Desensitization of the Isolated β_2-Adrenergic Receptor by β-Adrenergic Receptor Kinase, cAMP-Dependent Protein Kinase, and Protein Kinase C Occurs via Distinct Molecular Mechanisms†

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ABSTRACT: Exposure of β_2-adrenergic receptors (β_2ARs) to agonists causes a rapid desensitization of the receptor-stimulated adenylyl cyclase response. Phosphorylation of the β_2AR by several distinct kinases plays an important role in this desensitization phenomenon. In this study, we have utilized purified hamster lung β_2AR and stimulatory guanine nucleotide binding regulatory protein (G_s), reconstituted in phospholipid vesicles, to investigate the molecular properties of this desensitization response. Purified hamster β_2AR was phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), or βAR kinase (βARK), and receptor function was determined by measuring the β_2AR–agonist-promoted G_s-associated GTPase activity. At physiological concentrations of Mg^{2+} (<1 mM), receptor phosphorylation inhibited coupling to G_s by 60% (PKA), 40% (PKC), and 30% (βARK). The desensitizing effect of phosphorylation was, however, greatly diminished when assays were performed at concentrations of Mg^{2+} sufficient to promote receptor-independent activation of G_s (>5 mM). Addition of retinal arrestin, the light transduction component involved in the attenuation of rhodopsin function, did not enhance the uncoupling effect of βARK phosphorylation of β_2AR when assayed in the presence of 0.3 mM free Mg^{2+}. At concentrations of Mg^{2+} ranging between 0.5 and 5.0 mM, however, significant potentiation of βARK-mediated desensitization was observed upon arrestin addition. At a free Mg^{2+} concentration of 5 mM, arrestin did not potentiate the inhibition of receptor function observed on PKA or PKC phosphorylation. These results suggest that distinct pathways of desensitization exist for the receptor phosphorylated either by PKA or PKC or alternatively by βARK.

Receptor-mediated responses often become attenuated in the presence of continuous or repeated stimulation, a process that is termed desensitization. The mechanisms of desensitization have been extensively studied using the β_2-adrenergic receptor (β_2AR)[1] as a model system (Benovic et al., 1988). Binding of adrenergic agonists to this receptor leads to stimulation of adenylyl cyclase, via the guanine nucleotide regulatory protein, G_s, and the accumulation of cAMP (Gilman, 1987). Purified reconstituted β_2AR has been shown to be phosphorylated in vitro by βAR kinase (βARK) (Benovic et al., 1986), cAMP-dependent protein kinase (PKA) (Benovic et al., 1985), and protein kinase C (PKC) (Bouvier et al., 1987). Furthermore, in a reconstituted system using pure components and kinases, both βARK phosphorylation and PKA phosphorylation of β_2AR have been shown to modestly inhibit (~15–20%) receptor-stimulated G_s activation (Benovic et al., 1985, 1987a), consistent with a role for receptor phosphorylation in desensitization.

βARK, in contrast to PKA or PKC, phosphorylates only the agonist-occupied form of the receptor (Benovic et al., 1986) and would thus appear to be analogous to the retinal enzyme rhodopsin kinase. Rhodopsin kinase specifically phosphorylates bleached rhodopsin and is proposed to play an important role in the attenuation of the light signal (Wilden et al., 1986a). Phosphorylation is by itself insufficient to completely inhibit rhodopsin/transducin coupling. Phosphorylation, however, promotes the binding of arrestin to rhodopsin, and this appears to result in inhibition of rhodopsin function. It was proposed, by analogy to the light transduction pathway, that there might be an arrestin-like protein that serves to inhibit the function of βARK-phosphorylated β_2ARs. Indeed, the functional effects of βARK-mediated phosphorylation of β_2ARs could be potentiated by addition of the retinal protein arrestin (Benovic et al., 1987a). Recently, the cDNA for β-arrestin, a protein with a high degree of homology to retinal arrestin, has been cloned from a bovine brain library (Lohse et al., 1990). This protein has been proposed to act in concert with βARK to produce homologous desensitization of β_2ARs.

Desensitization of the β_2AR has been demonstrated both after PMA treatment of cells (Johnson et al., 1986, 1990) and in a cell-free system by incubation of a purified plasma membrane preparation with the catalytic subunit of PKA (Nambi et al., 1985; Clark et al., 1987). In both cases, desensitization of the β_2AR was most pronounced when adenylyl cyclase assays were performed at physiological Mg^{2+} concentrations [<1.0 mM (Gupta & Moore, 1980; Grubbs et al., 1984)]; high Mg^{2+} concentrations (5–10 mM) largely obscure both the PKC-mediated (Johnson et al., 1986) and the PKA-mediated (Kunkel et al., 1989) desensitization. It is interesting to note that the extent of desensitization observed in the plasma

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; G_s, stimulatory guanine nucleotide binding regulatory protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; βARK, β-adrenergic receptor kinase; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; PMA, 4β-phorbol 12-myristate 13-acetate.

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membrane preparation after PKA phosphorylation of β_2AR is much larger than previously observed using PKA-phosphorylated β_2AR in a reconstituted system (Benovic et al., 1985), studies which were performed at 10 mM Mg^{2+}. Such observations have raised important questions. Does β-arrestin or a β-arrestin-like protein bind to PKA- or PKC-phosphorylated β_2AR, or are such interactions limited to βARK-phosphorylated β_2AR? To what extent is βARK/β-arrestin-mediated desensitization Mg^{2+} dependent? To address these questions and thus gain information concerning the mechanism(s) whereby phosphorylation of β_2AR promotes receptor/G_s uncoupling, the functional consequences of phosphorylation of β_2AR by PKA, PKC, and βARK were examined in a reconstituted system in which the concentrations of retinal arrestin and Mg^{2+} were varied. Retinal arrestin rather than β-arrestin was used in this investigation since the latter protein is not yet available in sufficient quantities and purity to perform such studies.

Experimental Procedures

Protein Purification. β_2-Adrenergic receptors from hamster lung were purified to >95% homogeneity by sequential affinity and high-performance liquid chromatography as previously described (Benovic et al., 1984).

G_s was purified from a cholate extract of washed human erythrocyte membranes (Codina et al., 1984a). βARK (>70% homogeneity) was purified from bovine cerebral cortex (Benovic et al., 1987b) and the catalytic subunit of PKA from bovine heart according to published procedures (Bechtel et al., 1977). PKC was a generous gift of Dr. Perry Blackshear, Duke University Medical Center, Durham, NC. Arrestin was purified to >90% homogeneity from bovine retina using its reversible binding to phosphorylated rhodopsin (Wilden et al., 1986b).

Phosphorylation of βAR. Purified β_2AR (50–100 pmol) was reconstituted into phospholipid vesicles (Benovic et al., 1986; Cerione et al., 1983), and the protein–lipid pellets were resuspended in 20 mM Tris-HCl (pH 7.5)/2 mM EDTA before incubation with βARK, the catalytic subunit of PKA, or PKC. Typically ~5 pmol of reconstituted β_2AR was phosphorylated in a total volume of 40 μL. When being phosphorylated with βARK (0.1 μg), reaction mixtures contained 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$, 0.5 mM ascorbate, 20 μM (−)-isoproterenol, and 60 μM [γ-^{32}P]ATP (1000–5000 cpm/pmol) or 60 μM unlabeled ATP. In addition, a peptide inhibitor of PKA, PKI (Scott et al., 1985a,b), was included at a final concentration of 5 μg/mL. Similar conditions were used when phosphorylating β_2AR with PKA. Ascorbate, (−)-isoproterenol, and PKI were excluded, however, and βARK was replaced with 0.5 μg of the catalytic subunit of PKA. The phosphorylation of the β_2AR by PKC (0.1 μg) was performed in a buffer containing 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/mL phosphatidylserine, 0.025 mM NaH_2PO_4, 0.1 mM 4β-phorbol 12-myristate 13-acetate (PMA), and 60 μM [γ-^{32}P]ATP (~2500 cpm/pmol) or 60 μM unlabeled ATP. Phosphorylation reactions were incubated at 30 °C for 45 min and stopped, in the case of [γ-^{32}P]-ATP-containing incubations, with SDS sample buffer or alternatively by 10-fold dilution with ice-cold 100 mM NaCl/10 mM Tris-HCl, pH 7.2. Phosphorylation stoichiometries were determined as described previously (Benovic et al., 1986, 1987b). Using PKA, PKC, and βARK, the β_2AR was phosphorylated to a stoichiometry of 1.5 ± 0.2 (n = 5), 0.6 ± 0.2 (n = 4), and 6.0 ± 2.1 (n = 4) mol of P_i/mol of β_2, respectively. [γ-^{32}P]ATP-containing samples were electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (1970). The gels were immediately dried prior to autoradiography at −90 °C.

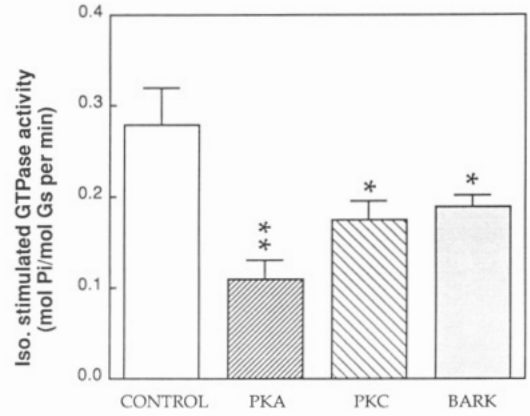

FIGURE 1: Phosphorylation-induced inhibition of β_2AR/G_s coupling. Purified hamster β_2AR phosphorylated with PKA, PKC, or βARK was co-reconstituted with G_s into phospholipid vesicles. The ability of control (unphosphorylated) and phosphorylated receptor preparations to couple to G_s was subsequently assessed by measuring the isoproterenol-stimulated GTPase activity at a free Mg^{2+} concentration of 0.3 mM. The results are means ± SEM of four separate experiments for the unphosphorylated and the PKA- and βARK-phosphorylated β_2AR and three separate experiments for the PKC-phosphorylated β_2AR. An asterisk denotes statistical significance from control (unphosphorylated) values: one asterisk, $p < 0.01$; two asterisks, $p < 0.001$.

The diluted samples were centrifuged (350000g for 40 min), and the resultant pellets were resuspended in 300 μL of 100 mM NaCl/10 mM Tris-HCl, pH 7.2. Samples were incubated with G_s and, where appropriate, arrestin before being assayed for isoproterenol-stimulated GTPase activity.

GTPase Assay. Fifty microliters of vesicles containing 50 fmol of β_2AR, 25 fmol of G_s, and, where appropriate, between 0 and 25 pmol of arrestin was incubated in a total volume of 100 μL containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 2 μg of bovine serum albumin/mL, 0.5 mM ascorbic acid, 0.2 mM App(NH)p, 0.2 μM [γ-^{32}P]GTP, and either 50 μM (−)-isoproterenol or 50 μM (±)-alprenolol. $MgCl_2$ was included in the assay such that the free Mg^{2+} concentration, as calculated by the method of Iyengar and Birnbaumer (1982), was as indicated in the text. After incubation at 30 °C for 20 min, reactions were stopped by addition of 20 μL of 100 mM NaH_2PO_4 (pH 2.0) and 500 μL of a 5% solution of Norit-A decolorizing charcoal. Following centrifugation the inorganic [^{32}P]phosphate in the supernatant was determined by extraction with molybdate/2-methyl-1-propranolol/toluene (Cerione et al., 1984). β_2AR-stimulated GTPase activity was calculated as the difference between the activity in the presence of isoproterenol and that in the presence of alprenolol and generally represented a ~2.5-fold stimulation by agonist.

Results

Reconstituted β_2AR was phosphorylated with either βARK, the catalytic subunit of PKA, or PKC, and the ability of the phosphorylated receptors to couple to G_s was subsequently compared to unphosphorylated, control, β_2AR. When GTPase assays were performed at a low concentration of free Mg^{2+} (0.3 mM), a pronounced and statistically significant inhibition of receptor/G_s coupling was observed on phosphorylation of β_2AR by any of the three kinases (Figure 1). The inhibition of coupling for the PKA-, PKC-, and βARK-phosphorylated receptor, as compared to unphosphorylated (control) β_2AR, was ~61% ($p < 0.001$), ~38% ($p < 0.01$), and ~33% ($p < 0.01$), respectively. This phosphorylation-induced inhibition

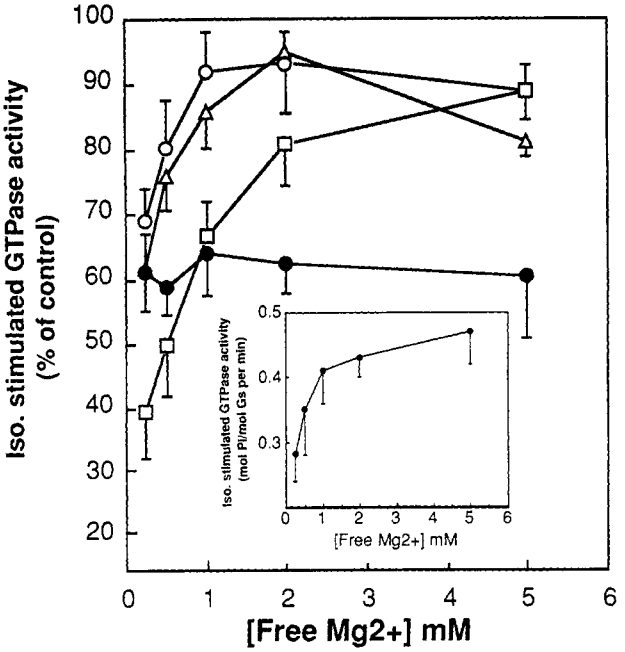

FIGURE 2: Effects of β_2AR phosphorylation and the concentration of free Mg^{2+} on inhibition of receptor/G_s coupling. Phospholipid vesicles containing purified β_2AR were phosphorylated with PKA (□, $n = 4$), PKC (△, $n = 3$), or βARK (○, $n = 4$). The stoichiometries of phosphorylation are given under Experimental Procedures. Control [unphosphorylated ($n = 5$)] and phosphorylated β_2AR preparations were subsequently assayed for isoproterenol-stimulated GTPase activity, at various concentrations of free Mg^{2+}. GTPase assays of βARK-phosphorylated β_2AR were additionally performed after incubation with retinal arrestin (arrestin:G_s molar ratio, 200:1) and purified G_s for 15 min at 4 °C (●, $n = 3$). The isoproterenol-stimulated GTPase activity of the unphosphorylated (control) β_2AR [mol of P_i (mol of G_s)$^{-1}$ min^{-1}] is shown in the inset. The activity of the phosphorylated receptors is expressed as a percentage of this control activity. The results are means ± SEM for the number of experiments given in parentheses.

of coupling could, however, be largely overcome by increasing the free concentration of Mg^{2+} (open symbols, Figure 2). At 5 mM Mg^{2+}, the coupling efficiency of the phosphorylated as compared to the unphosphorylated β_2AR was impaired by only 15–20%.

Retinal arrestin has been shown, in the presence of high concentrations of Mg^{2+}, to potentiate βARK-mediated β_2AR/G_s uncoupling (Benovic et al., 1987a). Figure 2, in addition to showing the inhibition of β_2AR/G_s coupling observed upon receptor phosphorylation by the various protein kinases (open symbols), also shows the ability of arrestin to potentiate βARK-mediated β_2AR/G_s uncoupling at various concentrations of Mg^{2+} (filled circles). In marked contrast to receptor phosphorylation, which has a more pronounced inhibitory effect at low Mg^{2+} concentrations, the inhibition due to βARK phosphorylation of β_2AR in the presence of arrestin was independent of Mg^{2+} concentration. When incubated in the presence of 50 nM arrestin (arrestin:G_s molar ratio, 200:1), the mean receptor-promoted GTPase activity for βARK-phosphorylated β_2AR was 61.5 ± 9.5% of control values. While arrestin did not potentiate desensitization of βARK-phosphorylated receptor at the lowest Mg^{2+} concentration tested (0.3 mM), arrestin addition did significantly enhance the inhibition of receptor/G_s coupling observed upon βARK phosphorylation when assayed at all concentrations of Mg^{2+} tested between 0.5 and 5.0 mM ($p < 0.01$ at Mg^{2+} concentrations between 0.5 and 5 mM).

To determine if protein cofactors similar to arrestin play a role in β_2AR desensitization mediated by PKA and PKC,

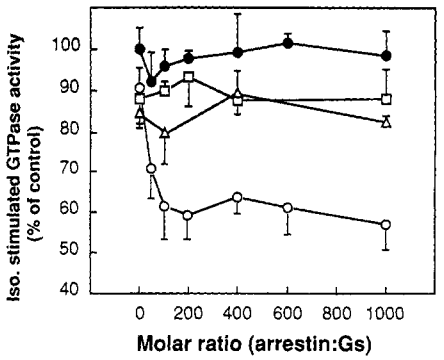

FIGURE 3: Inhibition of β_2AR/G_s coupling by retinal arrestin. Phospholipid vesicles containing unphosphorylated (control) receptor (●, $n = 4$) or β_2AR phosphorylated with PKA (□, $n = 4$), PKC (△, $n = 3$), or βARK (○, $n = 4$) were incubated with retinal arrestin and purified G_s for 15 min at 4 °C, prior to assay of isoproterenol-stimulated GTPase activity. Each assay contained 50 fmol of β_2AR, 25 fmol of G_s, 5 mM Mg^{2+}, and between 0 and 25 pmol of arrestin, and was performed as described under Experimental Procedures. The results are expressed as a percentage of the control (unphosphorylated) β_2AR activity which for these experiments was 0.38 ± 0.07 ($n = 4$) mol of P_i (mol of G_s)$^{-1}$ min^{-1}. The results are the means ● SEM for the number of experiments given in parentheses.

the receptor-stimulated G_s-associated GTPase activity of β_2AR phosphorylated with either of these kinases was assayed under conditions in which arrestin had a maximal inhibitory effect on βARK-phosphorylated β_2AR/G_s coupling, i.e., in the presence of high concentrations (5 mM) of Mg^{2+} (see Figure 2). Addition of arrestin to the reconstituted system had no effect on receptor function under nonphosphorylating conditions (Figure 3). The mean activity of the agonist-stimulated G_s-associated GTPase in the presence of varying concentrations of arrestin was 98 ± 6% ($n = 28$) of the value in the absence of arrestin. Similarly, addition of arrestin to the reconstituted system containing β_2AR phosphorylated with either PKA or PKC did not affect receptor/G_s coupling. In contrast, addition of arrestin leads to a progressive reduction in receptor/G_s interaction for βARK-phosphorylated β_2AR. At high arrestin concentrations (arrestin:G_s molar ratio, >100:1), only 61 ± 8.0% ($n = 20$) of the control activity is observed ($p < 0.001$). Thus, of the three kinases utilized in this study, only βARK-mediated desensitization of the receptor can be potentiated by arrestin.

Discussion

The results presented in this study serve to demonstrate the marked effect phosphorylation of β_2AR plays in the attenuation of receptor function, and consequently the important role this covalent modification of the receptor may have in the process of desensitization. Furthermore, these results suggest that distinct mechanisms of desensitization exist for PKA- or PKC-phosphorylated receptor as compared to βARK-phosphorylated receptor. At submillimolar concentrations of Mg^{2+}, concentrations which are likely to be physiological, PKA-phosphorylated β_2ARs were uncoupled by approximately 60% and βARK-phosphorylated β_2ARs by approximately 30%, significantly larger effects than those previously reported (Benovic et al., 1985; Lohse et al., 1990). Furthermore, PKC phosphorylation of β_2AR, the functional effects of which have not been previously reported, uncouples the receptor by approximately 40% under these conditions. Phosphorylation of β_2AR by any of these three kinases would thus be expected to cause significant desensitization in vivo.

Increasing the concentration of Mg^{2+} largely obscures the uncoupling effect of receptor phosphorylation (Figure 2).

Similar observations have previously been reported using membranes prepared from cells treated with low concentrations of agonists (~10 nM) (Johnson et al., 1990; Hausdorff et al., 1989). Under these conditions, in which desensitization is believed to be chiefly PKA-mediated, submillimolar concentrations of Mg^{2+} are required to observe desensitization of the β_2AR-stimulated adenylyl cyclase response. The results of the current study indicate that Mg^{2+}, at least in part, must exert its effects at the level of the receptor/G_s interaction, facilitating coupling. Nanomolar concentrations of Mg^{2+} are required for G_s and G_i GTPase activities (Sunyer et al., 1984; Brandt & Ross, 1986); however, evidence for a second lower affinity Mg^{2+} binding site which could potentially play a role in enhancing receptor/G_s coupling also exists. High concentrations of Mg^{2+} (5–100 mM) stimulate the rate of binding of GTPγS to both G_s and G_i (Higashijima et al., 1987a) and dramatically increase the rate of GTP hydrolysis by purified G_o (Higashijima et al., 1987b). Furthermore, high Mg^{2+} concentrations (~10 mM) have also been implicated in inducing a conformational change in both G_s and G_i (Codina et al., 1984b).

It is interesting to note that there are two potential sites at which either PKA or PKC could phosphorylate the β_2-adrenergic receptor, Ser-262 and Ser-347 located in the third cytoplasmic loop and the COOH-terminal tail of the receptor, respectively (Dixon et al., 1986). The functional consequences of phosphorylating the same residues with different kinases would be expected to be identical. The fact that PKC-mediated phosphorylation of β_2AR inhibited coupling to G_s to a lesser extent than PKA phosphorylation of the receptor may simply reflect the lower stoichiometry of phosphorylation achieved in these experiments (see Experimental Procedures).

In the present study, retinal arrestin was utilized to probe the generality of the involvement of protein cofactors in desensitization. Retinal arrestin rather than the apparently more specific β-arrestin was used because of the current unavailability of the latter protein in sufficient quantities. We found that addition of retinal arrestin has no effect on the G_s coupling of unphosphorylated or PKA- or PKC-phosphorylated β_2AR but does potentiate the inhibition of receptor/G_s coupling observed upon βARK-mediated phosphorylation in the presence of a wide range of Mg^{2+} concentrations (0.5–5.0 mM, Figure 2). Arrestin did not significantly potentiate βARK-mediated desensitization at 0.3 mM Mg^{2+} (the lowest concentration of free Mg^{2+} utilized in this study, Figure 2). However, since estimates for the physiological concentration of Mg^{2+} vary widely [0.1–0.6 mM (Gupta & Moore, 1980; Grubbs et al., 1984)], an in vivo role for protein cofactors, such as β-arrestin, in the potentiation of βARK-mediated desensitization cannot be ruled out. In this context, it is important to note that the molar ratios of arrestin/G_s (>100:1) required for inactivation of phosphorylated β_2AR are significantly higher than the arrestin:transducin molar ratio (<10:1) required for comparable effects on phosphorylated rhodopsin (Wilden et al., 1986a). Moreover, recent studies with partially purified β-arrestin suggest that it may have a much higher affinity for phosphorylated receptor than does retinal arrestin (Lohse et al., 1990). β-Arrestin might thus have a more profound effect than retinal arrestin at inhibiting the βARK-phosphorylated β_2AR/G_s coupling under physiological conditions.

The sites for PKA and PKC phosphorylation on the β_2AR are the same (Ser-262 and -347) and are located in close proximity to regions of the third cytoplasmic loop and proximal carboxyl terminus known to be involved in G-protein coupling (O'Dowd et al., 1988). Since these regions contain a number of charged residues, and may form amphipathic helices, the phosphorylation may alter coupling by altering charge distribution in these regions and thus the ability to couple to the G proteins. In contrast, the phosphorylation sites for βARK are concentrated at the distal part of the carboxy-terminal cytoplasmic tail of the receptor, well removed from these G-protein coupling regions. Accordingly, a functional role for β-arrestin in physically uncoupling the receptor may be required. β-Arrestin may also act in vivo to maintain the desensitized (phosphorylated) state of the receptor. Palczewski et al. (1989) have reported that binding of arrestin to phosphorylated rhodopsin severely inhibits the rate of dephosphorylation of rhodopsin. The inhibition of the phosphatase presumably reflects steric protection of the rhodopsin kinase phosphorylation sites on rhodopsin by arrestin since arrestin does not inhibit the dephosphorylation of model substrates to which it does not bind.

In conclusion, β_2AR phosphorylation by PKA, PKC, or βARK has been shown to have a significantly desensitizing effect at low, apparently physiological concentrations of Mg^{2+}. Furthermore, arrestin which specifically interacts only with βAR kinase phosphorylated receptor potentiates the inhibition of receptor/G_s coupling over a wide range of Mg^{2+} concentrations. These data indicate that inhibition of β_2-adrenergic receptor function by phosphorylation occurs via two distinct mechanisms: (1) a PKA- or PKC-mediated pathway that does not seem to utilize a cofactor and which can be masked by high Mg^{2+} concentrations; (2) a βARK-mediated pathway that utilizes an arrestin-like molecule as cofactor. Whether β-arrestin in vivo plays a role in functionally uncoupling the receptor, acts to maintain its phosphorylation state, or both remains to be resolved.

Acknowledgments

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Registry No. βARK, 102925-39-3; Mg, 7439-95-4; protein kinase, 9026-43-1.

References

Bechtel, P. J., Beavo, J. A., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 2691–2697.

Benovic, J. L., Shorr, R. G. L., Caron, M. G., & Lefkowitz, R. J. (1984) *Biochemistry 23*, 4510–4518.

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1985) *J. Biol. Chem. 260*, 7094–7101.

Benovic, J. L., Strasser, R. H., Caron, M. G., & Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2797–2801.

Benovic, J. L., Kuhn, M., Weyand, I., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8879–8882.

Benovic, J. L., Mayor, F., Jr., Staniszewski, C., Lefkowitz, R. J., & Caron, M. G. (1987b) *J. Biol. Chem. 262*, 9026–9032.

Benovic, J. L., Bouvier, M., Caron, M. G., & Lefkowitz, R. J. (1988) *Annu. Rev. Cell Biol. 4*, 405–428.

Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987) *J. Biol. Chem. 262*, 3106–3113.

Brandt, D. R., & Ross, E. M. (1986) *J. Biol. Chem. 261*, 1656–1664.

Cerione, R. A., Strulovici, B., Benovic, J. L., Lefkowitz, R. J., & Caron, M. G. (1983) *Nature (London) 306*, 562-566.
Cerione, R. A., Codina, J., Benovic, J. L., Lefkowitz, R. J., Birnbaumer, L., & Caron, M. G. (1984) *Biochemistry 23*, 4519-4525.
Clark, R. B., Friedman, J., Johnson, J. A., & Kunkel, M. W. (1987) *FASEB J. 1*, 289-297.
Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, R., Iyengar, R., & Birnbaumer, L. (1984a) *J. Biol. Chem. 259*, 5871-5886.
Codina, J., Hildebrandt, J. D., & Birnbaumer, L. (1984b) *J. Biol. Chem. 259*, 11408-11418.
Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J., & Strader, C. D. (1986) *Nature (London) 321*, 75-79.
Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615-624.
Grubbs, R. D., Collins, S. D., & Maguire, M. E. (1984) *J. Biol. Chem. 259*, 12184-12192.
Gupta, R. K., & Moore, R. D. (1980) *J. Biol. Chem. 255*, 3987-3993.
Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., & Lefkowitz, R. J. (1989) *J. Biol. Chem. 264*, 12657-12665.
Higashijima, T., Ferguson, K. M., Sternweiss, P. C., Ross, E. M., Smigel, M. D., & Gilman, A. G. (1987a) *J. Biol. Chem. 262*, 752-756.
Higashijima, T., Ferguson, K. M., Sternweiss, P. C., Smigel, M. D., & Gilman, A. G. (1987b) *J. Biol. Chem. 262*, 762-766.
Iyengar, R., & Birnbaumer, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5179-5183.
Johnson, J. A., Goka, T. J., & Clarke, R. B. (1986) *J. Cyclic Nucleotide Protein Phosphorylation Res. 11(3)*, 199-215.
Johnson, J. A., Clark, R. B., Friedman, J., Dixon, R. A. F., & Strader, C. D. (1990) *Mol. Pharmacol. 38*, 289-293.
Kunkel, M. W., Friedman, J., Shenolikar, S., & Clark, R. B. (1989) *FASEB J. 3*, 2067-2074.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1990) *Science 248*, 1547-1550.
Nambi, P., Peters, J. R., Sibley, D. R., & Lefkowitz, R. J. (1985) *J. Biol. Chem. 260*, 2165-2171.
O'Dowd, B. F., Hnatowich, M., Regan, J., Leader, M. W., & Lefkowitz, R. J. (1988) *J. Biol. Chem. 263*, 15985-15992.
Palczewski, K., McDowell, J. H., Jakes, S., Ingebritsen, T. S., & Hargrave, P. A. (1989) *J. Biol. Chem. 264*, 15770-15773.
Scott, J. D., Fischer, E. M., Demaille, J. G., & Krebs, E. G. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4379-4383.
Scott, J. D., Fischer, E. M., Takio, D., Demaille, J. G., & Krebs, E. G. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5732-5736.
Sunyer, T., Codina, J., & Birnbaumer, L. (1984) *J. Biol. Chem. 259*, 15447-15451.
Wilden, U., Hall, S. W., & Kuhn, M. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174-1178.
Wilden, U., Wust, E., Weyand, I., & Kuhn, M. (1986b) *FEBS Lett. 207*, 292-295.

The DNA-Binding Domain of the Cys-3 Regulatory Protein of *Neurospora crassa* Is Bipartite†

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ABSTRACT: *Cys-3*, the major sulfur regulatory gene of *Neurospora crassa*, encodes a regulatory protein that is capable of sequence-specific interaction with DNA. The interaction is mediated by a region within the CYS3 protein (the bzip region) which contains a potential dimer-forming surface, the leucine zipper, and an adjacent basic DNA contact region, NH_2-terminal to the leucine zipper. To investigate the bipartite nature of the bzip region, a series of *cys-3* mutants obtained by oligonucleotide-directed mutagenesis were expressed and tested for dimer formation as well as DNA binding and in vivo function. The results demonstrate that CYS3 protein exists as a dimer in the presence and absence of the target DNA and that dimerization of CYS3 is mediated strictly by the leucine zipper, which is required for both *cys-3* function in vivo and DNA-binding activity in vitro. Furthermore, a truncated CYS3 protein corresponding to just the bzip region was found to mediate dimer formation and to possess DNA-binding activity. A CYS3 mutant protein with a pure methionine zipper showed significant, although reduced, function in vivo and in vitro.

Cys-3, the major sulfur regulatory gene of *Neurospora crassa*, controls the expression of a set of unlinked structural genes which encode sulfur catabolic enzymes (Fu et al., 1989; Burton & Metzenberg, 1971; Marzluf & Metzenberg, 1968). Detailed studies of *cys-3* mutants, including null and ts mutants, suggest that the *cys-3* gene specifies a regulatory protein that is required for expression of the sulfur-related enzymes, presumably by binding DNA element(s) upstream of each structural gene (Fu et al., 1990; Paietta et al., 1987). DNA footprinting and mobility shift analysis showed that an *Escherichia coli* expressed CYS3 protein binds specifically to 5′-upstream DNA sequences of *cys-14* (sulfate permease gene) and the *cys-3* gene itself (Fu et al., 1989). *Cys-3* encodes a protein of 236 amino acid residues which shows considerable

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