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β-Adrenergic receptor kinase: Identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor

(S49 lymphoma cells/kin⁻ mutant/purification/desensitization/adenylate cyclase)

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ABSTRACT **Agonist-promoted desensitization of adenylate cyclase is intimately associated with phosphorylation of the β-adrenergic receptor in mammalian, avian, and amphibian cells. However, the nature of the protein kinase(s) involved in receptor phosphorylation remains largely unknown. We report here the identification and partial purification of a protein kinase capable of phosphorylating the agonist-occupied form of the purified β-adrenergic receptor. The enzyme is prepared from a supernatant fraction from high-speed centrifugation of lysed kin$^-$ cells, a mutant of S49 lymphoma cells that lacks a functional cAMP-dependent protein kinase. The β-agonist isoproterenol induces a 5- to 10-fold increase in receptor phosphorylation by this kinase, which is blocked by the antagonist alprenolol. Fractionation of the kin$^-$ supernatant on molecular-sieve HPLC and DEAE-Sephacel results in a 50- to 100-fold purified β-adrenergic receptor kinase preparation that is largely devoid of other protein kinase activities. The kinase activity is insensitive to cAMP, cGMP, cAMP-dependent kinase inhibitor, Ca^{2+}-calmodulin, Ca^{2+}-phospholipid, and phorbol esters and does not phosphorylate general kinase substrates such as casein and histones. Phosphate appears to be incorporated solely into serine residues. The existence of this novel cAMP-independent kinase, which preferentially phosphorylates the agonist-occupied form of the β-adrenergic receptor, suggests a mechanism that may explain the homologous or agonist-specific form of adenylate cyclase desensitization. It also suggests a general mechanism for regulation of receptor function in which only the agonist-occupied or "active" form of the receptor is a substrate for enzymes inducing covalent modification.**

Desensitization is a general phenomenon in biology in which prolonged exposure of a cell to a hormone or drug results in a reduced responsiveness to further challenge by that agent. Two major types of desensitization have been described (1, 2). Homologous, or agonist-specific, desensitization results in an attenuated response only to the desensitizing agent. Heterologous desensitization, on the other hand, results in a more general decrease in responsiveness to a wider variety of hormonal agents as well as to nonhormonal stimulators.

One of the most thoroughly studied model systems for desensitization is the β-adrenergic receptor-coupled adenylate cyclase (3). This receptor mediates catecholamine stimulation of adenylate cyclase via interaction with the stimulatory guanine nucleotide regulatory protein (4). Both heterologous and homologous forms of desensitization have been characterized. While clear distinctions between the two forms exist, one central feature that is common to both is that the β-adrenergic receptor is stoichiometrically phosphorylated during desensitization (5–7). Studies of heterologous desensitization carried out largely with avian erythrocytes have demonstrated that the cAMP-dependent protein kinase is involved in this process (8–10). Moreover, it has been shown *in vitro* that the isolated β-adrenergic receptor can be stoichiometrically phosphorylated by the pure catalytic subunit of the cAMP-dependent protein kinase. Furthermore, such phosphorylated receptors are functionally impaired in their coupling to the effector components of the adenylate cyclase system as assessed in reconstituted systems (11).

In contrast, homologous desensitization of the β-adrenergic receptor is clearly not cAMP-mediated. β-Adrenergic receptor phosphorylation has been demonstrated to accompany homologous desensitization not only in frog erythrocytes (6) and wild-type S49 cells (7) but also in mutants [cyc$^-$ (coupling-protein deficient) and kin$^-$ (cAMP-dependent protein kinase deficient)] of the S49 cells that cannot mount an agonist-promoted cAMP-dependent protein kinase response (7). This demonstrates that cAMP production and the cAMP-dependent protein kinase are not required for receptor phosphorylation and homologous desensitization in these cells. Thus, the relevant kinase(s) must either be stimulated by a second messenger other than cAMP or preferentially phosphorylate the agonist-occupied form of the receptor. In the present work we have identified a novel protein kinase in lysates of kin$^-$ cells that phosphorylates the β-adrenergic receptor in an almost totally agonist-dependent fashion.

MATERIALS AND METHODS

Materials. Most chemicals were from sources previously described (11, 12). Histone (types II-AS, V-S, and VII-S), casein, protein kinase inhibitor (type II), phorbol 12-myristate 13-acetate (PMA), and calmodulin were from Sigma. Dulbecco's modified Eagle's medium, phosphate-buffered saline, and horse serum were from GIBCO.

Purification and Reconstitution of Receptor. The β-adrenergic receptor from hamster lung was purified to >95% homogeneity by sequential affinity and HPLC as described (12). The purified receptor (typically 30–60 pmol) was reconstituted by incubating with bovine serum albumin (1 mg), sonicated soybean phosphatidylcholine (2.5 mg), and octyl glucoside (5 mg) before chromatography on 1-ml Extracti-gel columns as described (11). The eluates (2 ml) were incubated with 0.6 ml 50% polyethylene glycol for 10 min at 22°C, diluted with ice-cold 100 mM NaCl/10 mM Tris·HCl, and centrifuged at 300,000 × g for 1.5 hr at 4°C. The protein–lipid pellets were resuspended in 50–100 μl of 100 mM NaCl/10 mM Tris·HCl, pH 7.2. The efficiency of receptor insertion

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; N_s, stimulatory guanine nucleotide regulatory protein; kin$^-$, cAMP-dependent protein kinase deficient.

into the phosphatidylcholine vesicles, as determined by [^{3}H]dihydroalprenolol binding, was typically 20–30%.

Cell Fractionation. The kin$^-$ variant of S49 lymphoma cells were grown in Dulbecco's modified Eagle's medium with 10% horse serum supplemented with 10 mM Hepes. Cells were harvested by centrifugation (800 × *g* for 3 min) and washed three times with phosphate-buffered saline. The packed cells were lysed by resuspension in 20 mM Na phosphate, pH 7.2/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (1 ml of buffer per 2 ml of packed cells) followed by Polytron disruption. The lysed cells were centrifuged at 350,000 × *g* for 1 hr at 4°C. Typically, ≈1 ml of supernatant was obtained per ml of initial packed cells.

Phosphorylation of Reconstituted Receptor. Reconstituted β-adrenergic receptor (2–6 pmol) was phosphorylated with the various kinase preparations (1–400 μg of protein) in a total volume of 100–140 μl. The incubations contained 25 mM Tris·HCl, pH 7.4, 20 mM NaCl, 1.5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM NaF, 0.05 mM Na_3VO_4, 0.05 mM [γ-^{32}P]ATP (3–15 cpm/fmol), and 0.2–0.6 mg of soybean phosphatidylcholine. When HPLC or DEAE fractions were assayed, 2 μM (−)-isoproterenol was also included. Except where indicated, the incubations were for 30 min at 30°C. Reactions were stopped by the addition of 20 ml of ice-cold buffer A (10 mM Tris·HCl, pH 7.2/100 mM NaCl/10 mM NaF/0.1 mM Na_3VO_4). Samples were then centrifuged at 350,000 × *g* for 1 hr, and the resultant pellets were solubilized for 30 min at 4°C with 1 ml of buffer A containing 1% digitonin. After a 5-min centrifugation in Eppendorf tubes, the solubilized receptor was diluted 1:1 with buffer A and incubated with 0.75 ml of Sepharose-alprenolol for 1 hr at 22°C. The affinity resin was then washed once with 10 ml of 50 mM Tris·HCl, pH 7.2/500 mM NaCl/10 mM NaF/0.1 mM Na_3VO_4/0.05% digitonin and twice with 10 ml of buffer A containing 0.05% digitonin at 4°C. Receptor was then eluted for 1 hr at 22°C with 2 ml of 5 mM Tris·HCl, pH 7.2/50 mM NaCl/5 mM NaF/0.05 mM Na_3VO_4/0.025% digitonin/0.1 mM alprenolol. Samples (typically 1.5 ml containing 0.2–0.8 pmol of receptor as assessed by [^{125}I]iodocyanopindolol binding) were lyophilized and dissolved in $NaDodSO_4$ sample buffer before electrophoresis. Receptor purification after the phosphorylation incubation was required when using kin$^-$ supernatant or HPLC-purified kinase preparations because they contain many endogenous protein kinases and substrates. For consistency, receptor repurification was also used with DEAE-Sephacel-purified kinase preparations. However, we have been able to demonstrate that receptor phosphorylated by DEAE-Sephacel-purified kinase can be visualized on $NaDodSO_4$/polyacrylamide gels without the need for repurification.

Protein Kinase C and Histone 2B Kinase Assays. Protein kinase C activity was measured by using a procedure modified from Kikkawa *et al.* (13). Briefly, 10–20 μl of sample was incubated with 20 mM Tris·HCl (pH 7.4) containing 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (≈400 cpm/pmol), 50 μg of histone H1, and either 5 mM EGTA or 0.25 mM $CaCl_2$, 10 μg of phosphatidylserine, and 1 μM PMA. Reactions were carried out for 30 min at 30°C and were stopped by the addition of 25% trichloroacetic acid. Precipitated protein was collected and washed on Millipore GF/C filters. Protein kinase C activity was calculated by subtracting the EGTA value from that obtained in the presence of Ca^{2+}. Histone 2B kinase activity was measured by incubating 25–50 μl of sample with 20 mM Tris·HCl (pH 7.4) containing 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, and 50 μg of histone 2B for 30 min at 30°C. Reactions were stopped with 25% trichloroacetic acid, followed by filtration on GF/C filters.

Phosphoamino Acid Determination. The ^{32}P-labeled receptor was excised from a $NaDodSO_4$/polyacrylamide gel, and the protein was extracted by homogenization in 2 ml of 50 mM NH_4HCO_3, followed by a 12-hr incubation at 22°C. The gel pieces were sedimented by centrifugation, and the supernatant was lyophilized to dryness. The residue was taken up in 0.1 ml of 6 M HCl and transferred to tubes, which were then evacuated, sealed, and incubated for 2 hr at 110°C. After lyophilization, the residue was dissolved in 100 μl of 10 mM K phosphate (pH 3.5) before chromatography on a Partisil PXS 10/25 SAX column. The ^{32}P-labeled phosphoamino acid was identified by comigration with authentic phosphoamino acids detected by absorbance at 214 nm.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed by the method of Laemmli (14) using 10% homogeneous slab gels. Sample buffer contained 8% $NaDodSO_4$, 10% glycerol, 5% 2-mercaptoethanol, 25 mM Tris·HCl (pH 6.5), and 0.003% bromphenol blue. After electrophoresis, gels were dried with a Bio-Rad gel dryer prior to autoradiography (8–24 hr) at −90°C.

Receptor Assay. The receptor was assayed with either [^{3}H]dihydroalprenolol or [^{125}I]iodocyanopindolol as described (11). Protein was determined by the amidoschwarz assay (15) using bovine serum albumin as standard.

RESULTS

Phosphorylation of the β-Adrenergic Receptor by kin$^-$ Cell Lysates. Initially we attempted to determine whether kin$^-$ cell lysates, separated into particulate and soluble fractions, contained any kinase activity capable of phosphorylating the β-adrenergic receptor. This cell line was chosen for initial study because it exhibits typical homologous (agonist-specific) desensitization and β-adrenergic receptor phosphorylation while lacking the cAMP-dependent protein kinase (7, 16). Incubation of reconstituted hamster lung β-adrenergic receptor with the kin$^-$ soluble fraction under phosphorylating conditions resulted in barely detectable phosphorylation of the receptor (Fig. 1, lane 1). However, when the receptor was occupied with the β-agonist isoproterenol, the phosphorylation was enhanced 5 ± 1-fold ($n = 8$, lane 2) under these experimental conditions. The agonist effect could be completely blocked by coincubation with the β-antagonist alprenolol (lane 3). Attempts at phosphorylating reconstituted receptor with the particulate fraction of kin$^-$ cells proved unsuccessful, suggesting that the kinase is predominantly soluble under the conditions of isolation. In preliminary experiments we also demonstrated that the β-adrenergic receptor kinase is present in a variety of tissues, including rat lung, heart, and brain as well as a hamster smooth muscle cell line (data not shown). Thus, the receptor kinase is not a peculiarity of the kin$^-$ cells.

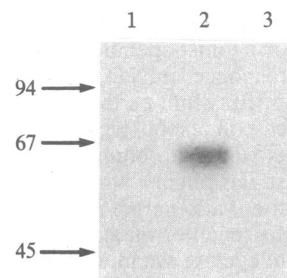

FIG. 1. Phosphorylation of purified hamster lung β-adrenergic receptor by the supernatant fraction of lysed kin$^-$ cells. Reconstituted receptor (6 pmol) was incubated with a supernatant fraction from high-speed centrifugation of lysed kin$^-$ cells (60 μg of protein) for 30 min at 30°C without (lane 1) or with additions of 10 μM (−)-isoproterenol (lane 2) and 10 μM (−)-isoproterenol/20 μM (±)alprenolol (lane 3). The phosphorylated receptor was then purified before electrophoresis on a 10% polyacrylamide gel. The molecular weight standards are shown × 10^{-3}. The result shown is representative of eight experiments.

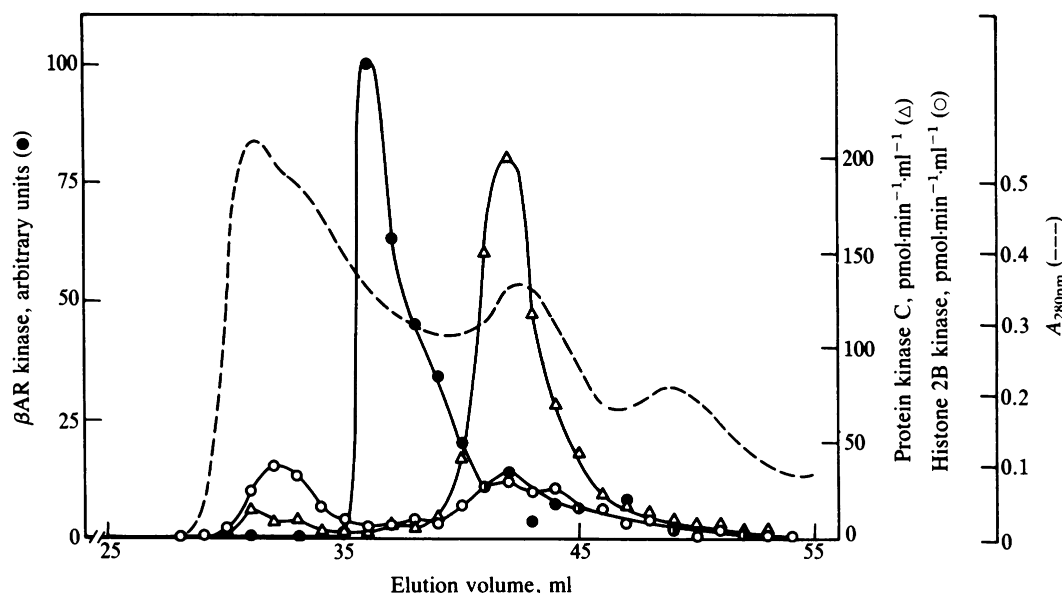

FIG. 2. High-performance steric exclusion chromatography of a soluble fraction of lysed kin⁻ cells. The supernatant fraction from high-speed centrifugation of lysed kin⁻ cells was chromatographed in 2-ml aliquots (7.5 mg of protein) on three tandem-linked TSK-3000 columns. The mobile phase was 20 mM Tris sulfate, pH 7.5/2 mM EDTA at a flow rate of 1 ml/min. β-Adrenergic receptor (βAR) kinase, protein kinase C, and histone 2B kinase were assayed as described. Maximum receptor phosphorylation in this experiment was ≈0.2 mol of ^{32}P per mol of receptor. The absorbance at 280 nm is also shown in this profile.

Partial Purification of β-Adrenergic Receptor Kinase. The soluble kin⁻ cell lysate was initially fractionated by molecular-sieve HPLC (Fig. 2). This resulted in virtually complete separation of the β-adrenergic receptor kinase activity from the major peaks of histone 2B kinase and protein kinase C. This step also resulted in an ≈3-fold increase in the specific activity of the β-adrenergic receptor kinase. The additional small peak of receptor phosphorylation coinciding with the protein kinase C activity most likely represents phosphorylation of the receptor by protein kinase C as it is enhanced by the inclusion of Ca^{2+} and phospholipid in the assay.

The peak of β-adrenergic receptor kinase activity from the HPLC was pooled and next chromatographed on a DEAE-Sephacel column (Fig. 3). The receptor kinase passed through the column unretarded, resulting in an apparent complete separation from the residual histone 2B kinase activity and an additional ≈25-fold increase in specific activity. This kinase preparation was used in all further studies. The fact that the receptor kinase did not interact with the DEAE column distinguishes it from a number of other kinases, including the cAMP-dependent and Ca^{2+}/phospholipid-dependent kinases. An additional small peak of receptor

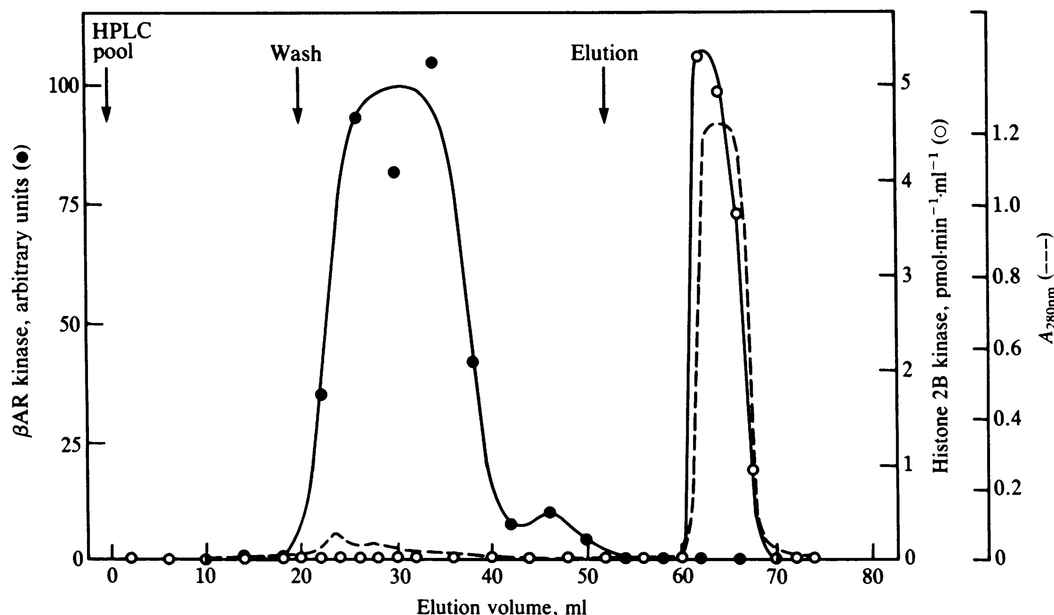

FIG. 3. DEAE-Sephacel chromatography of HPLC-purified β-adrenergic receptor (βAR) kinase. The peak receptor kinase fractions from five separate HPLC runs were pooled (20 ml total) and chromatographed on a 7-ml DEAE-Sephacel column equilibrated with 20 mM Tris·HCl, pH 7.5/2 mM EDTA. The column was washed with 32 ml of 20 mM Tris/2 mM EDTA before elution with this buffer containing 0.5 M NaCl. Fractions were assayed for β-adrenergic receptor kinase and histone 2B kinase as described. Maximum receptor phosphorylation in this experiment was ≈0.15 mol of ^{32}P per mol of receptor. The absorbance at 280 nm is also shown in this profile.

kinase activity was eluted after the major peak. This activity was seen in several preparations and appeared to increase when cells were lysed in the absence of EDTA.

Specificity of the β-Adrenergic Receptor Kinase. Receptor phosphorylation by DEAE-Sephacel-purified kinase was not affected by cAMP, cGMP, cAMP-dependent kinase inhibitor, Ca^{2+}/calmodulin or Ca^{2+}/phosphatidylserine/PMA (Table 1). These results clearly demonstrate that the receptor kinase is not a cAMP-dependent, cGMP-dependent, Ca^{2+}/calmodulin-dependent, or Ca^{2+}/phospholipid-dependent protein kinase. The specificity of the receptor kinase was further assessed by using mixed-type histones and casein as substrates. These proteins did not appear to be phosphorylated under conditions where the receptor was (data not shown). The receptor kinase appeared to be specific for serine residues, as phosphoamino acid determination revealed only phosphoserine.

Time Course of Receptor Phosphorylation. The time course of receptor phosphorylation in the presence or absence of isoproterenol is shown in Fig. 4. The striking increase in phosphorylation promoted by agonist (11-fold at 1 hr) clearly shows that agonist occupancy is virtually required for receptor phosphorylation. The increased fold stimulation of receptor phosphorylation by agonist seen with DEAE-Sephacel-purified vs. crude kinase preparations (11-fold vs. 5-fold) most likely reflects some agonist-independent phosphorylation of the receptor by protein kinase C or other kinases in the cruder preparations. These data are in contrast to previous work with the cAMP-dependent protein kinase (11). In those studies, β-adrenergic receptor phosphorylation was also enhanced by agonist occupancy; however, that effect was observed only as a 2- to 3-fold increase in the rate of phosphorylation, with no apparent change in the maximum stoichiometry obtained. Again this underscores the differences between the β-adrenergic receptor kinase and the cAMP-dependent protein kinase.

The relatively low stoichiometry obtained with the β-adrenergic receptor kinase (≈0.2 mol/mol) is likely due to several contributing factors. These include the relatively low amounts of kinase used in these studies (≈1–10% of that used in the cAMP-dependent kinase studies; ref. 11) along with the impurity of the kinase preparation. In addition, it is clear from the time course (Fig. 4) that the extent of phosphorylation was still increasing at 1 hr. Further purification of the kinase should enable us to significantly improve the extent of phosphorylation and, thus, permit a direct assessment of the functional significance of receptor phosphorylation.

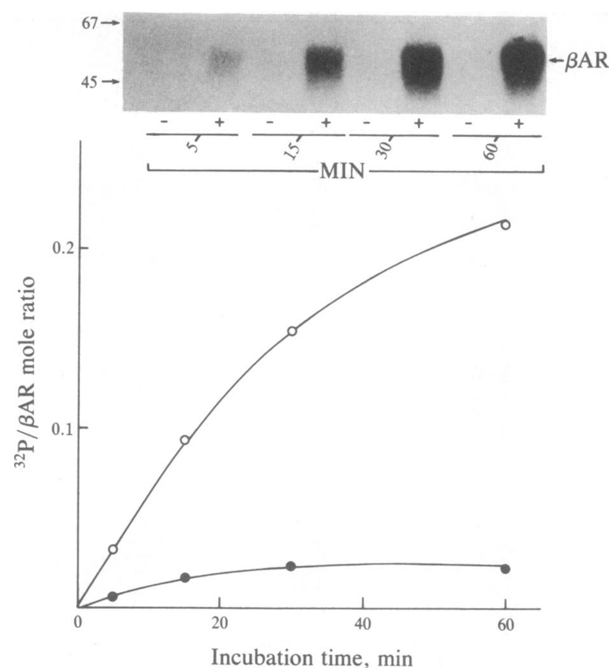

FIG. 4. Effect of isoproterenol on the time course of β-adrenergic receptor (βAR) phosphorylation by receptor kinase. Reconstituted β-adrenergic receptor (6 pmol) was incubated with DEAE-purified receptor kinase (1.5 μg of protein) in the presence (○; lanes + in *Inset*) or absence (●; lanes − in *Inset*) of 2 μM (−)-isoproterenol at 30°C for the indicated period of time. Phosphorylated receptor was then purified before electrophoresis on a 10% polyacrylamide gel. (*Inset*) Resulting autoradiogram after a 9-hr exposure of the dried gel. Phosphorylation stoichiometries were determined by counting the excised receptor bands as described (11). In calculating the stoichiometries, it was assumed that only half of the receptors in phosphatidylcholine vesicles were accessible to the kinase. This assumption was based on two lines of evidence. First, previous studies with the cAMP-dependent protein kinase showed that only ≈50% of the maximum phosphorylation could be achieved when the kinase was present only on the outside of the vesicles (11). In addition only ≈50% of the reconstituted receptors were accessible to deglycosylation by the enzyme endoglycosidase F (data not shown). The molecular weight standards are shown × 10^{-3}. The result shown is representative of two experiments.

DISCUSSION

These results provide evidence for a novel protein kinase that specifically phosphorylates the agonist-occupied β-adrenergic receptor. This kinase may represent a previously undiscovered protein kinase or it might represent a previously unrecognized specificity of a known kinase. Further purification and characterization of the β-adrenergic receptor kinase will be required to determine this. However, as described above, the β-adrenergic receptor kinase is clearly distinct from a number of other major kinases.

One of the most potentially interesting roles of the β-adrenergic receptor kinase might be its involvement in homologous desensitization. The mechanisms by which such receptor phosphorylation might lead to homologous desensitization are, however, currently unknown. One possibility is that the phosphorylated receptor has diminished ability to interact with the guanine nucleotide regulatory protein. Such a mechanism would be similar to that found for the cAMP-dependent protein kinase in heterologous desensitization (8–11). Studies on human astrocytoma cells (17, 18) demonstrating that desensitization precedes receptor sequestration would appear to support this hypothesis. Alternatively, receptor phosphorylation might trigger sequestration of the functionally intact receptors away from the effector guanine nucleotide regulatory protein (N_s)–adenylate cyclase complex. Recent data with the transferrin and epidermal growth factor receptors suggest that receptor phosphorylation might indeed be able to trigger receptor internalization (19–22).

Table 1. Effect of various protein kinase modulators on the β-adrenergic receptor kinase

Addition	Receptor phosphorylation, % of control
None (control)	100
cGMP (0.2 mM)	100 ± 3
cAMP (0.2 mM)	107 ± 9
cAMP-dependent kinase inhibitor (20 μg)	101 ± 16
Ca^{2+} (1.0 mM)/calmodulin (6 μg)	101 ± 5
Ca^{2+} (1.0 mM)/PtdSer (10 μg)/PMA (0.02 mM)	101 ± 6

The phosphorylation of β-adrenergic receptor (5 pmol) in the presence of 2 μM (−)-isoproterenol and DEAE-purified receptor kinase (1.5 μg) was studied in the presence or absence of various protein kinase modulators. Assay conditions were as described. Phosphorylation in the control was ≈0.15 mol of ^{32}P per mol of receptor. The data shown are the mean ± range of two experiments. PtdSer, phosphatidylserine.

These two mechanisms are, of course, not mutually exclusive. Thus, purification of the β-adrenergic receptor kinase may provide the key to ultimately elucidating the molecular events responsible for homologous desensitization.

The exquisite specificity of the β-adrenergic receptor kinase provides a simple mechanism for specific receptor phosphorylation and desensitization. Thus, the receptor is in essence only a substrate for the kinase when it is occupied by an agonist (i.e., stimulated). The notion that a protein kinase exists solely for phosphorylating the agonist-occupied β-adrenergic receptor, however, seems unlikely. Would every receptor that undergoes agonist-specific desensitization then have its own "personal" protein kinase? A more likely hypothesis is that a general receptor kinase exists that can phosphorylate different agonist-coupled receptors. Likely substrates for the β-adrenergic receptor kinase would be other adenylate cyclase-coupled receptors because the mechanism of receptor uncoupling and sequestration appears to be similar. It is not unreasonable to speculate that at least the "coupling" domains of all the adenylate cyclase-linked receptors might be structurally homologous. A single kinase could then act to phosphorylate and desensitize each of those receptors. However, the desensitization would in all cases be homologous or agonist-specific because only the agonist-occupied receptor would be phosphorylated.

Another aspect of receptor phosphorylation is the role, if any, of a receptor kinase in heterologous desensitization. At present only the cAMP-dependent protein kinase is known to be involved in heterologous desensitization (8–11, 23). However, only ≈50% of the total receptor phosphorylation and desensitization is accounted for by this kinase (8, 10, 11, 23). While the cAMP-dependent kinase provides a heterologous or nonselective effect on the system because of its broad substrate specificity, the receptor kinase might impart at least some agonist specificity.

A striking analogy can be drawn between the light-activated phosphodiesterase system of rod outer segments and the adenylate cyclase system. In the phosphodiesterase system, enzyme activity is mediated by the light-dependent interaction of rhodopsin, a receptor for light, with transducin, a guanine nucleotide regulatory protein (24). A potential role for phosphorylation in the regulation of this system was noted by a number of groups in the early 1970s when they discovered a light-dependent phosphorylation of rhodopsin (25–27). The kinase involved appears to be highly specific for bleached rhodopsin as a substrate (28, 29) and has been aptly named rhodopsin kinase. Shichi *et al.* have been able to demonstrate that phosphorylated rhodopsin has a reduced ability to interact with transducin, thus providing a mechanism for the light-induced "desensitization" observed in this system (30). Moreover, Somers and Klein have shown that the rat pineal gland contains high levels of a rhodopsin kinase-like activity while lacking the photoreceptor rhodopsin (31). They speculate that pineal rhodopsin kinase may be involved in regulating adrenergic responsiveness in these cells. The potential relationship between rhodopsin kinase, pineal rhodopsin kinase, and the β-adrenergic receptor kinase will be of interest to determine (11).

In summary, we have identified and partially purified a novel protein kinase that preferentially phosphorylates the agonist-occupied form of the β-adrenergic receptor. This kinase may play an important role in regulating the function of the β-adrenergic receptor as well as other adenylate cyclase-coupled receptors, as in homologous desensitization. Moreover, the existence of such a kinase suggests a general mechanism for regulating receptor function—i.e., that only the agonist-occupied form of the receptor is a substrate for relevant regulatory enzymes. Such a mechanism would explain the intimate linkage of receptor activation and desensitization. Future studies will focus on the determination of the ability of this kinase to phosphorylate other receptors and on an examination of the functionality of the phosphorylated receptors. However, such investigations will require further purification of the enzyme.

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