The \( \beta \)-adrenergic receptor kinase (\( \beta \)ARK) specifically phosphorylates the activated form of multiple receptors such as the \( \beta \)-adrenergic receptor (\( \beta \)AR) and rhodopsin. \( \beta \)ARK also phosphorylates synthetic peptides, albeit with an \( \sim 10^{-3} \) - fold lower \( V_{\text{max}}/K_m \) ratio as compared to receptors, with a clear preference for peptides containing acidic residues on the ammino-terminal side of a serine or threonine. To further characterize the mechanism of substrate phosphorylation by \( \beta \)ARK, we designed a series of analogue peptides containing a single amino acid change (serine, glutamic acid, or phosphoserine) situated 2 or 4 residues amino-terminal to the target serine. While \( \beta \)ARK weakly phosphorylated peptides lacking an acidic residue, peptides containing either a single phosphoserine or glutamic acid were substantially better substrates with a 3.5- to 8-fold increase in \( V_{\text{max}} \). Additional studies demonstrated that the interaction of \( \beta \)ARK with an activated receptor (\( \beta \)AR\(^*\) or Rho\(^*\)) also significantly enhanced peptide phosphorylation. Both Rho\(^*\) and a truncated rhodopsin lacking its carboxyl-terminal phosphorylation sites activated peptide phosphorylation to a similar extent with \( EC_{50} \) values for activation of 0.65 and 1.34 \( \mu \)M, respectively. In contrast, the agonist-occupied \( \beta \)AR activated peptide phosphorylation by \( \beta \)ARK with a substantially higher affinity (\( EC_{50} \) of 0.012 \( \mu \)M) compared to Rho\(^*\). The \( V_{\text{max}}/K_m \) ratio for \( \beta \)ARK phosphorylation of a poor peptide substrate such as RRASAAASAA was increased up to \( \sim 200 \)-fold by the activated receptor while the phosphorylation of a good peptide substrate (RRREEEEEEASAA) was increased only up to \( \sim 8 \)-fold. Our results suggest that acidic residues (glutamic acid or phosphoserine) localized on the amino-terminal side of target serines are important but not essential determinants in directing peptide phosphorylation. The substrate specificity of \( \beta \)ARK appears to rely more strongly on the overall topological structure of the activated receptor which promotes the specific binding and activation of \( \beta \)ARK.

The \( \beta \)-adrenergic receptor kinase (EC 2.7.1.126) catalyzes the agonist-dependent phosphorylation of the \( \beta \)AR, a critical step in regulating rapid agonist-specific or homologous desensitization of the receptor (1-3). \( \beta \)ARK phosphorylates the agonist-occupied \( \beta \)AR on its Ser/Thr-rich carboxyl-terminal tail to a maximal stoichiometry of \( \sim 8 \) (4). Receptor phosphorylation by \( \beta \)ARK promotes partial uncoupling of the \( \beta \)AR from interaction with the G protein \( \text{G}_i \), and also enhances the binding of an additional protein, termed \( \beta \)-arrestin, that further uncouples the \( \beta \)AR and \( \text{G}_i \) (5, 6). Similar mechanisms for regulating receptor function are found in the visual system (7, 8). In this system, light activation of rhodopsin promotes activation of the G protein transducin which then activates a cGMP phosphodiesterase. However, light-activated rhodopsin also serves as a substrate for phosphorylation by the specific enzyme rhodopsin kinase (9, 10). This light-dependent phosphorylation promotes binding of retinal arrestin (also termed S-antigen or 48-kDa protein) which effectively uncouples rhodopsin and transducin (11).

While the substrate specificity of rhodopsin kinase (RK) appears predominantly limited to rhodopsin (10, 12), the specificity of \( \beta \)ARK is more poorly defined. \( \beta \)ARK has a broad tissue distribution which does not correlate with any one receptor class (4, 13, 14). In fact, \( \beta \)ARK may well have a role in regulating multiple receptors given its ability to phosphorylate the human platelet \( \alpha_\text{IIb} \)-adrenergic receptor (15) and chick heart muscarinic receptor (16) to high stoichiometries in an agonist-dependent fashion. In addition, several studies have demonstrated that agonist activation of either the \( \beta \)AR, the prostaglandin \( \text{E}_2 \) receptor, or the somatostatin receptor promotes a decrease in the soluble and an increase in the membrane-associated \( \beta \)ARK activity (17, 18). Recent results suggest that this membrane association may involve the interaction of \( \beta \)ARK with G protein \( \beta \gamma \) subunits, a potential mechanism for further refining the substrate specificity of \( \beta \)ARK (19). Additional members of the G protein-coupled receptor kinase family have also recently been identified including bovine \( \beta \)ARK2 (20) and Drosophila GPRK1 and GPRK2 (21). Thus, this novel kinase family may well play a role in phosphorylating and regulating a wide variety of G protein-coupled receptors.

The mechanism by which rhodopsin kinase and \( \beta \)ARK specifically recognize and phosphorylate an activated receptor remains poorly understood. However, recent studies have demonstrated that both kinases have a preference for phosphorylating serines or threonines localized in an acidic environment (22). \( \beta \)ARK prefers acidic residues on the amino-
terminal side of a serine while RK has a preference for carboxyl-terminal acidic residues. The ability of these kinases to interact with multiple domains of their respective receptors has also been suggested. Studies have demonstrated that synthetic peptides from intracellular domains of the hamster βAR, predominantly the first and third intracellular loops, have the ability to inhibit βARK phosphorylation of the βAR (23). Moreover, two studies have demonstrated that rhodopsin kinase is directly activated by its specific binding to light-activated rhodopsin (24, 25). This activation is enhanced when the carboxyl-terminal sites of rhodopsin phosphorylation are proteolytically removed and inhibited when the third intracellular loop of rhodopsin is proteolyzed (25). In the present study, we have used various synthetic peptides to further define the amino acid specificity of βARK, in particular addressing whether the introduction of an amino-terminal phosphoserine enhances phosphorylation efficiency. Additional studies demonstrated that peptide phosphorylation by βARK is specifically and significantly activated by light-activated and either the agonist-occupied or light-activated rhodopsin. These results demonstrate that the overall topological structure of the activated receptor plays a key role in regulating signal-dependent receptor phosphorylation by βARK.

EXPERIMENTAL PROCEDURES

Materials—The chromatography resins S-Sepharose, heparin-Sepharose, and Mono S were from Pharmacia, while frozen bovine retinas were from George A. Hormel and Co. Kemptide was from Sigma, and purified bovine cAMP-dependent protein kinase was from Promega. [γ-32P]ATP was from Du Pont-Neenland England Nuclear, and endopeptidase Asp-N was from Boehringer Mannheim. All other reagents were from the best grade available.

Preparation and Assay of BARK—βARK was overexpressed and purified from infected Sf9 cells using the baculovirus expression system as described. Briefly, Sf9 cells were harvested 48 h postinfection by low speed centrifugation. The cells were washed and then homogenized in 20 mM Hepes, pH 7.2, 250 mM NaCl, 5 mM EDTA, 3 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine (50 ml of buffer per liter of cells). A high speed supernatant fraction was then diluted and loaded onto an S-Sepharose column which was then eluted with a linear gradient from 100 to 600 mM NaCl. For these studies, the βARK-containing fractions were pooled, diluted, and loaded onto a heparin-Sepharose column which was then eluted with a linear gradient from 100 to 600 mM NaCl. For these studies, the βARK-containing fractions were pooled and dialyzed against buffer A (20 mM Hepes, pH 7.4, 2 mM EDTA, 0.02% Triton X-100, 10% glycerol). The dialyzed enzyme was then further purified to >99% homogeneity by HPLC on a Mono S column using a 20-ml linear gradient from 0 to 0.5 M NaCl in buffer A. Purified βARK was routinely assayed using a mixture containing 20 mM Tris, pH 7.4, 2 mM EDTA, 7 mM MgCl2, 0.1 mM [γ-32P]ATP (300 to 1000 cpm/pmol), and 6 μM rhodopsin (urea-treated rod outer segments). Following incubation at 30 °C, reactions were quenched by the addition of an equal volume of sample buffer (8% sodium dodecyl sulfate (SDS), 25 mM Tris-HCl, pH 6.5, 10% glycerol, 5% mercaptoethanol, 0.003% bromphenol blue) followed by electrophoresis on a 10% SDS-polyacrylamide gel (27). After autoradiography, the 32P-labeled rhodopsin bands were excised and counted to determine phosphate incorporation. Protein concentrations were determined by the method of Bradford (28) using bovine serum albumin as standard. The final specific activity of the βARK preparation used in these studies was ~1 μmol/min/mg.

Preparation of Urea-treated Rod Outer Segments—Rod outer segments (ROS) were prepared from bovine retina as described (9, 29). All procedures were carried out either in the dark or under dim red light. Briefly, 50 bovine retinas were first suspended in 50 ml of 34% sucrose (w/v), 65 mM NaCl, 2 mM MgCl2, and 10 mM Tris acetate, pH 7.4. The mixture was shaken vigorously and centrifuged at 4,000 rpm for 4 min. The supernatant was diluted 3-fold with 10 mM Tris acetate, pH 7.4, and centrifuged for 20 min at 40,000 × g. The crude ROS pellets were resuspended in 20 ml of 0.77 mM sucrose, 10 mM Tris acetate, pH 7.4, and 1 mM MgCl2 (buffer B), Dounce-homogenized, drawn through a 20.5-gauge needle, and then purified on a stepwise sucrose gradient. The interface between 0.84 and 1.0 M sucrose was recovered, diluted with 25 ml of buffer B, and centrifuged for 30 min at 40,000 × g. The peak ROS were resuspended in 10 ml of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM urea, and sonicated on ice for 4 min. The ROS were diluted 6-fold with 50 mM Tris-HCl, pH 7.4, and centrifuged at 30,000 rpm for 45 min, and the pellet was then washed three times with 50 mM Tris and finally resuspended in 50 mM Tris, pH 7.4, in a final volume of 200 μl. The ROS showed negligible rhodopsin kinase activity and consisted of >90% rhodopsin as assessed by Coomassie Blue staining. The concentration of rhodopsin was determined by both protein assay and using the molar extinction coefficient of 40,000 at 438 nm in the presence of digitonin (30).

Preparation of [32P]-G-Rho—A truncated form of rhodopsin with 19 amino acid residues proteolytically removed from the carboxy terminus (32P-G-Rho) was made as previously described (25). Briefly, urea-treated ROS were incubated with Endopeptidase Asp-N in a ratio of 750C1 (w/w; rhodopsin/Endo Asp-N) in 10 mM Tris-HCl, pH 7.5, in the dark at 22 °C for 16 h. The reaction was quenched by the addition of 1 mM diethiothreitol and 1 mM EDTA, and the ROS membranes were recovered by centrifugation at 40,000 × g for 10 min. The ROS were resuspended in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM urea, sonicated, pelleted by centrifugation, washed three times with 50 mM Tris, and finally resuspended in 50 mM Tris, pH 7.4, 5 mM EDTA. Complete digestion to [32P]-G-Rho was validated by the presence of a single protein band of 38 kDa on a 12% SDS-polyacrylamide gel that was not phosphorylated by βARK (data not shown).

Preparation of Human βAR—The human β-adrenergic receptor was expressed using the baculovirus expression system and purified by affinity chromatography on an alpencolin Sepharose column as described (31). For phosphorylation studies, the purified receptor was reconstituted into phosphatidylcholine vesicles, pelleted by centrifugation, and resuspended in 20 mM Tris-HCl, pH 7.2, 2 mM EDTA (4). The concentration of receptor was determined using the β-adrenergic [125I]iodopindolol binding assay available commercially.

Preparation of Synthetic Peptides—Peptides were synthesized on an Applied Biosystems 430A synthesizer using t-butoxycarbonyl chemistry. Peptides were purified by HPLC on a Dynamax C-18 reverse phase column using a 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid. Phosphoserine-containing peptides were made in a 3:1 reaction mixture containing 20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 6 mM peptide (RRRASASA or RRRRASASAA), 10 mM MgCl2, 6 mM [γ-32P]ATP (1 cpn/pmol), and 7 μg of the cAMP-dependent protein kinase catalytic subunit. After a 6-h incubation at room temperature, 32P incorporation was determined by spotting 10-μl aliquots onto P81 paper followed by six washes with 75 mM phosphoric acid as described (32). The phosphoserine-containing peptides were purified by HPLC on a Mono S column using a linear gradient from 0.1 to 1.0 M ammonium acetate. This step effectively separated the more acidic phosphorylated peptide from the nonphosphorylated peptide. The concentration of phosphoserine-containing peptide was determined using the specific activity of the [γ-32P]ATP.

Peptide Assay—All peptide stock solutions were adjusted to pH 7.0 using Tris base (typically 50 mM Tris). Assays were routinely carried out by incubating the peptides (0 to 8 mM) with βARK at 30 °C in a mixture of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 7.5 mM MgCl2, and 0.1 mM [γ-32P]ATP (200–4000 cpm/pmol) in the presence or absence of rhodopsin or the βAR as indicated. Reactions were quenched by addition of 15% trichloroacetic acid, and denatured protein was removed by centrifugation for 10 min at 40,000 × g. Supernatant was spotted onto P81 paper and washed six times in 75 mM phosphoric acid. Peptide phosphorylation in the absence of kinase was found to be negligible. When receptor activation studies were performed, control incubations were carried out in the absence of peptide, and these values (typically <10% of the total phosphate incorporated) were subtracted from the actual values.

Data Analysis—Kinetic data were analyzed with the BASIC version of the SEQUEN program using the equation Δv = Vmax S/(K + S) (33). Activation data were analyzed using the equation Δv = v1 + [KA/(KA + v2)](v1 - v0). This equation is based on the assumption that E-A complex formation is rate-limiting. E and K represent the enzyme and enzyme-receptor complex; KA represents the dissociation constant of the E-A complex; v represents the apparent rate at each concentration of the activated receptor A, and v1 and v2 represent the rate of βARK phosphorylation at zero and 2 C. M. Kim, S. Dion, J. J. Onorato, and J. L. Benovic, submitted for publication.
infinite concentrations of A, respectively. Although some inhibition was observed at the higher concentrations of βARK and Rho', we only analyzed the activation observed at the lower receptor concentrations where the inhibition was considered to be negligible. The EC₅₀ for activation is reported as the concentration of receptor that gives half-maximal activation of peptide phosphorylation. All experiments were carried out in duplicate and repeated 3 to 9 times.

**RESULTS**

**Peptide Substrate Phosphorylation by βARK**—The agonist-dependent phosphorylation of the serine-threonine-rich C-terminal tail of the β₂AR by βARK plays an important role in rapid homologous desensitization (1–3). However, the mechanism by which βARK recognizes the substrate receptor remains poorly understood. Previous studies have demonstrated that βARK preferentially phosphorylates peptides containing acidic residues on the amino-terminal side of a serine or threonine (22). In the present study, we attempted to further elucidate the importance of an acidic environment for βARK phosphorylation by assessing whether phosphoserines can substitute for acidic residues. For these studies, the peptide RRRASAAASAA was synthesized and initially used as a substrate for βARK. As expected, this peptide was found to be a very poor substrate for purified βARK1 (Fig. 1, open circles). A comparison of the Vₘₐₓ/Kₘ ratios demonstrates that this peptide is a 240-fold poorer substrate compared to the acid-rich peptide RRREEEESAAA (Table I). By contrast, RRREEEESAAA is itself a poor substrate for βARK compared to an activated receptor with a Vₘₐₓ/Kₘ ratio ~4000-fold lower than rhodopsin (22). Thus, βARK is only able to very weakly phosphorylate serine residues localized in a basic environment.

We next examined whether the presence of a phosphoserine residue enhanced the ability of RRRASAAASAA to serve as a substrate for βARK. Since this peptide contains the consens-sus sequence (RRXS) for phosphorylation by the cAMP-dependent protein kinase, it was incubated with protein kinase A to phosphorylate the first serine. The phosphoserine-containing peptide (RRRASpAAASAA), purified by HPLC, was found to be an improved substrate for βARK (Fig. 1, closed circles). Kinetic studies revealed that the addition of the phospho-group on the serine resulted in an ~8-fold increase in the Vₘₐₓ with no significant change in the Kₘ (Table I). Qualitatively similar results were obtained when the peptides RRRASAAASAA and RRRASpAAASAA were phosphorylated by βARK with a 3.5-fold increase in Vₘₐₓ being observed for the phosphoserine-containing peptide (Table I). Similar results were also observed when a glutamic acid was substituted for the serine with a predominant change in the Vₘₐₓ (3- to 22-fold) and no significant change in the Kₘ (Table I). Thus, these results suggest that individual amino-terminal acidic residues improve the catalytic efficiency for βARK phosphorylation but have very little effect on substrate binding. Although the kinetics for phosphorylation of RRRASAAASAA were not measurable due to its limited solubility, the overall differences between phosphoserine- and glutamic acid-containing peptides as substrates for βARK appear minimal. This suggests that phosphate incorporation into a serine residue might enhance the ability of βARK to phosphorylate carboxyl-terminal localized serine or threonine residues. A comparison of βARK phosphorylation of the single acid and five acid peptides showed both a significant decrease in Kₘ and increase in Vₘₐₓ for the more acidic peptide demonstrating that multiple amino-terminal acidic residues enhance both phosphorylation efficiency and substrate binding (Table I).

**Effect of Rhodopsin on Peptide Phosphorylation**—Previous studies on the phosphorylation of rhodopsin and the β₂AR by rhodopsin kinase (25) and βARK (23), respectively, have suggested that multiple receptor domains are involved in kinase interaction. A multisite interaction of receptor and kinase is also suggested by previous findings that peptide substrates have Kₘ values in the millimolar range, while receptor substrates have Kₘ values from 0.16 to 14.5 μM (4, 22–25, 34). Intracellular domains of the β₂AR and rhodopsin, including the carboxyl terminus and possibly the first and third intracellular loops, are thought to be involved in the specific binding of receptor and kinase (23, 25). Moreover, the interaction of rhodopsin kinase with rhodopsin has been demonstrated to stimulate the ability of the kinase to phosphorylate synthetic peptides (24, 25). Since the peptide RRRASAAASAA was found to be a poor substrate for βARK, we assessed whether a receptor could alter the ability of βARK to phosphorylate this peptide. As shown in Fig. 2, Rho was found to dramatically stimulate the phosphorylation of RRRASAAASAA by βARK. This light-dependent activation of βARK by rhodopsin was also observed when the peptide

**TABLE I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₘ (mM)</th>
<th>Vₘₐₓ (nmol/min/mg)</th>
<th>Vₘₐₓ/Kₘ (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRRREEEESAAA</td>
<td>9</td>
<td>0.72 ± 0.13</td>
<td>28.3 ± 2.96</td>
</tr>
<tr>
<td>RRRASAAASAA</td>
<td>3</td>
<td>3.3 ± 0.8</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>RRRASpAAASAA</td>
<td>3</td>
<td>4.6 ± 1.5</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>RRRREAASAA</td>
<td>3</td>
<td>12.2 ± 7.6</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>RRRASAA</td>
<td>3</td>
<td>5.4 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>RRRREAASAA</td>
<td>5</td>
<td>3.4 ± 0.9</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

*Sp = phosphoserine.

1 A Kₘ for this peptide could not be determined due to its limited solubility. The Vₘₐₓ value for this peptide was taken from Ref. 22.

**FIG. 1. Peptide phosphorylation by βARK.** The experiments were carried out with the peptides RRRASAAASAA (O) and RRRASpAAASAA (●). 1 mM peptide was incubated with 0.4–0.6 μM βARK in a mixture containing 20 mM Tris-Cl, pH 7.4, 2 mM EDTA, 7 mM MgCl₂, and 0.1 mM [γ³²P]ATP at 30 °C. At the indicated time, reactions were quenched and peptide phosphorylation was determined by spotting the sample on P81 paper as described under "Materials and Methods."
FIG. 2. Activation of βARK by light-activated rhodopsin. 1 mM RRRAsAAAAS was incubated with 0.34 μM βARK in a mixture containing 20 mM Tris-Cl, pH 7.4, 2 mM EDTA, 7 mM MgCl₂, and 0.1 mM [γ-32P]ATP at 30 °C. The experiment was carried out either in the absence of rhodopsin (O) or in the presence of 2 μM dark rhodopsin (●) or 2 μM light-activated rhodopsin (□). At the indicated times, reactions were quenched and peptide phosphorylation was determined by spotting the sample on P81 paper as described under "Materials and Methods."

FIG. 3. Comparison of the activation of βARK by light-activated rhodopsin (Rho') and truncated rhodopsin (329G-Rho'). 1 mM RRRAsAAAAS was incubated with 1 μM βARK in a mixture containing 20 mM Tris-Cl, pH 7.4, 2 mM EDTA, 7.5 mM MgCl₂, and 0.1 mM [γ-32P]ATP in the presence of various concentrations (0 to 13 μM) of light-activated Rho' (●) or light-activated 329G-Rho' (□) at 30 °C for 60 min. Reactions were quenched and peptide phosphorylation was determined by spotting the sample on P81 paper as described under "Materials and Methods."

TABLE II

<table>
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<tr>
<th>Substrate</th>
<th>n</th>
<th>Kᵣ</th>
<th>Vᵢn</th>
<th>Vᵢmax/Kᵣ</th>
<th>Vᵢmax/Kᵣ fold increase</th>
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<tbody>
<tr>
<td>RRRAsAAAAS</td>
<td>3</td>
<td>3.3</td>
<td>0.54</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>+Rho'</td>
<td>3</td>
<td>0.38</td>
<td>11.3</td>
<td>1.5</td>
<td>188</td>
</tr>
<tr>
<td>+329G-Rho'</td>
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<td>0.48</td>
<td>9.1</td>
<td>0.9</td>
<td>19</td>
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<tr>
<td>RRRREEEESAA</td>
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<td>0.72</td>
<td>28.3</td>
<td>2.3</td>
<td>39</td>
</tr>
<tr>
<td>+Rho'</td>
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<td>0.92</td>
<td>21.8</td>
<td>1.6</td>
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<tr>
<td>+329G-Rho'</td>
<td>6</td>
<td>0.10</td>
<td>32.6</td>
<td>1.1</td>
<td>326</td>
</tr>
</tbody>
</table>

Peptides at various concentrations (0.05 to 3 mM) were incubated with βARK in the absence or presence of 1.4 μM 329G-Rho' or 1.4 μM Rho' for 60 min at 30 °C as described in the text. The kinetic constants are expressed as the mean ± S.E.
190-fold increase in the $V_{max}/K_a$ ratio. In contrast, the activation of RRREEEESAAA phosphorylation by $^{32}$G-Rho resulted in only an 8.4-fold increase in the $V_{max}/K_a$ ratio.

**Effect of $\beta$AR on Peptide Phosphorylation**—Since activated rhodopsin proved to be a potent stimulator of peptide phosphorylation, we also studied the ability of the $\beta$AR, a physiological substrate for PARK, to activate the kinase. As shown in Fig. 4, purified reconstituted human $\beta$AR also effectively stimulated peptide phosphorylation by $\beta$ARK. This stimulation was totally dependent on the receptor being occupied by a $\beta$-agonist such as isoproterenol, since the presence of receptor alone had no effect on peptide phosphorylation.

Previous studies have demonstrated that $\beta$ARK phosphorylates the activated $\beta$AR and rhodopsin with a similar $V_{max}$ but with a 25- to 50-fold difference in their $K_a$ values ($\beta$AR $\sim 0.16-0.25 \mu M$; rhodopsin $\sim 6-14.5 \mu M$) (4, 23). Thus, we would predict that when compared to Rho, the $\beta$AR should stimulate $\beta$ARK to a similar extent but at substantially lower concentrations. A comparison of the ability of $\beta$AR and Rho to activate the phosphorylation of RRREEEESAAA by $\beta$ARK at low receptor concentrations (0-0.05 $\mu M$) is shown in Fig. 5. At 0.25 $\mu M$ $\beta$ARK, the $\beta$AR stimulates peptide phosphorylation 14 ± 2-fold with an EC$_{50}$ of 0.012 ± 0.001 $\mu M$, which is $\sim 50$-fold lower than the EC$_{50}$ for Rho (Fig. 3). At a concentration of 0.05 $\mu M$, Rho activates $\beta$ARK phosphorylation of the peptide only 2-fold. These differences in EC$_{50}$ values correlate very well with the $K_a$ differences for phosphorylation of these receptors by $\beta$ARK as noted above. The differences in the maximum activation induced by either Rho or $\beta$AR is likely due to the different ratios of receptor to $\beta$ARK being used. In the Rho activation studies, the ratio of [Rho]/[\betaARK] was much greater than 1 and, thus, most of the $\beta$ARK should be bound to Rho. In contrast, in the $\beta$AR studies, the ratio of [$\beta$AR]/[\betaARK] was <0.2. Thus, $<20\%$

**Mechanism of $\beta$ARK Activation**

1 mM RRREEEESAAA was incubated with 0.9 $\mu M$ $\beta$ARK in a mixture containing 20 mM Tris-Cl, pH 7.4, 2 mM EDTA, 7.5 mM MgCl$_2$, and 0.1 mM [$\gamma$-$\text{32}$P] ATP and either light-activated rhodopsin (0 to 0.05 $\mu M$) (O) or reconstituted human $\beta$AR (0 to 0.05 $\mu M$) plus 80 $\mu M$ (-)-isoproterenol (>). Following a 60-min incubation at 30 °C, the reactions were quenched and peptide phosphorylation was determined by spotting the sample on P81 paper as described under "Materials and Methods."”

**Role of Acidic Residues in Peptide Phosphorylation by $\beta$ARK**—Previous results have demonstrated the importance of acidic residues in the ability of $\beta$ARK and rhodopsin kinase to phosphorylate synthetic peptides (22). For $\beta$ARK, the acidic residues needed to be localized on the amino-terminal side of a serine or threonine residue while RKO had a preference for carboxy-terminal acidic residues (22). Consistent with these results, we found that a single acidic residue 2 or 4 amino acids amino-terminal to a serine significantly enhanced the ability of a peptide to be phosphorylated by $\beta$ARK. This enhanced phosphorylation appeared to be predominantly due to an increase in the $V_{max}$ (Table I). Our results also demonstrate that the incorporation of a phosphoserine residue 2 or 4 amino acids amino-terminal to a serine significantly enhances phosphorylation by $\beta$ARK, again due to an increase in the $V_{max}$ (Table I). The importance of acidic residues in substrate recognition has also been demonstrated for a number of other protein...
kinases, most notably casein kinases 1 and 2 (35–38) and glycogen synthase kinase 3 (26). Casein kinase 2 phosphorylates synthetic peptides containing a cluster of acidic residues with the most critical position being 3 residues carboxy-terminal to a serine or threonine (35, 36). Phosphoserines in position 3, but not phosphothreonines, appear to be able to replace the acidic residue (37). In contrast, casein kinase 1 preferentially phosphorylates a serine near an amino-terminal phosphoserine with the best consensus sequence being Sp/Tp-X-X-S/T (38). If aspartate or glutamate residues are substituted for the phosphoserine, the peptide does not serve as a substrate for casein kinase 1. Glycogen synthase kinase 3 also preferentially phosphorylates peptides containing a phosphoserine; however, this residue needs to be on the carboxy-terminal side of a serine with a consensus sequence of S-X-X-X-Sp (26). This recognition of phosphoserine by glycogen synthase kinase 3 plays an important role in the sequential phosphorylation of glycogen synthase by this kinase (28). Our results with βARK do not suggest an absolute role for a phosphoserine in substrate recognition such as appears to be the case for casein kinase 2 and glycogen synthase kinase 3. However, our results do demonstrate that the phosphorylation of a serine residue enhances the ability of βARK to phosphorylate additional residues on the carboxy-terminal side of the first phosphoserine, suggesting the possibility of sequential substrate phosphorylation.

**Role of Receptor Binding in βARK Activation**—Previous studies have demonstrated that rhodopsin kinase is directly activated by its specific binding to light-activated rhodopsin (24, 25). This activation is enhanced when the carboxy-terminal sites of rhodopsin phosphorylation are proteolytically removed and inhibited when the third intracellular loop of rhodopsin is proteolyzed (25). In the present study, we have found that both the agonist-occupied βAR and light-activated rhodopsin also significantly activate the ability of βARK to phosphorylate synthetic peptides. A comparison of βARK activation by these two receptors demonstrated that the βAR has an ∼50-fold higher affinity for βARK as compared to Rho. However, the maximum phosphorylation efficiency induced by either the βAR or Rho are similar, with both decreased K_m values and increased V_max values measured for various peptide substrates. Our results are consistent with previous kinetic studies which demonstrated that βARK phosphorylates the activated βAR and rhodopsin with a similar V_max but with a 25- to 50-fold difference in their K_m values (βARK ∼ 0.2 μM; rhodopsin 6–14 μM) (4, 29). Moreover, while our studies are consistent with βARK being activated by its specific receptor binding, it is clear that the intact receptor still serves as a much better substrate for βARK compared to synthetic peptides (23, this study). This is likely due to the fact that βARK binding to the βAR will not only activate the kinase but it will also increase the local concentration of the carboxy-terminal phosphorylation domain of the receptor. As previously proposed for rhodopsin kinase (25), this weak association of substrate peptides (e.g, the carboxy terminus of the βAR) with βARK may be important for the catalysis of multiple phosphorylations on the βAR.

One difference between our results and those of Palczewski et al. (25) is the ability of intact rhodopsin and truncated β>G-Rho to activate peptide phosphorylation. Our results demonstrate that both Rho' and β>G-Rho' are comparable at activating βARK phosphorylation of the peptide RRRA-SAAASAA, a poor peptide substrate for βARK. In contrast, β>G-Rho' was significantly more potent than Rho' at activating the phosphorylation of the peptide DEASTTTSVKTE-TSQVARRR by rhodopsin kinase (25). This difference may be due to the different peptide substrates utilized in the two studies. Indeed, when we used the peptide RRREEEESAAA, a good peptide substrate for βARK, intact Rho' inhibited peptide phosphorylation by βARK while β>G-Rho' promoted an ∼8-fold increase in the V_max/K_m ratio. Another potential rationale for these differences is that rhodopsin kinase may have a higher affinity for rhodopsin than does βARK. Thus, rhodopsin kinase phosphorylation of the carboxy-terminal sites on intact Rho' would more effectively compete for peptide phosphorylation as compared to βARK phosphorylation of these sites.

Overall, the similarities between our studies and those of Palczewski et al. (25) are quite striking. The binding of a kinase (RK or βARK) to an activated receptor (Rho' or βAR) dramatically activates (>100-fold) the ability of the kinase to phosphorylate a peptide substrate. This enhanced phosphorylation results from both a decreased K_m (5–8-fold) and an increased V_max (18–50-fold). Moreover, both studies demonstrated that a truncated form of rhodopsin (β>G-Rho') also potently activates peptide phosphorylation by RK and βARK. This suggests that these kinases interact with domains on the receptor in addition to the carboxy-terminal sites of phosphorylation. While the precise sites of kinase binding to an activated receptor have not been identified, the third intracellular loop appears to be a major domain. This is borne out by the fact that while β>G-Rho' is a potent activator of RK and βARK, forms of rhodopsin that have been proteolyzed in their third intracellular loop substantially lose their ability to activate RK (25). βARK also appears to interact with multiple intracellular domains of the βAR since synthetic peptides derived from the βAR sequence are able to inhibit the ability of βARK to phosphorylate the βAR (23). The most potent inhibitors include the first (ΔC_30 ~ 40 μM) and amino-terminal portion of the third (ΔC_7 ~ 75 μM) intracellular loops.

Since all enzymatic reactions are initiated by substrate binding, this represents one of the most critical steps in determining substrate specificity. The binding of both βARK and rhodopsin kinase to their respective substrates appears to depend predominantly on the topologic structure of the activated receptor rather than on the actual amino acid sequence surrounding the phosphorylation site. Moreover, since both βARK and rhodopsin kinase can be activated by their corresponding receptor substrates in a signal-dependent fashion, it is likely that this important property will also be shared by other G-protein-coupled receptor kinases. Thus, the ability of G protein-coupled receptor kinases to specifically bind to multiple domains of an activated receptor likely plays an important role in substrate recognition. Future studies in this area will aim to further elucidate the structural domains involved in the highly specific binding and phosphorylation of activated receptors by G protein-coupled receptor kinases.

**Acknowledgments**—We are very grateful to C. Sanders for her technical expertise and Drs. R. Sterne Marr and K. Palczewski for their helpful comments.

**References**

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