phosphotyrosine (anti-pTyr) (Fig. 3A) or antibody to Grb2 (Fig. 3C). EGF stimulated immunoreactivity to anti-pTyr (Fig. 3A) equally, irrespective of prior treatment with forskolin (Fig. 3A). GRB2 was not bound detectably to EGFRs in untreated cells (Fig. 3C) but was bound to receptors after EGF stimulation, and this effect was not altered by prior treatment with forskolin.

Because EGF-dependent MKK activation was blocked by forskolin or IBMX, we considered the possibility that PKA might directly phosphorylate and inactivate MKK1 and MKK2. However, MKK1 and MKK2 were poor substrates for PKA (*10*), which prompted us to examine the effect of forskolin on EGF-dependent activation of Raf-1 (Fig. 4). The activation of Raf-1 was measured in immune complexes of antibodies to Raf-1 (anti–Raf-1) (*11*), with [γ-^{32}P]-adenosine triphosphate (ATP) and nearly homogeneous baculovirus-expressed MKK1 (*17*) as substrates. EGF stimulated Raf-1 kinase activity nearly fivefold, and stimulation was ablated by prior treatment with forskolin. Taken together, our data (Figs. 1 through 4) localize the point or points of action of forskolin (and presumptively, of IBMX) to steps between Grb2 binding and activation of Raf-1.

Ras appears to interact directly with Raf-1 (*3*, *4*). Raf-1 binds to immobilized Ras·GMP-PNP (guanylyl-imidodiphosphate) or Ras^{Val12}·GMP-PNP but not to a modified form of Ras with a mutation in the effector domain (Ras^{Ala36}) (*3*). Other studies have used a yeast genetic system or recombinant proteins expressed in *Escherichia coli* to demonstrate that NH_2-terminal fragments of Raf-1 interact with Ras (*4*). Expressed fragments of Raf-1 bind to Ras in a manner dependent on GTP and an intact Ras effector domain. Because expression of an NH_2-terminal fragment of Raf-1 acts as a dominant inhibitor of Ras effects (*18*), the Ras:Raf-1 interaction is presumed to have mechanistic significance. For these reasons, we tested the ability of PKA to phosphorylate Raf-1 and regulate Ras:Raf-1 binding.

To this end, kinase-defective $(His)_6$–Raf-1 in which Lys^{375} is changed to methionine [Raf(K375M)] was expressed in Sf9 insect cells and partially purified by nickel-chelate affinity chromatography (*17*). Incubation of Raf(K375M) with PKA and [γ-^{32}P]ATP resulted in phosphorylation of a 75-kD band in the Raf-1 preparation (Fig. 5A) that was not detectable in control reactions containing PKA or the preparation of Raf(K375M) incubated alone with [γ-^{32}P]ATP. The 75-kD phosphoprotein was identified as Raf-1 by protein immuno-

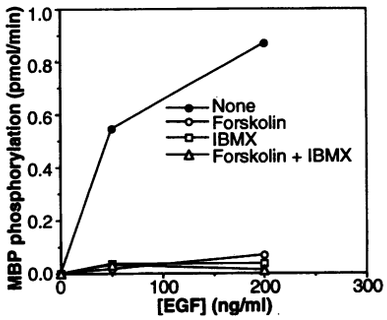

Fig. 1. Inhibition of EGF-dependent activation of $p42^{mapk}$ by forskolin and IBMX. Rat1hER cells were grown in Dulbecco's modified Eagle's minimum essential medium and 10% fetal calf serum. Confluent cells (one 6-cm plate per condition) were incubated (at 37°C for 50 min) in Kreb's Ringer bicarbonate–Hepes (KRB-Hepes) buffer (*29*) and then left untreated (vehicle added) or treated with 50 μM forskolin, 0.5 mM IBMX, or 50 μM forskolin plus 0.5 mM IBMX for 15 min. After EGF stimulation (5 min), cells were washed with cold phosphate-buffered saline. Each plate of cells was lysed in 0.5 ml of ice-cold buffer A (*30*) and centrifuged at 16,000*g* for 20 min. Portions (0.1 ml) of each supernatant were immunoprecipitated with anti-$p42^{mapk}$ (TR2) coupled to protein A–Sepharose beads, and $p42^{mapk}$ activity in the immune complex was determined. MBP kinase assays were done essentially as described (*12*), except that 2 μM PKI (Gibco-BRL, Gaithersburg, Maryland) was included in the reaction mixture and reactions were carried out at 30°C for 12 min. The data shown are the average of two experiments.

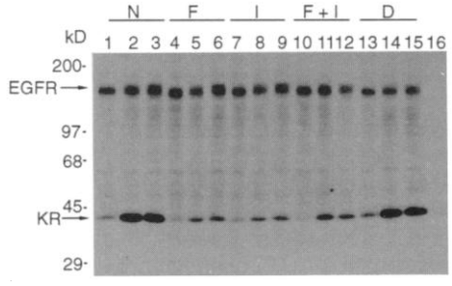

Fig. 2. Inhibition of MKK activation by forskolin and IBMX. Cells were incubated and supernatants were prepared from treated cells as described (Fig. 1). Supernatants were diluted to one-fifth the original concentration, and portions (5 μl) were assayed for MKK activity as described (*12*) with $p42^{mapk}$-K52R as the substrate. Phosphorylation was analyzed by autoradiography after SDS-PAGE (on 10% gels). Cells were incubated without (N) or with forskolin (F), IBMX (I), forskolin plus IBMX (F + I), or DMSO (D) (solvent for forskolin and IBMX) before stimulation with EGF for 5 min. Lanes 1, 4, 7, 10, and 13: nonstimulated cells; lanes 2, 5, 8, 11, and 14: cells stimulated with 50 ng/ml EGF; lanes 3, 6, 9, 12, and 15: cells stimulated with 200 ng/ml EGF; lane 16: no cell sample added (control reaction). Arrows indicate the KR substrate and autophosphorylated EGF receptors (EGFRs). A duplicate experiment showed similar results. Band densities on the autoradiographs were quantitated with a densitometer (Molecular Dynamics, Sunnyvale, California).

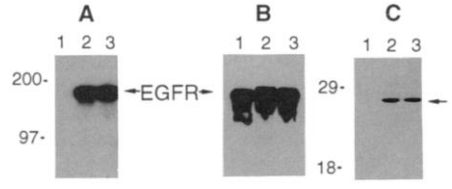

Fig. 3. Autophosphorylation of EGFRs and GRB2 binding. Quiescent Rat1hER cells were pretreated with DMSO (lanes 1 and 2) or forskolin (lanes 3) for 15 min and then left unstimulated (lanes 1) or were stimulated with EGF (200 ng/ml) for 5 min (lanes 2 and 3). Cells were lysed in buffer containing detergent, and EGFRs were immunoprecipitated as described (*31*) from equal portions of the lysate supernatants with RK-2, a rabbit polyclonal antibody to the EGFR. Immunoprecipitated proteins were separated by SDS-PAGE (10% gel) and transferred to a nitrocellulose membrane. The top half of the membrane was immunoblotted with anti-pTyr (*32*) (**A**); after the antibody was removed, the membrane was reprobed with antibody to the EGFR (**B**). The bottom half of the membrane was immunoblotted with a monoclonal antibody to Grb2 (arrow) (Transduction Laboratories, Lexington, Kentucky) (**C**). Molecular size markers are shown on the left in kilodaltons.

blotting (*10*). Thus, Raf-1 is an in vitro substrate for PKA.

We next compared the abilities of PKA-phosphorylated Raf(K375M) and nonphosphorylated Raf(K375M) to bind to immobilized Ras^{Val12}. Phosphorylated and nonphosphorylated Raf(K375M) were prepared and were treated identically except for addition of PKA at the initiation or conclusion of the phosphorylation reaction, respectively (Fig. 5A). Equal portions of phosphorylated and nonphosphorylated Raf(K375M) were incubated with immobilized Ras^{Val12} loaded with either GMP-PNP or guanosine diphosphate (GDP), and proteins retained after washing were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and protein immunoblotting with antibody to Raf-1 (Fig. 5B). Nonphosphorylated Raf(K375M) bound preferentially to Ras^{Val12}·GMP-PNP (*3*, *4*). However, the binding of PKA-phosphorylated Raf(K375M) to Ras^{Val12}·GMP-PNP and to Ras^{Val12}·GDP was reduced approximately 75% in comparison to that of nonphosphorylated Raf. Reciprocal changes in the amount of Raf(K375M) were detected in supernatants from the binding reactions (*10*). These experiments were repeated three times with similar results. Thus, these data demonstrate that phosphorylation of Raf-1 in vitro by PKA can inhibit binding of Raf-1 to Ras.

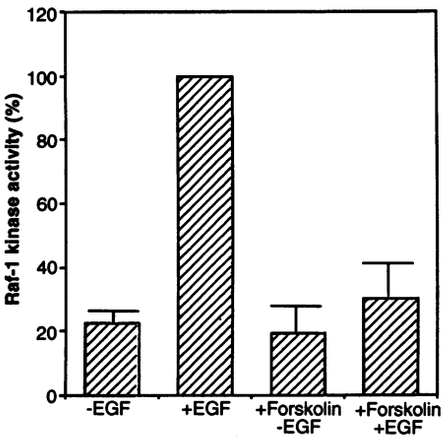

Fig. 4. Raf-1 kinase activity in cells treated with forskolin. Rat1hER cells (one 15-cm plate per condition) were grown to confluence, rendered quiescent, incubated with DMSO (vehicle) or forskolin, and stimulated with EGF as described (Fig. 1). Cell lysis, immunoprecipitation of Raf-1, and an assay of Raf-1 kinase activity in the immune complexes were done as described (*11*) by transfer of ^{32}P incorporation into homogenous, baculovirus–rat MKK1 as the substrate (*17*). MKK1 was separated by SDS-PAGE (on 10% gels), and the ^{32}P incorporated into MKK1 was quantitated by Cerenkov radiation by counting of excised MKK1 bands. Values represent the mean of four experiments. Data were corrected for MKK1 autophosphorylation and for background radioactivity in the Raf-1 immune complex; the corrected incorporation (+EGF) was set to 100% (range = 1000 to 3000 cpm). Bars indicate standard error.

We conducted studies to determine whether Raf-1 from forskolin-treated cells also has decreased binding affinity for Ras. Rat1hER cells were treated or not treated with forskolin and then stimulated with and without EGF. Equal portions of supernatants from lysates of cells treated under the different conditions were analyzed for their ability to bind to immobilized Ras (Fig. 6). Raf-1 from cells not treated with forskolin bound preferentially to Ras·GMP-PNP. In contrast, the binding of Raf-1 from cells treated with forskolin to Ras·GMP-PNP was decreased by approximately 70 ± 10% (average of two experiments ± range). The use of phosphatase inhibitors and the use of subsaturating concentrations of lysate appeared to be important for the detection of the difference in binding. Thus, increased concentrations of cAMP in vivo decrease the apparent affinity of Raf-1 for Ras. Because similar inhibition of Raf-1 binding is caused by phosphorylation of Raf-1 in vitro by PKA, a plausible mechanism for the reduction of Raf:Ras affinity that occurs in intact cells is phosphorylation of Raf-1 by PKA.

To determine whether forskolin induces Raf-1 phosphorylation in intact cells, we labeled Rat1hER cells with ^{32}P and treated them with or without forskolin. Raf-1 was isolated by immunoprecipitation and SDS-PAGE. The ^{32}P content of Raf-1, quantitated by Cerenkov radiation, was increased 1.5 ± 0.2 times (average of two experiments ± range) by forskolin. Three major tryptic phosphopeptides (α, β, and γ) were observed upon analysis of the ^{32}P-labeled Raf-1 protein band from Rat1hER cells treated with forskolin that corresponded to three phosphopeptides (a, b, and c) of Raf-1 from untreated cells (Fig. 7). The ^{32}P content of phosphopeptides α, β, and γ was 4.7 ± 0.9, 1.5 ± 0.2, and 2.1 ± 0.2 times that of phosphopeptides a, b, and c (averages of two experiments ± range), respectively. Assuming that the modest increase in ^{32}P content of phosphopeptide b is not significant, the ^{32}P content of phosphopeptide b provides an internal control. With this assumption, forskolin treatment increased the ^{32}P content of phosphopeptide a threefold.

Phosphopeptides a, b, and c appear to correspond to previously described (*19*) phosphopeptides A, B, and X identified as in vivo sites of Raf-1 phosphorylation in NIH 3T3 cells. The sites of phosphorylation in phosphopeptides A and B were identified as Ser^{43} and Ser^{621}, respectively (*19*). To confirm that forskolin treatment induced phosphorylation of Ser^{43}, we phosphorylated a synthetic peptide containing Ser^{43} in vitro with PKA and cleaved it with trypsin (*19*). The tryptic peptide ($RAS^{43}DDG$) (*20*) contains only one po-

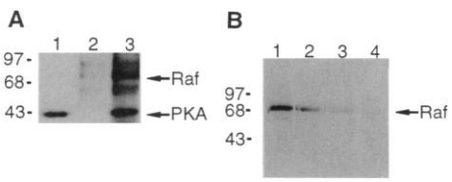

Fig. 5. Effect of PKA phosphorylation of Raf-1 on binding to Ras. (**A**) Phosphorylation of kinase-defective Raf-1 by PKA. Kinase-defective Raf(K375M), tagged with $(His)_6$ at the COOH-terminus, was expressed in Sf9 cells by a recombinant baculovirus and partially purified by nickel-chelate affinity chromatography. Raf(K375M) preparation (10 μg of total protein; estimated 1% purity) was phosphorylated (at 30°C for 30 min) in a total volume of 100 μl containing 20 mM tris-Cl (pH 7.5 at 4°C), 0.1 mM [γ-^{32}P]ATP (10 cpm/fmol), 10 mM magnesium acetate, and 1 μg of the catalytic subunit of PKA. Reactions were terminated with sample buffer and subjected to SDS-PAGE (on a 10% gel) and autoradiography [for 45 min with Xomat AR5 (Kodak, Rochester, New York)]. Lane 1: reaction omitting Raf; lane 2: reaction omitting PKA; lane 3: complete reaction mixture. (**B**) Binding of Raf to immobilized Ras. The K375M preparation was incubated with or without PKA as above, except radioactive ATP was omitted and 20 mM EDTA (final concentration) was added at the end of the incubation to quench the reaction. PKA was added to the tube containing quenched, non–PKA-phosphorylated Raf(K375M). Aliquots of the phosphorylated and nonphosphorylated Raf were analyzed for binding to immobilized Ras^{Val12} prebound to GMP-PNP (lanes 1 and 3) or GDP (lanes 2 and 4) as described (*3*). Bound Raf was detected by protein immunoblotting with anti–Raf-CR2 by enhanced chemiluminescence. Lanes 1 and 2, nonphosphorylated Raf; lanes 3 and 4, phosphorylated Raf. Molecular size markers are shown on the left in kilodaltons.

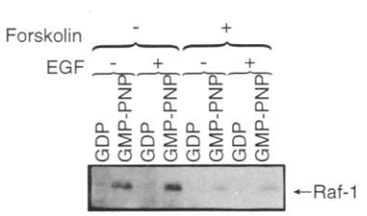

Fig. 6. Binding of Raf from cells treated with or without forskolin to immobilized Ras. Confluent plates of Rat1hER cells (four 15-cm plates per condition) were cultured without serum (18 hours), incubated with or without forskolin, and treated with or without EGF (100 ng/ml, final concentration) as described (Fig. 1). Portions of supernatant protein from each treatment were analyzed (*32*) for Raf binding to immobilized c-Ha-Ras loaded with GMP-PNP or GDP. The arrow indicates Raf detected by protein immunoblotting.

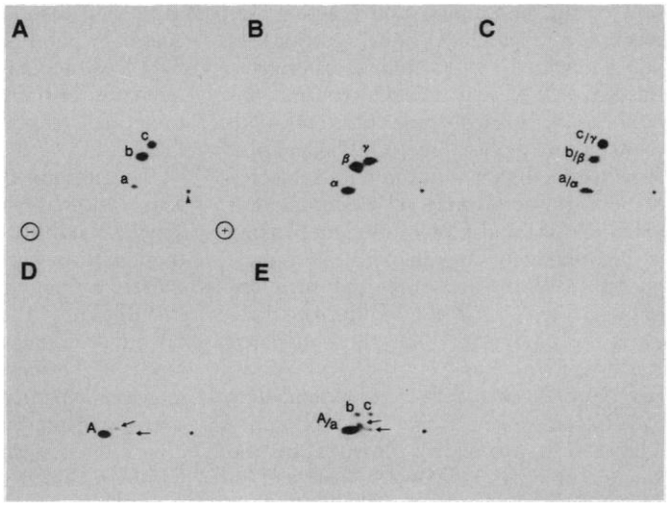

Fig. 7. Analysis of forskolin-induced phosphorylation of Raf-1 in Rat1hER cells by phosphopeptide mapping. Confluent Rat1hER cells in 15-cm plates were washed three times with KRB-Hepes buffer and then incubated for 2.5 hours with 10 ml per plate of KRB-Hepes buffer containing 5 mCi of [^{32}P]-orthophosphate. Forskolin or DMSO (vehicle control) was added directly to the labeling medium, and the cells were incubated for 20 min at 37°C before lysis and immunoprecipitation of Raf-1 (Fig. 4). Immunoprecipitated proteins were separated by SDS-PAGE; Raf-1 proteins were processed for tryptic phosphopeptide mapping as described (*19*). Radiolabeled phosphopeptides were visualized and quantitated with a PhosphorImager (Molecular Dynamics). (**A**) Raf-1 from DMSO-treated cells. (**B**) Raf-1 from forskolin-treated cells. Samples in (A) and (B) were processed identically. (**C**) Mixture of phosphopeptides used in (A) and (B). (**D**) Tryptic phosphopeptide mapping of a synthetic peptide (ISPTIVQQFGYQRRAS^{43}DDGKLTD) (*20*) containing Ser^{43} (*19*). The peptide was phosphorylated by PKA and purified by SDS–Tricine gel electrophoresis before trypsin digestion as described (*19*). (**E**) Mixture of Ser^{43} peptide (D) and Raf-1 peptides (A). The labels a, b, and c and α, β, and γ denote three major phosphopeptides derived from Raf-1 of DMSO- and forskolin-treated cells, respectively. A, tryptic peptide containing Ser^{43}; arrowhead, origin; arrows, two partially digested peptides derived from the Ser^{43} peptide. This experiment was repeated with similar results.

tential phosphorylation site, Ser^{43}. Phosphopeptides a (α) comigrated with this tryptic phosphopeptide, which identifies Ser^{43} as the principal site of Raf-1 phosphorylation in cells containing increased concentrations of cAMP. Ser^{43} resides in a sequence, RRXS^{43} (*20*), favorable (*21*) for phosphorylation by PKA; Ser^{43} is located in the NH_2-terminal regulatory domain of Raf-1 that binds to Ras (*4*). Thus, it is reasonable to ascribe to Ser^{43} phosphorylation the cAMP-induced reduction in binding affinity of Raf-1 to Ras, but contributions from other PKA sites (or targets) cannot be excluded. Increased concentrations of cAMP induced by forskolin might also activate cyclic guanosine monophosphate–dependent protein kinase, an enzyme with overlapping but not identical substrate specificity to PKA (*22*). Thus, our data do not resolve the relative contribution of these two enzymes for the mediation of the effect of forskolin or IBMX.

Previous studies in a variety of systems suggest that one site of action of cAMP lies downstream of Ras, which is consistent with our data. Forskolin inhibits the effect of v-Ras on the transcription of a rat prolactin promoter construct in GH_4 pituitary cells (*23*). In addition, a revertant line of v-Ras–transformed NIH 3T3 cells contains enhanced PKA activity, and increased cAMP content in the parent line causes loss of some characteristics of the transformed phenotype (*24*). Forskolin treatment does not dissociate the GRB2-Sos complex in Rat1hER cells (*25*).

Although these data are consistent with our suggestion that phosphorylation of Raf-1 on Ser^{43} may be one mechanism by which cAMP inhibits signaling from tyrosine kinases, other targets such as phospholipase C-γ1 (*26*) and Sos are not excluded. Increased phosphorylation of Sos was observed in cells treated with forskolin (*25*). Raf-1 was expressed in all cells examined, and activation of MAPK by ligands for other receptor tyrosine kinases is also dependent on Ras (*3, 4, 27*). Thus, it is likely that similar antagonism between cAMP and MAPK will occur for insulin and platelet-derived growth factor and possibly other ligands whose receptors are tyrosine kinases. Additional pathways for MKK activation independent of Ras and mediated by MAP or ERK kinase (MEK) kinase (*28*), a mammalian homolog of the yeast protein STE11, are likely. Thus, the possibility remains that some pathways for MKK activation may not be inhibited by cAMP elevation. Our demonstration that cAMP elevation negatively regulates Raf-1 activation begins the dissection of the complex interdigitation of the MAPK pathways with other signal-transducing pathways.

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30. Buffer A consists of 10 mM tris-acetate (pH 7.5 at 4°C), 40 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM benzamidine, 0.5% (v/v) Triton X-100, phenylmethylsulfonylfluoride (0.1 mg/ml), 1 μM pepstatin A, leupeptin (2 μg/ml), and aprotinin (2 μg/ml).
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33. For the in vivo studies, cells were quickly chilled and washed twice with ice-cold phosphate-buffered saline, sedimented, and resuspended in p21 buffer (*3*) (100 μl per 15-cm plate). In later experiments, additional phosphatase inhibitors (10 mM Na_2VO_4, 10 mM *p*-nitrophenyl phosphate, 40 mM β-glycerol phosphate, 25 mM NaF, 1 μM microcystin-LR, 0.01 μM calyculin A, and 0.2 mM sodium pyrophosphate) were included in all buffers and appear to be important for preserving the effect of forskolin on Raf-1. Resuspended cells were homogenized (*27*), and the lysates were centrifuged (100,000*g* at 4°C for 30 min). Approximately 40 μg of supernatant protein was incubated (200 μl total volume at 4°C for 30 min) with 50 μg of immobilized c-Ha-Ras, loaded with GMP-PNP or GDP (*3*). Beads were sedimented by brief centrifugation (15,000*g* for 1 min), and supernatants were removed. Beads were resuspended in 200 μl of p21 buffer containing CHAPS (0.1% w/v) and layered onto 5× p21 buffer (1 ml in a 1.5-ml tube) and centrifuged. Recovered beads were washed (4× with 1 ml of p21 buffer with CHAPS). Adsorbed proteins were released with SDS sample buffer and processed by SDS-PAGE (on 8% gels) for protein immunoblotting with antibodies to Raf-CR2 (conserved region 2) (anti–Raf-CR2); immunoblots were developed by enhanced chemiluminescence. Raf band intensities of immunoblots were quantitated on the basis of the local background by a densitometer (Molecular Dynamics, Sunnyvale, CA).
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Inhibition by cAMP of Ras-Dependent Activation of Raf

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Activation of the Raf and extracellular signal–regulated kinases (ERKs) (or mitogen-activated protein kinases) are key events in mitogenic signaling, but little is known about interactions with other signaling pathways. Agents that raise levels of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) blocked DNA synthesis and signal transduction in Rat1 cells exposed to epidermal growth factor (EGF) or lysophosphatidic acid. In the case of EGF, receptor tyrosine kinase activity and association with the signaling molecules Grb2 and Shc were unaffected by cAMP. Likewise, EGF-dependent accumulation of the guanosine 5′-triphosphate–bound form of Ras was unaffected. In contrast, activation of Raf-1 and ERK kinases was inhibited. Thus, cAMP appears to inhibit signal transmission from Ras by preventing Ras-dependent activation of Raf-1.

Cyclic adenosine 3′,5′-monophosphate (cAMP) was the first second messenger to be identified, and its role in regulating physiological processes is well established (*1*). Hormone receptors increase the intracellular concentration of cAMP by increasing the amount of the free α subunit of the guanosine triphosphate (GTP) binding protein G_s ($G\alpha_s$·GTP), which activates adenylyl cyclase (*2, 3*). Despite literature dating back 25 years (*3*), the precise role of cAMP in regulating cell growth and proliferation remains a matter of considerable debate (*3, 4*). In some cells, such as Swiss 3T3 cells and thyrocytes, cAMP is a mitogenic messenger and promotes the G_1 to S phase transition in the cell cycle (*4*). In contrast, cAMP inhibits the proliferation of many cell lines, including fibroblasts, T cells, neuroblastoma and astrocytoma cells, and cells transformed by Ras, Src, and polyoma middle T antigen (*3*).

Kinetic analysis indicates cAMP exerts a cytostatic effect in the early G_0 to G_1 phase as well as mid-G_1 phase (*5*) and, in a few cases, G_2 (*3*). Cyclic AMP inhibits proliferation by growth factors irrespective of their ability to activate the polyphosphoinositide (PI) pathway; therefore, the ability of cAMP to inhibit full activation of the PI pathway is unlikely to explain its effects (*5*). Because cAMP inhibits proliferation stimulated by receptor tyrosine kinases or GTP binding protein–coupled or "serpentine" receptors, it is more likely to exert its effect downstream of receptor activation and second messenger generation, where signal transduction pathways converge.

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A common point of convergence for many, if not all, growth factors is the activation of the Ras proteins (*6*), which regulate signals to the mitogen-activated protein kinases ERK1 and ERK2 (*7*). These are serine-threonine kinases that require phosphorylation at both tyrosine and threonine for activation; both phosphorylation events are catalyzed by the same dual-specificity mitogen-activated protein (MAP) kinase kinase called MAP or ERK kinase (MEK) (*8*). MEK is in turn regulated by at least two upstream kinases: the c-Raf-1 proto-oncogene product (*9*) or MEK kinase (*10*). The stimulation of quiescent cells with various growth factors or phorbol esters or the introduction of oncogenic Ras proteins leads to the activation of ERKs within minutes (*7*). Indeed, Ras is required for growth factors to fully activate the Raf-MEK-ERK pathway (*7, 11*), which suggests that Ras is a common target for growth factors and that this kinase cascade is important for regulating the G_1 to S phase transition. In Rat1 cells, Ras is a point of convergence for two different classes of growth factor, lysophosphatidic acid (LPA) or epidermal growth factor (EGF), to fully stimulate DNA synthesis and ERK activation (*11*). Here, we have examined the effect of cAMP on growth factor stimulation of ERK1 and ERK2.

Quiescent Rat1 cells were incubated with 1 mM dibutyryl cAMP for 10 min before stimulation with LPA or EGF, and ERK activation was assayed on protein immunoblots by the appearance of activated, hyperphosphorylated forms with retarded mobility on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Dibutyryl cAMP alone had no effect on the activation of ERK1 and ERK2 in Rat1 cells, but a 10-min treatment with dibutyryl cAMP completely inhibited the LPA- or EGF-induced mobility shift (Fig. 1A); 8-bromo–cAMP was also effective (*12*). With a more sensitive immune complex kinase assay (*13*), we found that the ability of the phorbol ester PMA (phorbol 12-myristate 13-acetate) to activate MAP kinases, albeit a submaximal activation compared with that of EGF, was also inhibited by dibutyryl cAMP (Fig. 1B). These effects were not due to contamination of dibutyryl cAMP with butyrate or metabolic release of butyrate within the cell because butyrate itself (up to 1 mM) did not inhibit ERK1 (Fig. 1B) or DNA synthesis (*12*). Furthermore, a 10-min exposure to dibutyryl cAMP did not affect the amount of immunoreactive ERK1 and ERK2 (Fig. 1A), so the loss of ERK activity (Fig. 1B) did not result from a decreased amount of the enzyme. The 50% inhibition concentration (IC_{50}) of dibutyryl cAMP upon LPA- or EGF-stimulated ERK1 was approximately 0.1 to 0.2 mM, which agreed with that for inhibition of DNA synthesis (*12*).

The adenosine diphosphate (ADP) ribo-