



**Functional Desensitization of the Isolated  $\beta$ -adrenergic Receptor by the  $\beta$ -adrenergic Receptor Kinase: Potential Role of an Analog of the Retinal Protein Arrestin (48-kDa Protein)**

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*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 84, No. 24 (Dec. 15, 1987), 8879-8882.

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Wed Oct 8 18:51:16 2003

## Functional desensitization of the isolated $\beta$ -adrenergic receptor by the $\beta$ -adrenergic receptor kinase: Potential role of an analog of the retinal protein arrestin (48-kDa protein)

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Communicated by Harvey F. Lodish, August 27, 1987

**ABSTRACT** The  $\beta$ -adrenergic receptor kinase is an enzyme, possibly analogous to rhodopsin kinase, that multiply phosphorylates the  $\beta$ -adrenergic receptor only when it is occupied by stimulatory agonists. Since this kinase may play an important role in mediating the process of homologous, or agonist-specific, desensitization, we investigated the functional consequences of receptor phosphorylation by the kinase and possible analogies with the mechanism of action of rhodopsin kinase. Pure hamster lung  $\beta_2$ -adrenergic receptor, reconstituted in phospholipid vesicles, was assessed for its ability to mediate agonist-promoted stimulation of the GTPase activity of coreconstituted stimulatory guanine nucleotide-binding regulatory protein. When the receptor was phosphorylated by partially ( $\approx 350$ -fold) purified preparations of  $\beta$ -adrenergic receptor kinase, as much as 80% inactivation of its functional activity was observed. However, the use of more highly purified enzyme preparations led to a dramatic decrease in the ability of phosphorylation to inactivate the receptor such that pure enzyme preparations ( $\approx 20,000$ -fold purified) caused only minimal ( $\approx 16 \pm 7\%$ ) inactivation. Addition of pure retinal arrestin (48-kDa protein or S antigen), which is involved in enhancing the inactivating effect of rhodopsin phosphorylation by rhodopsin kinase, led to partial restoration of the functional effect of  $\beta$ -adrenergic receptor kinase-promoted phosphorylation ( $41 \pm 3\%$  inactivation). These results suggest the possibility that a protein analogous to retinal arrestin may exist in other tissues and function in concert with  $\beta$ -adrenergic receptor kinase to regulate the activity of adenylate cyclase-coupled receptors.

Transmembrane signaling systems for converting extracellular stimuli as divergent as hormones, drugs, or photons of light into intracellular metabolic changes have been remarkably conserved through evolution. Such systems generally consist of three major components: a receptor, such as the  $\beta$ -adrenergic receptor ( $\beta$ AR) or the visual "light receptor" rhodopsin; a guanine nucleotide-binding regulatory protein, such as the stimulatory guanine nucleotide-binding regulatory protein ( $G_s$ ) or transducin; and an effector, such as adenylate cyclase or cGMP phosphodiesterase (1–4). Not only are such systems analogous, but individual components are actually homologous proteins such as the  $\beta$ AR and rhodopsin or  $G_s$  and transducin. Moreover, two analogous enzymes that may function to regulate the activities of such systems have been described. Rhodopsin kinase is a cytosolic enzyme that phosphorylates only the light-bleached form of rhodopsin on multiple serine and threonine residues (up to 9 mol of phosphate per mol of receptor) and is thought to be involved in deactivating the illuminated form of rhodopsin so that it does not continue to activate transducin (5, 6).  $\beta$ AR kinase is a ubiquitously distributed cytosolic enzyme that

phosphorylates only the agonist-occupied form of the  $\beta$ AR (up to 9 mol of phosphate per mol) (7). It is probably involved in phosphorylating and regulating other adenylate cyclase-coupled receptors as well (8, 9). We have proposed that  $\beta$ AR kinase may mediate the phosphorylation of the  $\beta$ AR that accompanies and may cause the "homologous" or agonist-specific form of adenylate cyclase desensitization.

Phosphorylation of rhodopsin by rhodopsin kinase only partially explains the diminished capacity of phosphorylated rhodopsin to activate transducin. In addition, another cytosolic retinal protein, variably termed 48-kDa protein, S antigen, or arrestin, binds specifically to phosphorylated rhodopsin and, thereby, inhibits its ability to interact with transducin (6).

In the present studies we have investigated the functional consequences of phosphorylation of purified mammalian  $\beta_2$ -adrenergic receptor with  $\beta$ AR kinase by assessing the ability of these receptors to mediate the agonist-promoted stimulation of the GTPase activity of  $G_s$  in a reconstituted system. Moreover, a functionally important interaction of purified retinal arrestin with this system is documented.

### METHODS

**Protein Purification.**  $\beta$ -Adrenergic receptor from hamster lung was purified to  $>95\%$  homogeneity by sequential affinity chromatography and HPLC as described (10).

$G_s$  was purified from a cholate extract of washed human erythrocyte membranes as described (11). The preparations used in these experiments were from step 8A in ref. 11 and were  $>95\%$  pure as judged by Coomassie blue staining of polyacrylamide gels.

$\beta$ AR kinase was purified from bovine brain by  $(\text{NH}_4)_2\text{SO}_4$  precipitation of a high-speed supernatant fraction as described (12). The precipitate was then chromatographed on Ultrogel AcA 34, DEAE-Sephacel, CM-Fractogel, and hydroxylapatite. This yielded preparations in which  $\beta$ AR kinase represented  $\approx 0.8\%$  of the total protein after Ultrogel AcA 34,  $\approx 1.5\%$  after DEAE-Sephacel, 10–20% after CM-Fractogel, and  $>90\%$  after hydroxylapatite.

Arrestin was purified to  $>90\%$  homogeneity from bovine retina using its reversible binding to phosphorylated rhodopsin (13). Some preparations were further purified to apparent homogeneity by FPLC using a Mono Q column (13). Both preparations gave identical results.

**Phosphorylation.** Purified  $\beta$ AR (25–50 pmol) was initially reconstituted in soybean phosphatidylcholine vesicles as described (7, 14). The protein-lipid pellets were resuspended in 20–30  $\mu\text{l}$  of 20 mM Tris-HCl, pH 7.5/2 mM EDTA before incubation with  $\beta$ AR kinase. These reaction mixtures con-

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Abbreviations: p[NH]ppA, adenosine 5'-[ $\beta$ , $\gamma$ -imido]triphosphate;  $\beta$ AR,  $\beta$ -adrenergic receptor;  $G_s$ , stimulatory guanine nucleotide-binding regulatory protein.

tained, in a total volume of 40  $\mu$ l, reconstituted  $\beta$ AR (2–5 pmol), 20  $\mu$ M (–)-isoproterenol, and  $\beta$ AR kinase in 20 mM Tris-HCl, pH 7.5/2 mM EDTA/5 mM MgCl<sub>2</sub>/0.5 mM ascorbic acid and either 50  $\mu$ M ATP (phosphorylated) or 50  $\mu$ M adenosine 5'-[ $\beta$ , $\gamma$ -imido]triphosphate (p[NH]ppA) (control). In addition, to inhibit endogenous cAMP-dependent protein kinase present in the crude  $\beta$ AR kinase preparations a peptide inhibitor of this kinase was included in most experiments at a concentration of 5  $\mu$ g/ml. This inhibitor is a synthetic peptide comprising the 24 amino-terminal residues of the specific cAMP-dependent protein kinase inhibitor (15, 16). In some experiments (see Fig. 1), 20 ml of cold 100 mM NaCl/10 mM Tris-HCl, pH 7.2/2 mM EDTA was added after the incubation period. The samples were then centrifuged (350,000  $\times$  *g* for 60 min), and the resultant pellets were resuspended in 300  $\mu$ l of 100 mM NaCl/10 mM Tris-HCl, pH 7.2. Samples were incubated with G<sub>s</sub> before assaying for GTPase activity. In other experiments (see Figs. 2 and 3), following a 1-hr incubation at 30°C, the samples were diluted with 250  $\mu$ l of cold 100 mM NaCl/10 mM Tris-HCl, pH 7.2. The diluted samples containing either control or phosphorylated  $\beta$ AR were then incubated with G<sub>s</sub> and retinal arrestin (see Fig. 3) for 20 min on ice. Samples were then assayed for GTPase activity. In these studies, the stoichiometry of phosphorylation varied depending on the particular  $\beta$ AR kinase preparation used. With Ultrogel Aca 34- or DEAE-Sephacel-purified  $\beta$ AR kinase, only 3–4 mol of phosphate per mol of  $\beta$ AR could be obtained with a 1-hr incubation due to the relatively lower concentration of  $\beta$ AR kinase in these preparations. More concentrated  $\beta$ AR kinase preparations (CM-Fractogel or hydroxylapatite) are able to maximally phosphorylate  $\beta$ AR (8–9 mol of phosphate per mol) during a 1-hr incubation or less.

**GTPase Assay.** Samples were assayed for GTPase activity at 30°C by incubating 50  $\mu$ l of vesicles in a total volume of 100  $\mu$ l containing 10 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, bovine serum albumin at 2 mg/ml, 0.5 mM ascorbic acid, 0.2 mM p[NH]ppA, 0.1  $\mu$ M [ $\gamma$ -<sup>32</sup>P]GTP, and either 100  $\mu$ M (–)-isoproterenol or 100  $\mu$ M (±)-alprenolol. Reactions were stopped by the addition of trichloroacetic acid, and, following centrifugation, the inorganic [<sup>32</sup>P]phosphate in the supernatant was determined by extraction with molybdate/2-methyl-1-propanol/benzene (17). Isoproterenol-stimulated GTPase activity was calculated as the difference between the activity in the presence of isoproterenol and in the presence of alprenolol and generally represented an  $\approx$ 3-fold stimulation by the agonist.

**Electrophoresis.** Gel electrophoresis was performed by the method of Laemmli (18) using 10% polyacrylamide gels. Sample buffer consisted of 8% (wt/vol) NaDodSO<sub>4</sub>, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 25 mM Tris-HCl (pH 6.5), and 0.003% bromphenol blue. After electrophoresis, gels were immediately dried prior to autoradiography at –90°C.

## RESULTS

Initially, partially purified preparations of  $\beta$ AR kinase were used to phosphorylate the reconstituted purified  $\beta$ AR. These enzyme preparations, which were  $\approx$ 1% pure, had been chromatographed on an Ultrogel Aca 34 column after (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and were purified  $\approx$ 350-fold from the starting material (12). As shown in Fig. 1, as phosphate was incorporated into the receptor, a progressive diminution of the receptor-promoted activation of G<sub>s</sub> was observed. At a stoichiometry of  $\approx$ 3 mol of phosphate per mol of  $\beta$ AR, an  $\approx$ 80% reduction in receptor activity was observed when compared with the control (nonphosphorylated). This loss of receptor function was not due to a reduced ability of the receptor to bind agonist ligands since, as shown in Table 1,

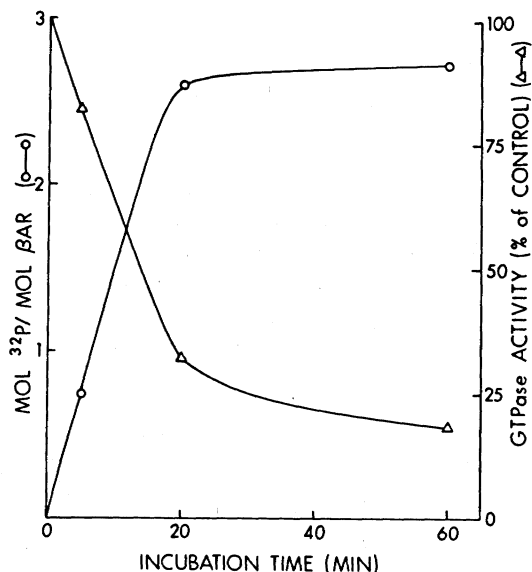


FIG. 1. Isoproterenol-promoted GTPase activity in phospholipid vesicles containing G<sub>s</sub> and receptor phosphorylated by  $\beta$ AR kinase. Phospholipid vesicles containing purified  $\beta$ AR were incubated at 30°C for 5, 20, or 60 min with Ultrogel Aca 34-purified  $\beta$ AR kinase in a buffer of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 4 mM MgCl<sub>2</sub>, 10  $\mu$ M (–)-isoproterenol, 4 mM sodium phosphate, 4 mM NaF, and either 75  $\mu$ M p[NH]ppA (control) or 75  $\mu$ M ATP (phosphorylated) or 75  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP ( $\approx$ 2 cpm/fmol). Reactions were stopped by the addition of 20 ml of cold 100 mM NaCl/10 mM Tris-HCl, pH 7.2/2 mM EDTA. After centrifugation, the pellets from the [ $\gamma$ -<sup>32</sup>P]ATP samples were resuspended in NaDodSO<sub>4</sub> sample buffer before electrophoresis on a 10% polyacrylamide gel. Stoichiometries of phosphorylation were determined as described (7, 12). Pellets from the p[NH]ppA and ATP incubations were resuspended in 300  $\mu$ l of 100 mM NaCl/10 mM Tris-HCl, pH 7.2. Samples (containing  $\approx$ 0.4 pmol of  $\beta$ AR) were then incubated with  $\approx$ 1.5 pmol of G<sub>s</sub> for 20 min at 4°C before assaying for isoproterenol-stimulated GTPase activity.

the affinity of several ligands for binding to control and phosphorylated receptors was not altered. The loss of function was also not due to proteolysis since, as assessed by NaDodSO<sub>4</sub>/PAGE, the molecular weight of the receptor is unaltered (data not shown).

While phosphorylation of the receptor by partially purified  $\beta$ AR kinase preparations was accompanied by a parallel reduction in receptor activity, this effect was substantially lost as the kinase was further purified. Fig. 2 presents results obtained with  $\beta$ AR kinase preparations of various levels of purity. When the enzyme preparations used for the experiments shown in Fig. 1 were chromatographed on DEAE-Sephacel columns, a further  $\approx$ 2-fold purification occurred. As described (12), in this step of purification  $\beta$ AR kinase

Table 1. Comparison of the EC<sub>50</sub> values of control and phosphorylated  $\beta$ AR

Agent	EC <sub>50</sub> , nM	
	Control	Phosphorylated
(–)-Alprenolol	8.3 $\pm$ 0.6	6.8 $\pm$ 0.3
(–)-Isoproterenol	1725 $\pm$ 548	1750 $\pm$ 299
(–)-Epinephrine	6450 $\pm$ 2692	5500 $\pm$ 997

EC<sub>50</sub> values for the ligands were calculated from competition curve data at a concentration of 50 pM [<sup>125</sup>I]-labeled (–)-cyanopindolol and various concentrations of agonist or antagonist. Data were analyzed by computer modeling methods (19). The  $\beta$ AR used in this experiment was phosphorylated by CM-Fractogel-purified  $\beta$ AR kinase. The purity of the  $\beta$ AR kinase preparation had no appreciable influence on the results of such studies. The data shown are mean  $\pm$  SD of two experiments.

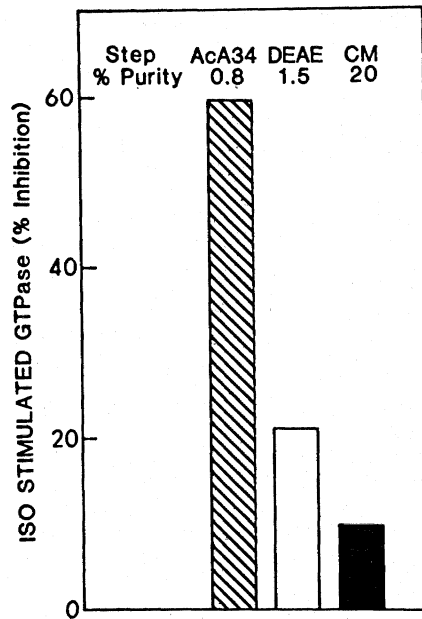


FIG. 2. Effect of receptor phosphorylation by various  $\beta$ AR kinase preparations on inhibiting receptor- $G_s$  coupling. Phospholipid vesicles containing purified  $\beta$ AR were incubated for 60 min with Ultrogel AcA 34-purified, DEAE-Sephacel-purified, or CM-Fractogel-purified  $\beta$ AR kinase. The percent purity at each step is shown at the top of the figure. Control (p[NH]ppA) and phosphorylated (ATP)  $\beta$ AR preparations ( $\approx 0.5$  pmol) were then incubated with  $G_s$  ( $\approx 1.5$  pmol) before assaying for isoproterenol-stimulated GTPase activity. The results are expressed as the percent inhibition of phosphorylated vs. control receptor preparations. The stoichiometry of phosphorylation in this particular experiment was 3–4 mol of phosphate per mol of receptor with all  $\beta$ AR kinase preparations. These results are representative of three independent experiments.

passes directly through the DEAE-Sephacel column. Such enzyme preparations, which are about 1.5% pure, caused only  $\approx 20\%$  inactivation of receptor-promoted GTPase activity. Additional chromatography on CM-Fractogel and hydroxylapatite columns produced essentially homogeneous enzyme preparations that, while phosphorylating the receptors to a comparable stoichiometry in this experiment (3–4 mol of phosphate per mol), caused only 10–15% receptor inactivation. These results suggest that, in addition to receptor phosphorylation, the participation of some other factor that is lost during the purification of  $\beta$ AR kinase is required for receptor function to be regulated.

Since it has been demonstrated (6) that inactivation of light-promoted phosphodiesterase activity is enhanced by binding of arrestin to phosphorylated rhodopsin, we tested the effects of purified retinal arrestin in the  $\beta$ AR system. The results are shown in Fig. 3. In the absence of arrestin, the phosphorylated receptor stimulated  $G_s$ -associated GTPase activity to  $84.4 \pm 6.8\%$  ( $n = 5$ ) of that obtained with the unphosphorylated receptor. Addition of increasing amounts of arrestin to the reconstituted  $\beta$ AR/ $G_s$ /pure  $\beta$ AR kinase system has no effect on receptor activity under nonphosphorylating conditions. Thus, the mean activity of control receptor in the presence of various levels of arrestin was  $98.1 \pm 3.5\%$  ( $n = 13$ ) of the value in the absence of arrestin. However, following the phosphorylation of  $\beta$ AR by ATP and pure  $\beta$ AR kinase, addition of various amounts of arrestin leads to a progressive reduction in receptor- $G_s$  interaction, such that at high arrestin (arrestin/ $G_s$  molar ratio,  $>100:1$ ) only  $59.3 \pm 2.6\%$  ( $n = 9$ ) of control activity is observed ( $P < 0.001$ ). This effect was shown to be specific as it required the simultaneous presence of  $\beta$ AR kinase-phosphorylated  $\beta$ AR,  $G_s$ , and arrestin. Arrestin could not be replaced by bovine

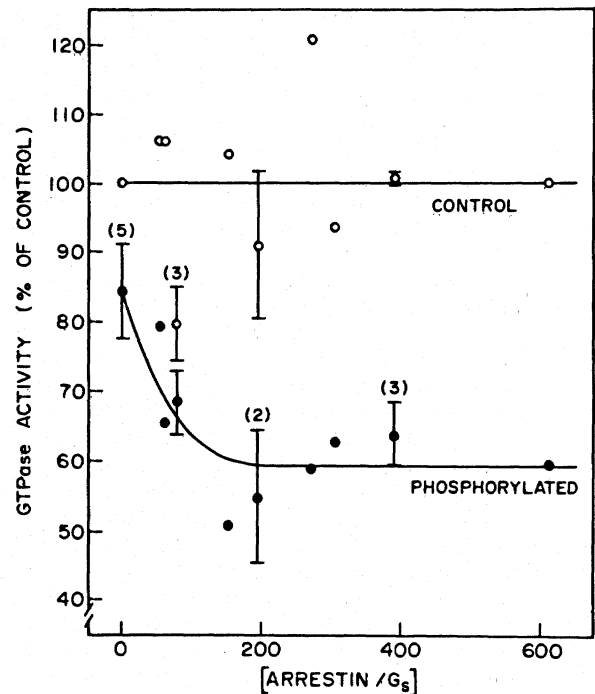


FIG. 3. Effect of retinal arrestin on inhibiting  $\beta$ AR- $G_s$  coupling. Phospholipid vesicles containing purified  $\beta$ AR were incubated for 60 min with purified  $\beta$ AR kinase ( $>90\%$ ). The stoichiometry of phosphorylation was  $\approx 8$  mol of phosphate per mol of receptor. Control (p[NH]ppA) and phosphorylated (ATP)  $\beta$ AR preparations ( $\approx 0.4$  pmol) were then incubated with purified retinal arrestin (0–1500 pmol) and pure  $G_s$  ( $\approx 2.4$  pmol) for 20 min at  $4^\circ\text{C}$ . Samples were then assayed for isoproterenol-stimulated GTPase activity. The results are data from five separate experiments. The error bars represent means  $\pm$  SEM from two to five independent experiments as shown in parentheses; individual points are data from single experiments.

serum albumin or heat-denatured arrestin. The maximum reduction achieved was about one-half of that observed with the crude  $\beta$ AR kinase preparations (40% vs. 80%). Thus, addition of pure arrestin to  $\beta$ AR phosphorylated by pure  $\beta$ AR kinase partially restores the ability of the phosphorylation to inactivate receptor function.

## DISCUSSION

These results have important implications for understanding the possible mechanisms by which covalent modification of adenylate cyclase-coupled receptors may regulate their function. Phosphorylation of the  $\beta$ AR by partially purified preparations of  $\beta$ AR kinase clearly leads to receptor inactivation, apparent as an impairment of receptor coupling to  $G_s$ . In clear analogy with the retinal system for inactivating rhodopsin (6), the functional effect of  $\beta$ AR kinase appears to be significantly enhanced by some other cytosolic factor(s) that is separated from  $\beta$ AR kinase during its purification. The existence of such a factor that may be similar to the retinal protein is suggested, because authentic retinal arrestin can augment the effect of  $\beta$ AR kinase. Possibly, with the nonretinal homolog of this protein, even more dramatic effects on the function of the receptor would be observed. In this connection it can also be noted that the molar ratios of arrestin/ $G_s$  ( $>100:1$ ) required for inactivation of phosphorylated  $\beta$ AR are significantly higher than the arrestin/transducin molar ratio ( $<10:1$ ) required for comparable effects on phosphorylated rhodopsin (6). Since antibodies to arrestin do not identify cross-reactive proteins in tissues other than the pineal (20), there may be significant differences in the structure of any such putative arrestin-like protein in nonretinal tissues.

An obvious starting point for efforts to identify such a protein would be side fractions from the purification of  $\beta$ AR kinase. Inasmuch as a major decrease in the receptor-inactivating ability of  $\beta$ AR kinase occurs after the DEAE-Sephacel purification step, it may be that the relevant proteins are adsorbed to this column. The ability of any such fraction to restore to highly purified  $\beta$ AR kinase preparations receptor inactivating properties would provide an assay for identifying and purifying the putative homolog of the retinal arrestin.

Several studies have demonstrated that  $\beta$ AR phosphorylation occurs during homologous desensitization. Treatment of frog erythrocytes with isoproterenol leads to an increase in  $\beta$ AR phosphorylation from 0.6 to 1.9 mol of phosphate per mol of receptor (21). Studies in S49 lymphoma cells (from 0.2 to 0.8 mol of phosphate per mol of receptor) and DDT<sub>1</sub>-MF2 cells (from 0.4 to 2.0 mol of phosphate per mol of receptor) also show increased receptor phosphorylation with agonist treatment (8, 22). These whole-cell studies suggest that relatively low stoichiometries of phosphorylation are obtained (1–2 mol of phosphate per mol of receptor) as compared with *in vitro* phosphorylation of purified  $\beta$ AR by  $\beta$ AR kinase (8 mol of phosphate per mol of receptor) (12). These results are potentially explained in several ways. First, it is possible in the whole-cell studies that during the receptor-purification procedures, which are required for subsequent visualization of the phosphorylated receptor on NaDodSO<sub>4</sub>/PAGE, a significant amount of phosphate is lost due to contaminating phosphatase activity. A second point to be considered is that these measurements of phosphorylation stoichiometry are only average values, i.e., they do not provide information about individual receptor populations. Thus an average of 2 mol of phosphate per mol of receptor may result from receptors phosphorylated to various degrees from 0 to 8 mol of phosphate per mol of receptor. This issue may be approachable using techniques such as isoelectric focusing. Another possibility is that the stoichiometry of phosphorylation in whole cells is indeed relatively low. The data in Fig. 1, which demonstrate that 2–3 mol of phosphate per mol of  $\beta$ AR inhibits its interaction with G<sub>s</sub>  $\approx$ 80%, suggest that a high stoichiometry is not required for uncoupling. Additional studies will be necessary to determine the correlation between phosphorylation stoichiometry and desensitization.

The concerted effects of  $\beta$ AR kinase and retinal arrestin on the function of the  $\beta$ AR further extend the growing list of analogies between the visual and hormonal membrane sig-

naling systems. These analogies must now be extended to include the mechanisms of regulation of receptor function at the molecular level.

We thank Claudia Staniszewski for her excellent technical assistance and Mary Holben for her skillful help in preparing this manuscript.

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