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A Novel Catecholamine-Activated Adenosine Cyclic 3′,5′-Phosphate Independent Pathway for β-Adrenergic Receptor Phosphorylation in Wild-Type and Mutant S_{49} Lymphoma Cells: Mechanism of Homologous Desensitization of Adenylate Cyclase†

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ABSTRACT: Virtually all known biological actions stimulated by β-adrenergic and other adenylate cyclase coupled receptors are mediated by cAMP-dependent protein kinase. Nonetheless, "homologous" or β-adrenergic agonist-specific desensitization does not require cAMP. Since β-adrenergic receptor phosphorylation may be involved in desensitization, we studied agonist-promoted receptor phosphorylation during homologous desensitization in wild-type S_{49} lymphoma cells (WT) and two mutants defective in the cAMP-dependent pathway of β-agonist-stimulated protein phosphorylation (cyc^- cannot generate cAMP in response to β-adrenergic agonists; kin^- lacks cAMP-dependent kinase). All three cell types demonstrate rapid, β-adrenergic agonist-promoted, stoichiometric phosphorylation of the receptor which is clearly not cAMP mediated. The amino acid residue phosphorylated is solely serine. These data demonstrate, for the first time, that catecholamines can promote phosphorylation of a cellular protein (the β-adrenergic receptor) via a cAMP-independent pathway. Moreover, the ability of cells with mutations in the adenylate cyclase–cAMP-dependent protein kinase pathway to both homologously desensitize and phosphorylate the β-adrenergic receptors provides very strong support for the notion that receptor phosphorylation may indeed be central to the molecular mechanism of desensitization.

Hormones that elevate intracellular levels of cAMP induce their physiological effects through phosphorylation of specific target proteins mediated by cAMP-dependent protein kinase (Krebs & Beavo, 1979). Accordingly, key regulatory cellular proteins have been shown to become phosphorylated upon β-adrenergic agonist stimulation (Rubin & Rosen, 1985; Steinberg & Coffino, 1979; Steinberg & Agard, 1981; Nimmo & Cohen, 1977) and to be regulated in their functionality by such cAMP-mediated phosphorylation (Krebs & Beavo, 1979). It has also been shown that intracellular increases of cAMP achieved either through hormonal stimulation or by exposure of cells to membrane-permeable analogues of cAMP lead to identical protein phosphorylation (Steinberg & Coffino, 1979). All of these phosphorylation processes are catalyzed by cAMP-dependent protein kinases. In cells which are characterized by a transdominant regulatory mutation in the expression of the catalytic subunit of the cAMP-dependent protein kinase (i.e., the kin^- mutant of S_{49} lymphoma cells) (Steinberg et al., 1978) and which are therefore devoid of

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cAMP-dependent protein kinase activity, β-adrenergic agonist stimulation or cAMP analogues fail to induce protein phosphorylation (Steinberg & Coffino, 1979). These and other data have strongly suggested that all protein phosphorylation stimulated via the β-adrenergic receptor is mediated via the cAMP-dependent protein kinase.

Prolonged exposure of cells to β-adrenergic agonists leads to desensitization, the process whereby agonist stimulation of receptor-coupled effectors such as adenylate cyclase becomes attenuated with time (Harden, 1983; Lefkowitz et al., 1983). One form of desensitization termed *heterologous* is largely cAMP mediated and involves a general blunting of responsiveness not only to stimulation by β-adrenergic agonists but also to other hormones as well (Stadel et al., 1981). Current information indicates that at least one mechanism of heterologous desensitization involves phosphorylation and uncoupling of the β-adrenergic receptor which are mediated at least in part by the cAMP-dependent protein kinase (Stadel et al., 1983; Sibley et al., 1984). In contrast, *homologous* desensitization, which is characterized by a loss of responsiveness to β-adrenergic agonists only, is not cAMP mediated (Perkins, 1983). Thus, mutants of the S_{49} lymphoma cells which lack the guanine nucleotide regulatory protein which couples the β-adrenergic receptor to adenylate cyclase (cyc^-) (Bourne et al., 1975, 1981; Ross & Gilman, 1977) and which hence cannot raise cAMP levels in response to agonists such as isoproterenol nonetheless rapidly desensitize upon exposure to β-agonists (Green & Clark, 1981; Green et al., 1981; Clark et al., 1985). The same is true of the kin^- mutant which lacks the cAMP-dependent protein kinase (Shear et al., 1976; Steinberg et al., 1978; Mahan et al., 1985). Moreover, a recent report has indicated that phosphorylation of the β-adrenergic receptor is associated with homologous desensitization in frog erythrocytes (Sibley et al., 1985).

The hypothesis that β-adrenergic receptor stimulation might be coupled to a hitherto unappreciated non-cAMP-dependent pathway which might phosphorylate the receptors and lead to homologous desensitization forms the basis for the present studies. In this work, we utilize mutants of the S_{49} lymphoma cells (cyc^-, kin^-) deficient in the various components of the cAMP-dependent pathway of protein phosphorylation to document the existence of just such a novel β-adrenergic receptor-coupled cAMP-independent protein kinase pathway.

Experimental Procedures

Cells and Incubations. S_{49} lymphoma cells, wild type (clone 24.3.2) and cyc^- and kin^- (clone 24.6.1), have been described previously [Bourne et al., 1975; for a review, see Johnson et al. (1980)]. The phenotype of the cyc^- cells was confirmed by adenylate cyclase assays as discussed below (see Figure 1). The phenotype of the kin^- cells to exclude revertants (Van Daalen Wetters et al., 1983) was verified by cAMP-dependent protein kinase assays as described below. The cells were grown in Dulbecco's modified Eagle's medium with 10% horse serum supplemented with 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES).[1] Cells were harvested by centrifugation (800*g*, 3 min) and washed 3 times with phosphate-free Dulbecco's modified Eagle's medium. The cells were incubated at a density of (1–2) × 10^8 cells/mL at 37 °C for 5 min. The ATP pool of the cells was ^{32}P labeled by adding carrier-free [^{32}P]P_i (0.3 mCi/mL) to the incubation (10 min, 37 °C). Desensitization was started by adding the β-agonist (−)-isoproterenol to a final concentration of 10^{-5} M or other drugs as indicated. Unless otherwise stated, the desensitization was performed for 20 min at 37 °C. Aliquots for determination of the specific activity of the ATP pool were taken at the end of the incubation (see below). The incubation was stopped by adding ice-cold phosphate-buffered saline with propranolol (10^{-6} M) and sedimentation of the cells (800*g*, 5 min).

Plasma Membrane Preparation and Purification of the β-Adrenergic Receptor. The cells were washed in ice-cold phosphate-buffered saline 3 times by centrifugation. The cells were lysed in 30 volumes of HEPES buffer (5 mM HEPES, 15 mM $MgSO_4$, 5 mM NaH_2PO_4, and 2 mM EDTA, pH 7.3) by 20 strokes of a Dounce homogenizer on ice. The unbroken cells and nuclei were sedimented (800*g*, 5 min). The supernatant was then recentrifuged at 48000*g* for 20 min. The resulting membrane pellet was washed 3 times in HEPES buffer. Aliquots of the plasma membrane preparation were taken for adenylate cyclase assays and β-adrenergic receptor binding (see below). The final plasma membranes were resuspended in 20 volumes of 100 mM NaCl, 10 mM Tris, 5 mM NaH_2PO_4, 5 mM EDTA, and 0.05% digitonin, pH 7.4, and resedimented at 48000*g* for 20 min. The membranes were then solubilized in 20 mL of 100 mM NaCl, 10 mM Tris, 5 mM NaH_2PO_4, 5 mM EDTA, and 1.25% digitonin with 20 strokes of a Dounce homogenizer on ice and stirring for 60 min at 4 °C. After centrifugation (48000*g* for 30 min), the supernatant was tested for β-adrenergic receptor binding. Affinity chromatography of soluble β-adrenergic receptor preparations was performed by using a Sepharose–alprenolol gel prepared as previously described (Caron et al., 1979). The soluble preparations (3–6 pmol per incubation condition) were applied to the gel at 25 °C. Since sequestered as well as nonsequestered receptors migrate with the membrane preparation, applied control and desensitized cells yielded equal amounts of β-adrenergic receptors. The columns were extensively washed at 4 °C [2.5 bed volumes of 100 mM NaCl, 10 mM Tris, 5 mM NaH_2PO_4, 2 mM EDTA, and 0.05% digitonin, pH 7.4 (buffer I), then 3 bed volumes of buffer I with 0.05% digitonin, then 3 bed volumes of buffer I with 500 mM NaCl, and finally 10 bed volumes of buffer I]. The β-adrenergic receptor was eluted with a gradient from 0 to 10^{-5} M alprenolol at 25 °C. Ten-milliliter fractions were collected and tested for β-adrenergic receptor binding. The two fractions containing the highest receptor concentration were pooled and concentrated down to 2 mL in Amicon stirring cells, desalted on Sephadex G_{50} columns, and tested again for β-adrenergic receptor binding before lyophilization. The recoveries of the receptors ranged between 45 and 60% and were equal for control and desensitized receptors. The lyophilized samples were resuspended in SDS–PAGE buffer. Equal amounts of β-adrenergic receptors were separated on 8% SDS–PAGE gels according to Laemmli (1970) and autoradiographed for 8–48 h. The receptor bands on the gel were cut out and after rehydration counted in a Packard Tri-Carb 4640 scintillation counter with 100% counting efficiency.

Amino Acid Determination. After autoradiography, the ^{32}P-labeled receptor band was excised from the dried gels and extracted in 2 × 2 mL of 50 mM $NaHCO_2$ for 12 h at 30 °C. After lyophilization, the sample was taken up in 200 μL of 6 N HCl and hydrolyzed under vacuum for 2 h at 110 °C.

[1] Abbreviations: [^{125}I]CYP, [^{125}I]iodocyanopindolol; [^3H]CGP 12177, [^3H]-4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one; [^{125}I]pABC, [^{125}I]iodo-*p*-azidobenzylcarazolol; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; N_s, guanine nucleotide binding stimulatory protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane; HPLC, high-pressure liquid chromatography.

After lyophilization, the sample was analyzed on a strong anion-exchange HPLC column (Partisil 10 SAX) utilizing unlabeled phosphohydroxy amino acids as internal standards (Swarup et al., 1981).

β-Adrenergic Binding: [^{125}I]CYP Binding. β-Adrenergic receptor number in plasma membranes and soluble fractions was determined by saturating levels (300 pM) of [^{125}I]iodocyanopindolol ([^{125}I]CYP) binding as previously described (Shorr et al., 1982). Nonspecific binding was determined in the presence of 10^{-5} M alprenolol. Bound radioligand was separated from free radioligand in membranes by the filter assay, in soluble preparations by Sephadex G_{50} columns (Caron et al., 1979).

[^3H]CGP 12177 Binding. To quantitate β-adrenergic receptors on the cell surface, [^{3}H]CGP 12177 binding on whole cells was performed (Staehelin & Simon, 1982). In parallel incubations (without the addition of [^{32}P]P_i), cells were desensitized and washed as described previously. The cells were then resuspended to a concentration of 5×10^7 cells/mL in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. Binding was performed in 8-point saturation curves with 0.05–4 nM [^{3}H]CGP 12177 in the absence or presence of 10^{-5} M alprenolol at 4 °C for 16 h. The incubation was stopped by rapid filtration on GF/A Whatman filters and washing with 12 mL of phosphate-buffered saline. The cell-bound radioactivity was measured in a Packard liquid scintillation counter with 50% counting efficiency. Saturation curves were analyzed by computer assisted techniques utilizing nonlinear least-squares curve-fitting techniques based on the law of mass action (De Lean et al., 1980).

Adenylate Cyclase Assay. Adenylate cyclase assays were performed as previously described (Strasser & Lefkowitz, 1985). Final concentrations in the incubations were 5 mM HEPES, 15 mM $MgSO_4$, 5 mM NaH_2PO_4, and 2 mM EDTA (pH 7.4).

ATP Determination. In order to determine the phosphorylation state of the β-adrenergic receptor, we labeled the ATP pool of intact S_{49} lymphoma cells by incubating them with 0.3 mCi/mL carrier-free [^{32}P]P_i. The specific [^{32}P]ATP activity increased rapidly during the first 10 min and remained stable for at least the next 20 min (data not shown). All subsequent incubations were done during this steady-state period of the specific [^{32}P]ATP activity. The specific [^{32}P]ATP activity was not altered by the addition of drugs (β-agonists and β-antagonists) for various periods of time. On the basis of data from human erythrocytes and rat liver, the specific activity of [γ-^{32}P]ATP was assumed to be half of the total specific activity of [^{32}P]ATP (Niehaus & Hammerstedt, 1976; Mayer & Krebs, 1970). The phosphorylation of the β-adrenergic receptor was quantitated by slicing the receptor-containing section of the gel (see above). The specific counts were corrected for the amount of receptor loaded on the gel and then divided by the specific activity of [γ-^{32}P]ATP to give the stoichiometry of the phosphorylation process in moles of phosphate per mole of receptor.

Determination of the cAMP-Dependent Protein Kinase. In order to verify that the kin$^-$ mutant cell line is in fact devoid of the cAMP-dependent protein kinase, we compared the activity of the cAMP-dependent protein kinase in wild-type, cyc$^-$, and kin$^-$ cell lysates under basal conditions and after stimulation with 10^{-4} M cAMP. The cAMP-dependent protein kinase was determined in the cell lysate by the method of Roskoski (1983) using the synthetic Ser-peptide (Leu-Arg-Arg-Ala-Ser-Leu-Glu) as substrate. The addition of cAMP induces a 4–6-fold stimulation of the cAMP-dependent protein kinase in the wild-type and cyc$^-$ cells (data not shown). In the kin$^-$ mutant cell line, however, cAMP does not stimulate the phosphorylation of the Ser-peptide (data not shown). These findings are consistent with the notion that the kin$^-$ mutant is lacking the cAMP-dependent protein kinase which is in accordance with previously published data (Steinberg & Coffino, 1979; Van Daalen Wetters et al., 1983).

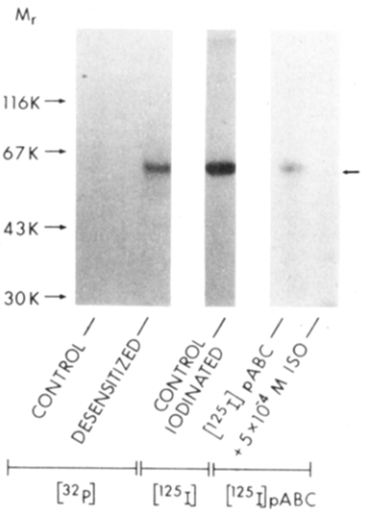

FIGURE 1: Agonist-promoted phosphorylation of the β-adrenergic receptor of WT S_{49} lymphoma cells. Wild-type S_{49} lymphoma cells were prelabeled with 0.3 mCi/mL carrier-free [^{32}P]P_i and then incubated for 20 min in the absence (controls) or presence of 10^{-5} M (−)-isoproterenol (desensitized). Shown is the autoradiography of the affinity-purified receptors after SDS gel electrophoresis (lanes 1 and 2). The partially purified preparation from controls (lane 1) was radioiodinated and separated on SDS gel electrophoresis (lane 3). For comparison, in lanes 4 and 5 the specifically [^{125}I]pABC photoaffinity-labeled β-adrenergic receptors from plasma membranes of S_{49} lymphoma cells are shown (see Experimental Procedures). Indicated on the left side are the relative mobilities (molecular weights) of standard proteins. The arrow indicates the receptor peptides.

Photoaffinity Labeling of the β-Adrenergic Receptors with [^{125}I]pABC. For the photoaffinity labeling of the β-adrenergic receptors with [^{125}I]iodo-*p*-azidobenzylcarbazolol ([^{125}I]pABC) (Lavin et al., 1982), plasma membranes were utilized. The receptor, at a concentration of 50 pM, was incubated with [^{125}I]pABC at a concentration of 50–60 pM in 50 mM Tris and 5 mM EDTA (pH 7.2) for 90 min at 25 °C, in the presence of protease inhibitors (10^{-4} M PMSF, 100 μg/mL soybean trypsin inhibitor, 10^{-5} M pepstatin A, 2.5 μg/mL leupeptin, and 5 μg/mL benzamidine). The incubations were carried out in the absence or presence of 10^{-5} M alprenolol. Plasma membranes were washed 3 times in the same buffer with the inclusion of 0.1% bovine serum albumin. The samples were photolyzed for 90 s utilizing UV light (wavelength ~350 nm). The samples were then solubilized in SDS–PAGE buffer and separated on SDS–PAGE gel electrophoresis according to Laemmli (1970).

Radioiodination of the β-Adrenergic Receptor. Radioiodination of the affinity-purified preparations derived from control cells after the phosphorylation experiments was performed as previously described (Caron et al., 1979).

Results

Wild-type (WT) S_{49} cells, prelabeled with [^{32}P]P_i (see Experimental Procedures), were briefly exposed to the β-agonist isoproterenol (20 min). Following the treatment, the β-adrenergic receptors were solubilized with digitonin, purified by affinity chromatography, and electrophoresed on SDS gels. As shown in lanes 1 and 2 of Figure 1, the β-agonist treatment led to a dramatic increase in the phosphorylation of the re-

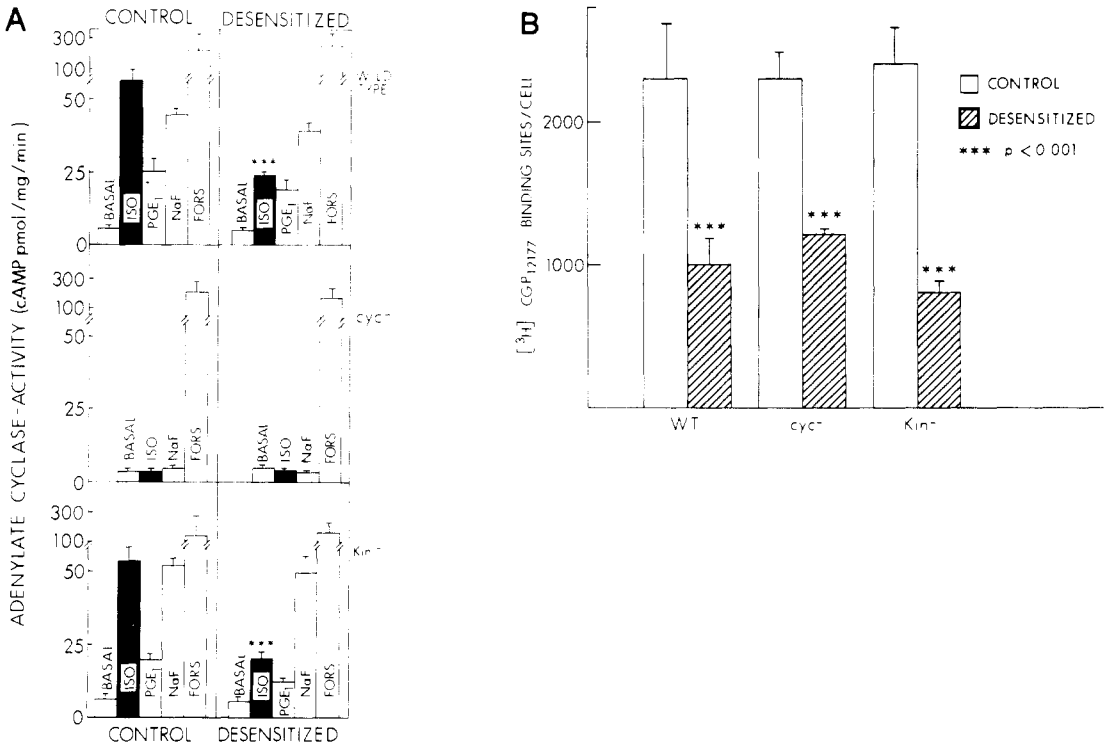

FIGURE 2: (A) Adenylate cyclase activity in plasma membranes from control and isoproterenol-desensitized S_{49} lymphoma cells. The adenylate cyclase activity was measured in plasma membranes of the three cell lines of the S_{49} lymphoma cells (WT, wild type; cyc$^-$, lacking functional N_s; kin$^-$, lacking cAMP-dependent protein kinase). After preincubation with [^{32}P]P_i and incubation in the absence (control) or presence of 10^{-5} M isoproterenol (desensitized) for 20 min at 37 °C (see Experimental Procedures), the adenylate cyclase activity was measured in the absence of any effectors (BASAL) or in the presence of 10^{-4} M (−)-isoproterenol (ISO), 3×10^{-4} M prostaglandin E_1 (PGE_1), 10 mM sodium fluoride (NaF), or 10^{-4} M forskolin (FORS). The data shown are the means ± SE of four sets of experiments with triplicate determinations. Statistical analysis was done by an analysis of variance. Indicated are the significant (***$p < 0.01$) changes after desensitization. (B) [^{3}H]CGP 12177 binding to control and isoproterenol-desensitized S_{49} lymphoma cells. Shown are the number of specific binding sites on the cell surface before and after desensitization. Cells of the three S_{49} lymphoma cell lines [WT (wild type), cyc$^-$, and kin$^-$] were incubated for 20 min at 37 °C in the absence (control) or presence of 10^{-5} M isoproterenol (desensitized). After three washes in cold PBS, the cells were incubated in saturation experiments at a density of 2×10^7 cells/mL in DMEM with 10% horse serum for 16 h at 4 °C with increasing concentrations of [^{3}H]CGP 12177 (0.05–4 nM). The nonspecific binding was determined in the presence of 10^{-5} M alprenolol. The total number of receptor binding sites was determined by computer-assisted analysis of the saturation binding isotherms as described elsewhere (De Lean et al., 1980). The affinity of the ligand did not change significantly throughout the experiments and ranged between 0.7 and 0.9 nM (not shown). Shown are the means ± SE of three sets of experiments with duplicate determinations at each point of the saturation isotherm (see Experimental Procedures). Indicated are the significant changes after desensitization (***$p < 0.001$).

ceptor with the stoichiometry increasing from 0.2 to 0.8 mol of PO_4/mol of β-adrenergic receptor. Phosphorylation of the receptor occurs solely on serine residues (data not shown).

To characterize the purified preparation (~1000-fold), the affinity-purified preparations from control cells (after the phosphorylation experiment, lane 1) were then radioiodinated and analyzed on SDS gel electrophoresis. The predominant protein in the affinity-purified preparation represents the receptor protein with a relative molecular weight of 64 000 (lane 3, Figure 1). The specifically photoaffinity-labeled β-adrenergic receptor peptides from plasma membranes of wild-type S_{49} lymphoma cells are shown for comparison (lanes 4 and 5, Figure 1). The photoaffinity-labeled β-adrenergic receptor migrates again as a single band at a relative molecular weight of about 64 000 with one minor proteolytic breakdown product at about M_r 55 000. After desensitization, the β-adrenergic receptor is unaltered in electrophoretic mobility. This is in agreement with the pattern seen previously (Rashidbaigi et al., 1983). It can be seen that the relative molecular weight of the specifically photoaffinity-labeled receptor peptide is identical with those of the phosphoproteins isolated by affinity chromatography, thus confirming the receptor nature of these isolated peptides.

The exposure of these cells to isoproterenol leads to a typical homologous desensitization of the adenylate cyclase (Figure 2A) (top panel) with a decreased response of the cyclase to the desensitizing hormone but not to other hormonal or nonhormonal stimulators. The typical homologous desensitization is also characterized by the sequestration of about 50% of the receptors away from the cell surface (Figure 2B). This is documented by whole cell binding with a hydrophilic β-adrenergic ligand, CGP 12177, which does not cross the plasma membrane and therefore only binds to the receptors at the cell surface.

Both mutant cell lines utilized in this study are also desensitized upon agonist exposure. First, as previously shown (Hertel et al., 1983; Clark et al., 1985; Mahan et al., 1985) and documented in Figure 2B, cyc$^-$ as well as kin$^-$ cells sequester about 50% of their receptors away from the plasma membranes. Moreover, incubation of kin$^-$ cells with the agonist (−)-isoproterenol results in a 40–50% decrease of the (−)-isoproterenol-stimulated adenylate cyclase activity. As with the wild-type cells, other parameters of enzyme activity such as that stimulated by prostaglandin E_1, sodium fluoride, and forskolin were not or were only minimally decreased (5–10%). cyc$^-$ cells, as documented in Figure 2A, middle panel, typically lack isoproterenol or sodium fluoride stimulated adenylate cyclase activities but retain forskolin-stimulated activity due to the missing guanine nucleotide binding protein (N_s). Although this pattern does not change with agonist preincubation, homologous desensitization in cyc$^-$ membranes can be documented by reconstituting cyc$^-$ membranes with

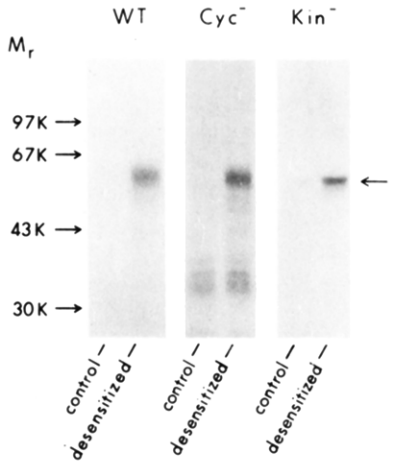

FIGURE 3: Agonist-promoted phosphorylation of the β-adrenergic receptor in S_{49} mutant cells. The three cell clones of S_{49} lymphoma cells (WT, cyc⁻, and kin⁻) were prelabeled with carrier-free [^{32}P]P_i (0.3 mCi/mL) and then incubated for an additional 20 min in the absence (controls) or presence of 10^{-5} M (−)-isoproterenol (desensitized). Shown is the autoradiograph of the affinity-purified β-adrenergic receptors after separation by SDS gel electrophoresis. Indicated on the left are the relative mobilities (molecular weights) of standard proteins. The experiments were repeated 3–4 times with similar results. The arrow indicates the receptor peptides.

normal N_s protein (Green & Clark, 1981). These data documenting the desensitization pattern in these cells are in excellent agreement with previously published results (Shear et al., 1976).

Although both mutant cell lines are defective in their β-agonist-stimulated cAMP-dependent pathway of protein phosphorylation, they can nonetheless desensitize in response to β-agonist stimulation. This might indicate that in these cells β-adrenergic receptor desensitization is not associated with phosphorylation or that receptor phosphorylation is occurring via a kinase pathway which bypasses the second messenger cAMP. To address this question, we incubated [^{32}P]P_i-prelabeled WT, cyc⁻, and kin⁻ cells in parallel with or without isoproterenol. In all three cells lines (Figure 3), wild type, cyc⁻, and kin⁻, the desensitization with the β-agonist isoproterenol increases the phosphorylation of the β-adrenergic receptor from about 0.2 pmol of phosphate/mol of receptor to about 0.8 mol of phosphate/mol of receptor. In each case, the amount of phosphate incorporated into the receptor is comparable (WT, 0.8 mol of phosphate/mol of β-adrenergic receptor, $n = 4$; cyc−, 0.85 mol/mol, $n = 2$; kin⁻, 0.80 mol/mol, $n = 8$). The receptors become phosphorylated solely on serine residues (data not shown).

These data document that isoproterenol-induced phosphorylation of the β-adrenergic receptor is not dependent on cAMP production (cyc⁻ mutant) and is not catalyzed by the cAMP-dependent protein kinase (kin⁻ mutant). They also show that uncoupling of the β-adrenergic receptor from the N_s–protein alone (cyc⁻) is not sufficient to increase the phosphorylation of the β-adrenergic receptor.

Such β-agonist-induced phosphorylation is pharmacologically specific as documented in Figure 4 and is closely associated with the desensitization of the β-adrenergic-coupled adenylate cyclase system. Equal doses of (−)-isoproterenol are more effective than (+)-isoproterenol in inducing phosphorylation as well as desensitization in kin⁻ cells. These effects can be blocked by the β-antagonist propranolol (Figure 4A,B).

Thus far then, these data document that a β-agonist is able to induce phosphorylation of a cellular protein, specifically its own cellular receptor in a pharmacologically specific manner

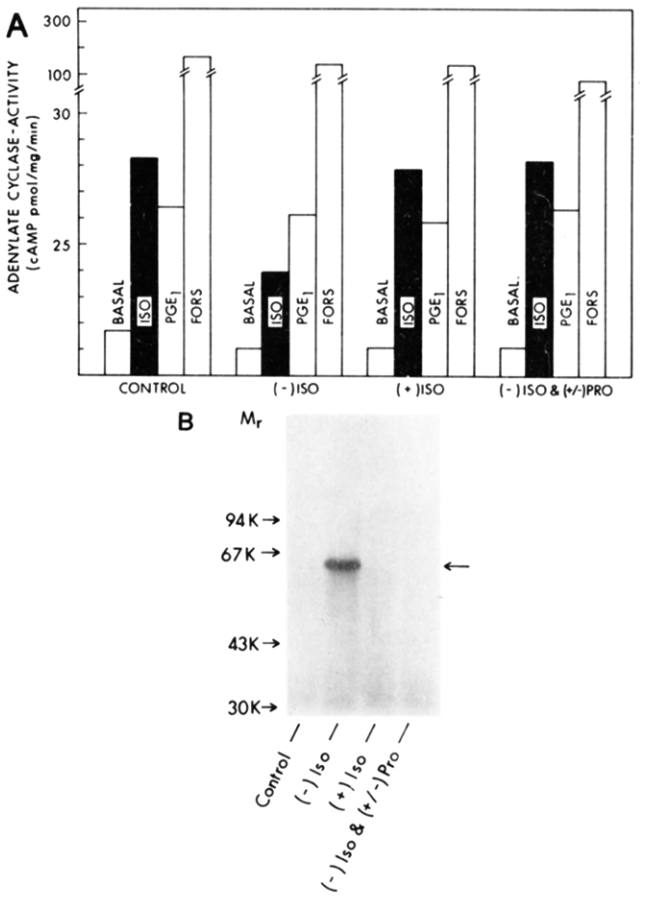

FIGURE 4: Specificity of β-agonist-promoted phosphorylation and desensitization of adenylate cyclase coupled β-adrenergic receptors. kin⁻ cells prelabeled with 0.3 mCi/mL carrier-free [^{32}P]P_i were incubated (37 °C, 20 min) with buffer alone (control), with 10^{-6} M (−)-isoproterenol, with 10^{-6} M (+)-isoproterenol, or with 10^{-6} M (−)-isoproterenol in the presence of 10^{-6} M (±)-propranolol. Panel A shows the adenylate cyclase activities in the plasma membranes (compare Figure 2A); panel B shows the autoradiograph of the affinity-purified β-adrenergic receptors. The data shown are representative of two experiments.

via a cAMP-independent pathway. Homologous desensitization is associated with this receptor phosphorylation.

In order to further investigate the relationship of receptor phosphorylation and adenylate cyclase desensitization, we determined the time course of both processes in the kin⁻ cells. As shown in Figure 5, the isoproterenol-stimulated adenylate cyclase activity decreases rapidly after cells are exposed to isoproterenol and is maximally desensitized by 5 min. The phosphorylation of the β-adrenergic receptor increases even more rapidly. At 1–2 min, phosphorylation of the β-adrenergic receptor is already maximal, reaching ~1 mol of phosphate/mol of β-adrenergic receptor. Upon longer desensitization (20 min), the phosphorylation of the β-adrenergic receptor gradually decreases, while desensitization of the adenylate cyclase remains constant. Wild-type S_{49} lymphoma cells show an identical pattern (data not shown).

Discussion

These results document for the first time that catecholamines can promote phosphorylation of a cellular protein, i.e., their own receptors, via a cAMP-independent pathway. Agonist activation of the β-adrenergic receptors in S_{49} lymphoma cels, or in mutants which are defective in different components of the adenylate cyclase–cAMP-dependent protein kinase pathway, leads to phosphorylation and desensitization of the receptors. All previous known phosphorylation events

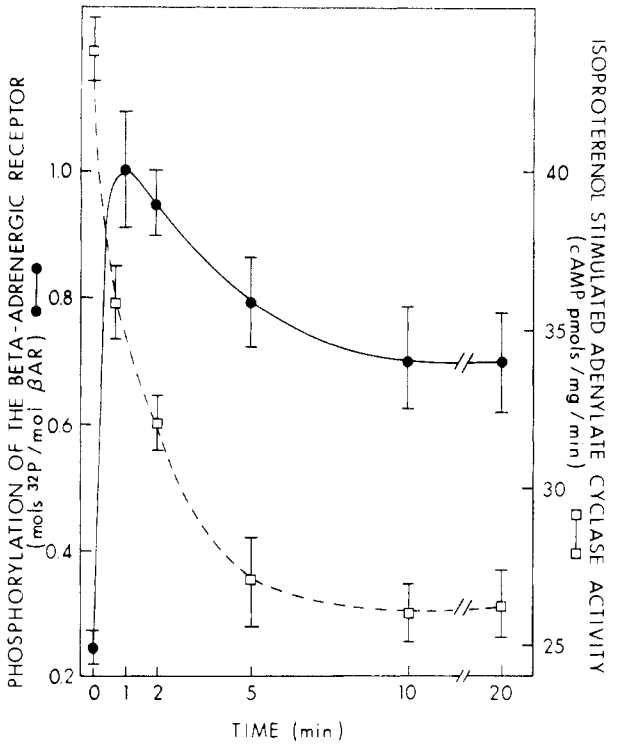

FIGURE 5: Time course of β-agonist-promoted desensitization of isoproterenol-stimulated adenylate cyclase activity and phosphorylation of the β-adrenergic receptor. The kin⁻ mutant cells were prelabeled with 0.3 mCi/mL carrier-free [^{32}P]P_i and then the β-agonist (−)-isoproterenol (10^{-5} M) was added for various periods of time (1–20 min). Shown is the stoichiometry of the phosphorylated β-adrenergic receptor (●) and the isoproterenol-stimulated adenylate cyclase activity (□). The data represent the mean of three experiments (±SE).

promoted by β-adrenergic receptors have been found to be cAMP mediated (Steinberg & Coffino, 1979).

Since isoproterenol stereospecifically stimulates and propranolol blocks the stimulation of β-adrenergic receptor phosphorylation during homologous desensitization, it can be concluded that the phosphorylation is itself a β-adrenergic receptor mediated event. This must mean either that the β-agonist occupying the receptor is stimulating a kinase via a messenger other than cAMP or that agonist occupancy of the receptor simply makes the receptor a much better substrate for the kinase. We cannot as yet distinguish between these two possibilities. We also have evidence which suggests that the purified β-adrenergic receptor does not autophosphorylate (Benovic et al., 1985).

The nature of the protein kinase(s) involved in β-adrenergic receptor phosphorylation in homologous desensitization remains unknown. It might be one of the known non-cAMP-dependent protein kinases or represent a hitherto undiscovered specific "receptor kinase". A potential analogy for this latter suggestion is "rhodopsin kinase" which functions to specifically phosphorylate and "desensitize" the "light receptor" of the rod outer segment, rhodopsin (Shichi & Somers, 1978). Whatever the nature of the non-cAMP-dependent kinase which leads to β-adrenergic phosphorylation and homologous desensitization, it may well play a role in the desensitization of other adenylate cyclase coupled receptors. Presumably, occupancy of these various receptors (e.g., prostaglandin receptors) by the appropriate agonist ligand would transform the receptor, by conformational changes, into an appropriate substrate for this kinase.

The mechanism(s) by which β-adrenergic receptor phosphorylation might lead to homologous desensitization can only be conjectured. There are at least two obvious possibilities which are not mutually exclusive. One is that the phosphorylated receptor is less able to interact with the guanine nucleotide regulatory protein; i.e., it is uncoupled. This would be analogous to recent findings bearing on the mechanism of heterologous desensitization where receptor phosphorylation by cAMP-dependent (Strulovici et al., 1984; Benovic et al., 1985) and possibly other protein kinases has been shown to uncouple the receptors from N_s as assessed in reconstituted systems. However, to date there have been conflicting results as to the functionality of homologously desensitized β-adrenergic receptors as assessed by reconstitution approaches (Kassis & Fishman, 1984; Kassis et al., 1985; Clark et al., 1985; Strulovici et al., 1983; Strasser et al., 1985).

A second possibility is that in homologous desensitization the key role of the receptor phosphorylation is to trigger the sequestration of the receptors away from the effector N_sC complex. Elsewhere (Strasser et al., 1985; Strasser & Lefkowitz, 1985), we have presented evidence that such sequestration of functionally intact receptors in fact contributes to this form of desensitization at late time points. Recent data with the transferrin receptor and epidermal growth factor (EGF) receptors have suggested that receptor phosphorylation might in some way trigger receptor internalization (Klausner et al., 1984; Shoyab et al., 1979). It is also, of course, possible that receptor phosphorylation initially uncouples the receptors and then leads to their sequestration. The observation that the phosphorylation of the β-adrenergic receptor is maximal even before maximal desensitization is reached is suggestive of such a sequential model for homologous desensitization of the β-adrenergic receptor. Also, recently published kinetic data support such a model (Waldo et al., 1983; Toews et al., 1984). Within the sequestered compartment, the β-adrenergic receptors might become dephosphorylated, thus resulting in the observed gradual decrease of phosphorylation of the total pool of receptors at later time points. This notion is in agreement with the recent finding that sequestered receptors from frog erythrocytes and S_{49} cells are functionally active (Clark et al., 1985; Strulovici et al., 1983; Strasser & Lefkowitz, 1985).

In summary, we have utilized mutants of the S_{49} murine lymphoma cells to document for the first time that β-agonists can promote the phosphorylation of a cellular protein via a cAMP-independent pathway. This novel pathway leads to rapid phosphorylation of the β-adrenergic receptors and to homologous desensitization of the β-adrenergic coupled adenylate cyclase system.

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Registry No. cAMP, 60-92-4; serine, 56-45-1; adenylate cyclase, 9012-42-4; isoproterenol, 7683-59-2.

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