Visual arrestin modulates the intracellular response of retinal rod cells to light by specifically binding to the phosphorylated light-activated form of the photoreceptor rhodopsin (P-Rh*). In order to characterize the molecular interaction between rhodopsin and arrestin, we have studied the ability of synthetic peptides from the proposed cytoplasmic loops of rhodopsin to inhibit arrestin binding. A third cytoplasmic loop peptide competed most effectively for arrestin binding to P-Rh*, exhibiting an $K_{D}$ of $-100$ µM, while a first cytoplasmic loop peptide weakly inhibited binding with an $K_{D}$ of $-1100$ µM. The first and third cytoplasmic loop peptides also inhibited P-Rh* interaction with both ARR1(2-16:404), an arrestin mutant that lacks residues 2-16, and ARR1(1-1911, a mutant that contains only the amino half of arrestin. However, the third loop peptide had an ~5-fold lower affinity at inhibiting the binding of ARR1(1-1911) to P-Rh*. While the first and third loop peptides also inhibited arrestin binding to light-activated rhodopsin and a truncated rhodopsin lacking its C-terminal sites of phosphorylation, the peptides modestly enhanced arrestin binding to phosphorylated dark rhodopsin. These results suggest that the third and, to a lesser extent, the first cytoplasmic loops of rhodopsin may play an important role in arrestin binding to light-activated forms of rhodopsin.

Phototransduction in retinal rod cells has proven to be an invaluable model system for characterizing G protein-mediated signal transduction (1). Upon absorption of light, the photoreceptor rhodopsin undergoes a conformational change that allows it to interact with and activate the G protein transducin. Transducin, in turn, activates a cGMP phosphodiesterase, ultimately leading to the closing of plasma membrane cation channels and hyperpolarization of the membrane. Inactivation of this cascade at the receptor level is initiated by the light-dependent phosphorylation of rhodopsin by the specific enzyme rhodopsin kinase (2-4). The subsequent binding of visual arrestin to the phosphorylated light-activated form of rhodopsin (P-Rh*)1 completely inactivates the receptor and stops further signal transduction (5, 6). A similar mechanism of desensitization appears to exist in hormonal transduction mediated by the $\beta$-adrenergic receptor, where inactivation occurs through the binding of $\beta$-arrestin to the phosphorylated agonist-activated receptor (7-12).

Several lines of investigation have been utilized to characterize the molecular interactions between arrestin and G proteins. Synthetic peptides from the second, third, and putative fourth cytoplasmic loops of rhodopsin have been shown to compete with metarhodopsin II for binding to transducin, implicating these domains in rhodopsin/transducin interaction (13). A fourth cytoplasmic loop rhodopsin peptide has also been shown to elicit fluorescence changes in fluorescently labeled transducin $\beta$ subunits (14). Further support for the involvement of the fourth cytoplasmic loop in rhodopsin/transducin interaction comes from the observation that disruption of this loop by de-palmitoylation with hydroxylamine stimulates the rhodopsin-stimulated GTPase activity of transducin (15). Mutagenesis studies have also demonstrated that the palmitoylation of rhodopsin is not required for rhodopsin/transducin interaction (16). Critical residues involved in rhodopsin/transducin interaction have also been identified in the second and third cytoplasmic loops of rhodopsin by mutagenesis (17-19). Similar lines of investigation have implicated the involvement of the second, third, and fourth cytoplasmic loops of the $\beta_2$-adrenergic receptor in $G_{o}$ interaction (20-23). Taken together, these results implicate a multi-site interaction between G proteins and the various cytoplasmic domains of the receptors.

The molecular interactions between G protein-coupled receptors and G protein-coupled receptor kinases have also been investigated. Rhodopsin kinase interaction with the cytoplasmic loops of rhodopsin has been demonstrated by the observation that truncated rhodopsin, lacking the C-terminal sites of phosphorylation, stimulates the ability of the kinase to phosphorylate peptide substrates (24, 25). Similar results have been observed for peptide phosphorylation by the $\beta$-adrenergic receptor kinase (26). Synthetic peptides corresponding to the various cytoplasmic loops of the receptors have also been used to characterize receptor/receptor kinase interaction. Peptides from the second and third cytoplasmic loops and the C terminus of rhodopsin inhibit the ability of rhodopsin kinase to phosphorylate bleached rhodopsin (27). The third cytoplasmic loop of rhodopsin has also been implicated in rhodopsin kinase interaction through the use of proteolytically cleaved forms of rhodopsin (24). Analogous studies have demonstrated that peptides from the first, second, and third cytoplasmic loops of the $\beta_2$-adrenergic receptor inhibit $\beta$ARK phosphorylation of the stimulatory guanine nucleotide-binding protein; HPLC, high performance liquid chromatography; Rh*, light-activated rhodopsin; ROS, rod outer segments; $\beta_2$G-Rh*, truncated rhodopsin lacking its C-terminal 19 amino acids; ARR, arrestin.

3226
receptor (28). Taken together, these results suggest a multi-site interaction of the receptor kinases with the cytoplasmic domains of the receptors.

Since previous studies of rhodopsin/arrestin interaction have mainly focused on the arrestin domains (29–32), little is currently known about the receptor domains involved in arrestin interaction. In the present study, we have characterized the ability of synthetic peptides from the cytoplasmic domains of rhodopsin to inhibit [3H]arrestin binding to different forms of rhodopsin. In addition, the corresponding arrestin sites involved in these interactions with rhodopsin were localized.

These data suggest a multi-site interaction between arrestin and the cytoplasmic loops of rhodopsin.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes, endopeptidase Asp-N, and molecular biology reagents were from Boehringer Mannheim. [γ-32P]ATP, [32]PdATP, and [3H]thymidine were from DuPont NEN, and RNAs was from Promega. Sepharose 2B and all other chemicals were from Sigma. Rabbit reticulocyte lysate (National Institutes of Health) (36). Sp6 RNA polymerase was purified from an overexpressing Escherichia coli strain HB101/PTISP6 (34). 11-cis-Retinal was provided by Dr. R. Crouch (National Institutes of Health).

**Plasmid Constructions**—The visual arrestin cDNA was generously provided by Dr. K. Shinozaki (Molecular Biology Institute of National Institutes of Health) (36). Both plasmid constructs were generated from a construct encoding bovine arrestin containing 6 additional amino acids at the N terminus (AKR[6-404]) (31). The arrestin open reading frame lacking the first 64 nucleotides (including the 18 nucleotides coding for the 6 additional amino acids) was excised with BgIII and HindIII and then purified on a 1% low melting agarose gel. The resulting fragment was subcloned into the vector pG236-I (digested with NcoI and HindIII), containing an Sp6 promoter and an idealized 5′-untranslated region (31), along with an oligonucleotide duplex encoding the natural N terminus of bovine arrestin. The resulting construct encodes a full-length bovine arrestin ARR[1-404]. In order to create an N-terminal deletion, the construct was partially digested with BgIII, blunted with mung bean nuclease, and the resulting fragment, starting with codon 17, was excised with HindIII. The excised fragment was subcloned into pG286-I digested with NcoI (this end was subsequently blunted with Klenow and then contains a starting ATG codon) and HindIII. The resulting construct lacks codons 2 to 16 and encodes the N-terminal arrestin mutant ARR[2-16-404]. The sequences of both constructs were confirmed by DNA sequencing using Sequenase (U. S. Biochemical Corp).

**In Vitro Transcription and Translation**—To generate full-length arrestins, the in vitro transcription were initially linearized with HindIII, which cuts 120 bases downstream from the stop codon. To generate a truncated arrestin containing only the first 191 amino acids, (ARRR[1-191]), the plasmid was linearized with StuI, which cuts within the open reading frame at nucleotide 905 (35). In vitro transcription and translation were carried out as described (31, 32). Briefly, 100–150 μg/ml RNA template was added to a translation mixture containing 70% rabbit reticulocyte lysate, 120 mmoles potassium acetate, 0.25 mM magnesium acetate, 30 mM creatine phosphate, 5 mM cAMP, 160 μg/ml creatine kinase, 200 units/ml RNasin, 0.1 μg/ml pepstatin, 0.1 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 20 μg/ml each of myristate and palmitate, 50 μM each of 19 unfolded amino acids, and 20–50 μM [3H]Thymidine (800,000–1,000,000 cpm/ml), and then the template was incubated at 22 °C for 2 h. Following translation, ATP and GTP were added to a final concentration of 0.8–1 mM, the samples were incubated for 10 min at 37 °C, and aggregated proteins were pelleted by centrifugation at 100,000 rpm for 1 h in a TL100 ultracentrifuge at 5 °C. The supernatants, which contained >90% of the synthesized protein, were then used for functional assays. Assays could be performed without purification of the expressed protein since the synthesized arrestin is the only radiolabeled protein in the translation mixture. The functional interaction was demonstrated by the fact that >90% of the expressed arrestin can bind to P-Rh*. Protein synthesis was determined by measuring the amount of [3H]thymidine radioactivity incorporated into a hot trichloroacetic acid-insoluble fraction (36). The specific activities of the in vitro synthesized proteins were calculated using the number of leucine residues in the corresponding peptide and the known specific activity of the radioactive leucine.

**Rhodopsin Preparations**—Urea-treated rod outer segment (ROS) membranes were prepared as described (37). Phosphorylated rhodopsin was prepared as described (31) using purified [3H]rhodopsin overexpressed in Sf9 cells (38). BARK has been shown to phosphorylate light-activated rhodopsin at the same sites (26, 39) and with a comparable stoichiometry as rhodopsin kinase. Briefly, 250–300 μg of rhodopsin (ROS membranes) was incubated with 25–30 μg of BARK, 2 mM ATP, 6 mM MgCl₂ in 1 ml of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA at 37 °C for 60 min. The reaction was stopped by dilution with ice-cold 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and centrifugation at 50,000 rpm for 30 min.

The resulting pellet was washed twice with 2.5 ml of the Tris buffer, thoroughly resuspended in 1 ml of the same buffer, and sonicated on ice for 1 min. Regeneration of the opsin was carried out by addition of a 3-fold molar excess of 11-cis-retinal and incubation in the dark at 37 °C for 40 min followed by addition of another 3-fold molar excess of 11-cis-retinal and incubation in the dark at 37 °C for 2 h. The membranes were aliquoted under dim red light and stored at −80 °C wrapped in foil. The regeneration efficiency was determined by absorbance at 456 nm and found to be 98 ± 3%. The stoichiometry of phosphorylation was determined by addition of 1–2 μCi of [γ-32P]ATP to a 20-μl aliquot of the initial reaction mixture followed by incubation as described above. This reaction was stopped by addition of 5 μl of 5 x concentrated SDS sample buffer followed by gel electrophoresis (40). The gel was dried and autoradiographed, and the labeled rhodopsin band was excised and collected. In this study, we utilized a rhodopsin preparation that was phosphorylated to a stoichiometry of ~2.2 mol of phosphate/mol of rhodopsin.

A truncated form of rhodopsin ([32G-Rh]) lacking its C-terminal sites of phosphorylation was prepared as described previously (24, 28). Briefly, urea-treated ROS membranes were incubated with endopeptidase Asp-N in a ratio of 750:1 (w/w, rhodopsin:endopeptidase Asp-N) in 10 mM Tris-Cl, pH 7.5, at 22 °C for 16 h in the dark. The reaction was stopped by addition of 1 mM dithiothreitol and 1 mM EDTA followed by centrifugation at 40,000×g for 10 min. The ROS pellet was resuspended in 50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 5 μg x-aminonucleotide, pelleted by centrifugation, washed three times in 50 mM Tris-Cl, pH 7.4, and finally resuspended in 50 mM Tris-Cl, pH 7.4. Complete digestion to [32G-Rh] was confirmed by the presence of a single protein band of ~38 kDa on a 12% SDS-polyacrylamide gel that was not phosphorylated by β-arrestin.

**Arrestin Binding Assays**—2 μM [3H]rhodopsin was incubated in 50 mM Tris-Cl, pH 7.5, 0.5 mM MgCl₂, 150 mM potassium acetate, 1.5 mM dithiothreitol with 150 μM of the various functional forms of rhodopsin in a final volume of 50 or 100 μl either in the light or in the dark. The incubations also contained various concentrations of the different synthetic peptides. Following a 5-min incubation at 37 °C, the samples were placed on ice and then loaded onto a 2-ml Sepharose 2B column equilibrated with 25 mM Tris-Cl, pH 7.5, 2 mM EDTA buffer. Arrestin bound to ROS membranes eluted in the void volume (0.5–1.1 ml for a 50-μl reaction and 0.4–1.0 ml for a 100-μl reaction). Nonspecific binding was determined in the absence of ROS membranes.

**Synthetic Peptide Synthesis and Purification**—Peptides corresponding to rhodopsin's cytoplasmic loops (loop 1, TVGQIKKLRTPLNYI; loop 2, KPSMNFRGFENAT; loop 3, VKEAAAQQQESAT, residues 230–242; loop 4, TQKAEKEVTR, residues 141–153; loop 5, VYKEAAAQQQESAT, residues 141–153; loop 6, Wb(2-16)-4041. The sequences of all of these fragments was confirmed by DNA sequencing using Sequenase (U. S. Biochemical Corp).

**RESULTS**

**Peptide Inhibition of Arrestin Binding to Phosphorylated Light-activated Rhodopsin**—The molecular interactions of rhodopsin with both transducin (13–16, 17–19, 41) and rhodopsin kinase (24, 25, 27) have previously been investigated. The cytoplasmic domains involved in rhodopsin interaction with ar-
restin, however, have not been studied. To this end, we have utilized synthetic peptides from the bovine rhodopsin sequence to inhibit rhodopsin/arrrestin interactions. The synthetic peptides used for this study are depicted in Fig. 1 and encompass the four proposed cytoplasmic loops of rhodopsin. A cDNA construct encoding full-length bovine arrestin (ARR[1-404]) in the vector pG2S6-I was first generated from a construct encoding an arrestin containing 6 additional amino acids at its N terminus (31). In vitro synthesis of the ARR[1-404] construct in the presence of [3H]leucine yielded a protein with the expected electrophoretic mobility (Fig. 2, lane 1). The resulting tritiated ARR[1-404] demonstrated high affinity and selectivity for binding to phosphorylated light-activated rhodopsin (P-Rh*), although a low level of binding to light-activated (Rh*) and phosphorylated dark rhodopsin (P-Rh) was also observed (data not shown). These results are similar to those found for full-length arrestins containing either 1 or 6 additional amino acids near the N terminus (31, 32).

The synthetic peptides encompassing the various proposed cytoplasmic loops of rhodopsin were then tested for their ability to inhibit ARR[1-404] binding to P-Rh*. As shown in Fig. 3, all four cytoplasmic loop peptides appeared to inhibit arrestin binding in a dose-dependent fashion. The third cytoplasmic loop peptide was the most potent, exhibiting an IC50 value of 34 nM (15-30-fold lower than any other peptide) (Table 1). In addition, a peptide from the proximal end of the third cytoplasmic loop weakly inhibited ARR[1-404]/P-Rh* interaction, while a peptide from the distal end of this loop did not inhibit arrestin binding (Table 1). The ability of the peptide from the proximal end of the third cytoplasmic loop to inhibit ARR[1-404]/P-Rh* interaction suggests that this portion contains critical residues for arrestin binding. However, this peptide was significantly less potent than the intact third cytoplasmic loop peptide, suggesting that arrestin interaction with the third cytoplasmic loop of rhodopsin depends on more than just critical residues in its N-terminal portion. It is possible that critical residues for arrestin interaction may also exist in the distal end of the third loop and/or that the interaction with this loop is conformation-dependent. Indeed, critical residues for transducin interaction exist in both the N-terminal and C-terminal portions of the third cytoplasmic loop (17-19).
loops of rhodopsin were found to inhibit arrestin binding to P-Rh* just as well as the native peptides (data not shown). Thus, while the inhibition by the first and third cytoplasmic loop peptides appears to be specific, our results suggest that the effects of the second and fourth loop peptides are probably nonspecific. It is interesting to note, however, that we have also identified several other peptides that have the overall basic and hydrophobic character of the second and fourth cytoplasmic loops that were able to weakly inhibit arrestin binding to P-Rh*. In contrast, several other peptides that were basic (and not hydrophobic), neutral, or acidic did not inhibit arrestin binding to P-Rh* (data not shown). Overall, these results suggest that the first and third cytoplasmic loops of rhodopsin may be involved in arrestin binding to rhodopsin, implicating a multi-site interaction similar to that concluded from synthetic peptide studies of rhodopsin interaction with both transducin (13) and rhodopsin kinase (27).

Localization of Arrestin Sites Interacting with the Rhodopsin Cytoplasmic Loops—Previous studies have demonstrated that a truncated arrestin containing only the first 191 residues partially retained the ability to recognize the phosphorylation state and, to a lesser extent, the activation state of rhodopsin. This led us to propose that the amino half of the arrestin molecule contains key receptor recognition sites (31, 32). We have therefore tested the ability of the various peptides to inhibit the interaction of in vitro synthesized ARR(1-191) (Fig. 2, lane 3) with rhodopsin. Since the effects of the first and third cytoplasmic loop peptides on ARR(1-404)/P-Rh* interaction appear to be specific, only these peptides were used for further investigation. The interaction of ARR(1-191) with P-Rh* is also inhibited by the first and third cytoplasmic loop peptides (Table I). While ARR(1-191) appears to still interact predominantly with the third cytoplasmic loop of rhodopsin, this peptide was 5-fold less effective at inhibiting the ARR(1-191)/P-Rh* interaction when compared to ARR(1-404)/P-Rh* interaction. These results suggest that the amino half of the arrestin protein contains most of the sites of interaction with the cytoplasmic loops of rhodopsin, consistent with the previous finding that this domain of arrestin contains key recognition sites for the light activation and phosphorylation states of rhodopsin (31, 32).

The Role of the Arrestin N Terminus in Binding to P-Rh*—Previous studies on an arrestin mutant lacking 15 N-terminal residues, ARR(Δ2-16)-404, demonstrated that it had a reduced affinity and selectivity for P-Rh* and an impaired ability to recognize both the light activation and phosphorylation states of rhodopsin (32). It was postulated that either residues 2-16 in arrestin are critical for the “activation-recognition” and “phosphorylation-recognition” functions of arrestin or the N terminus of arrestin maintains the proper conformation for selective binding to P-Rh*, possibly through interaction with its regulatory C-terminal domain (32). In order to address this question, we attempted to determine whether this deletion alters arrestin interaction with the cytoplasmic loops of rhodopsin by assessing the ability of the rhodopsin loop peptides to inhibit in vitro synthesized ARR(Δ2-16)-404 (Fig. 2, lane 2) binding to P-Rh*. As shown in Fig. 4, ARR(Δ2-16)-404/P-Rh* interaction is inhibited by the first and third cytoplasmic loop peptides with the third loop peptide again being the most potent. In general, the ability of these peptides to inhibit the ARR(Δ2-16)-404/P-Rh* interaction did not differ significantly from their ability to inhibit ARR(1-404) binding to P-Rh* (Table I). Thus, although the N-terminal deletion results in an impairment of arrestin interaction with rhodopsin, it appears that it does not disrupt the ability of arrestin to interact with the first and third cytoplasmic loops of rhodopsin. As previously proposed (32), the N-terminal region of arrestin may therefore be regulatory in nature, ensuring the proper conformation necessary for high affinity binding to P-Rh*. We cannot, however, rule out the possibility that this N-terminal deletion disrupts the ability of arrestin to interact with the phosphorylated C terminus of rhodopsin.

Synthetic Peptide Inhibition of Full-length Arrestin Interaction with Light-activated and Truncated Rhodopsins—Arrestin interacts predominantly with the phosphorylated form of light-activated rhodopsin (5, 31, 42), implicating the phosphorylated C terminus of rhodopsin in arrestin interaction. However, arrestin does not appear to directly interact with the non-phosphorylated C terminus of rhodopsin since it binds weakly but comparably to both light-activated rhodopsin (Rh*) and a truncated rhodopsin lacking its C terminus (32G-Rh*) (32). In order to determine whether the same cytoplasmic loops of rhodopsin

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**TABLE I**

IC₅₀ values for rhodopsin cytoplasmic loop peptide inhibition of full-length and truncated arrestin binding to phosphorylated light-activated rhodopsin

<table>
<thead>
<tr>
<th>Loop</th>
<th>IC₅₀ (μM)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-404</td>
<td>1.133 ± 0.20²</td>
<td>1.20</td>
</tr>
<tr>
<td>Δ(1-2)-404</td>
<td>1.202 ± 0.20²</td>
<td>1.20</td>
</tr>
<tr>
<td>1-191</td>
<td>1.133 ± 0.20²</td>
<td>1.20</td>
</tr>
</tbody>
</table>

² The values in parentheses represent the number of experiments run in duplicate to obtain the IC₅₀ value.
Peptide Inhibition of Arrestin-Rhodopsin Interaction

**Fig. 5A.** Comparison of peptide inhibition of [H]ARR[1-404] binding to P-Rh*, Rh*, and 329G-Rh*. Samples containing 150 nm of the various forms of rhodopsin, 2 nm [H]ARR[1-404] (1300 dpm/fmol), and a concentration of peptide known to inhibit 60-75% of the binding to P-Rh* (1000 µl for loop 1 and 70 µl for loop 3) were incubated in either a 50 µl (P-Rh* samples) or 100 µl (Rh* and 329G-Rh* samples) reaction at 37 °C for 5 min in the presence of light. Bound [H]ARR[1-404] was then determined by separation on a Sepharose 2B column as described under "Experimental Procedures." Control binding ranged from 24,000 to 36,000 dpm (−18−28 fmol) for the P-Rh* samples and from 4000 to 6000 dpm (−3−5 fmol) for the Rh* and 329G-Rh* samples. The means ± S.E. from two separate experiments run in duplicate are shown. B, comparison of peptide inhibition of [H]ARR[1-404] binding to P-Rh* and P-Rh. Samples containing 150 nm of the various forms of rhodopsin, 2 nm [H]ARR[1-404] (1600 dpm/fmol), and 1 nm of the first or third loop peptide were incubated in a total volume of 100 µl at 37 °C for 5 min in the light (P-Rh*) or in the dark (P-Rh). Bound [H]ARR[1-404] was then determined by separation on a Sepharose 2B column as described under "Experimental Procedures." Control binding ranged from 2000 to 3000 dpm (−2 fmol) for the P-Rh samples and 65,000 to 90,000 dpm (−40−55 fmol) for the P-Rh* samples. Nonspecific binding was always <50% of the overall binding for P-Rh samples and <5% of the overall binding for P-Rh* samples. In these particular experiments the non-acetylated and non-amidated form of the loop 3 peptide was used. The means ± S.E. of two separate experiments run in duplicate are shown.

**DISCUSSION**

Arrestin binds with high affinity and selectivity to the phosphorylated light-activated form of rhodopsin (6, 31, 32). Until recently, little was known about the nature of arrestin selectivity for P-Rh* other than the notion that arrestin undergoes a conformational change upon binding (43). Recently, the high affinity and selectivity of arrestin binding to P-Rh* has been proposed to be mediated by three domains in arrestin: an activation-recognition site(s) that interacts with those portions of rhodopsin which manifest its activation, a phosphorylation-recognition site that interacts with the phosphorylated C terminus of rhodopsin, and a hydrophobic "booster" site that appears to be mobilized upon arrestin binding to P-Rh* (32). If rhodopsin is only light-activated, "low affinity" binding of arrestin occurs through interaction between the activation-recognition site(s) of arrestin and the rhodopsin domains that change conformation upon activation. Low affinity arrestin binding would as well occur with phosphorylated dark rhodopsin through interaction between the phosphorylation-recognition site of arrestin and the phosphorylated C terminus of rhodopsin. The low affinity binding of arrestin to either Rh* or P-Rh is ionic in nature, thus enabling rapid dissociation of arrestin. However, if rhodopsin is both light-activated and phosphorylated, the activation-recognition and phosphorylation-recognition sites of arrestin interact with their rhodopsin counterparts promoting mobilization of the hydrophobic booster site for interaction. This three site interaction results in high affinity arrestin binding to P-Rh*, which has both hydrophilic and hydrophobic components that account for the strict selectivity of arrestin for P-Rh* (32).

In order to define the domains of rhodopsin involved in arrestin interaction, we have studied the ability of synthetic peptides from the proposed rhodopsin cytoplasmic domains to inhibit arrestin/rhodopsin interactions. Although all four
rhodopsin cytoplasmic loop peptides were found to inhibit arrestin binding to P-Rh* (Fig. 3), only the first and third loop peptides appeared to be specific since scrambled peptides from the second and fourth cytoplasmic loops inhibited arrestin binding as well as the corresponding native peptides. These results suggest that arrestin binding to rhodopsin involves a multi-site interaction with the first and third cytoplasmic loops of rhodopsin in addition to the phosphorylated C-terminal tail of rhodopsin. A predominant site of arrestin interaction appears to be the third cytoplasmic loop since this peptide was the most potent inhibitor with an IC50 of 34 μM (~30-fold higher affinity than the first loop peptide) (Fig. 3, Table I). However, the affinities of the first and third loop peptides for arrestin appear to be >1000-fold lower than the affinity of rhodopsin for arrestin (31,43). This affinity difference is likely due to a single-site interaction of arrestin with the peptides versus a multi-site interaction of arrestin with P-Rh* (first and third cytoplasmic loops and phosphorylated C-terminal tail).

Several lines of evidence have also implicated the involvement of multiple cytoplasmic loops in activation-dependent receptor interactions with both G proteins and receptor kinases. Multiple synthetic peptides have been demonstrated to inhibit receptor/G protein (13,14,20) and receptor/receptor kinase (27,28) interactions. Moreover, the third cytoplasmic loop of the receptor appears to be critical for interaction with both the G proteins and receptor kinases as well. For example, a third cytoplasmic loop peptide from rhodopsin was able to effectively compete with rhodopsin for transducin interaction (13) and was found to be the most effective peptide at inhibiting rhodopsin kinase phosphorylation of bleached rhodopsin (27). Mutagenesis studies have also demonstrated the critical importance of the third cytoplasmic loop in receptor/G protein coupling (17–19,21–23). Multiple deletions as well as point mutations in the third cytoplasmic loop of rhodopsin effectively disrupted rhodopsin/transducin interactions (17–19). Chimeric receptors have also been used to demonstrate the critical importance of the third cytoplasmic loop in receptor/G protein interactions (23,44–46). Moreover, the coexpression of a third cytoplasmic loop peptide with its native receptor resulted in receptor antagonism most likely by uncoupling receptor/G protein interactions (47).

The first and third cytoplasmic loops of rhodopsin appear to be involved specifically in arrestin binding to light-activated forms of rhodopsin as evidenced by the ability of the first and third loop peptides to disrupt arrestin binding to Rh* and 329G-Rh* (Fig. 5A). Since the second and fourth loop peptides were unable to disrupt arrestin binding to these forms of rhodopsin, their mechanism of inhibition may be through interaction with the phosphorylated C terminus of rhodopsin or possibly via an ability to mimic sites in arrestin that are only involved in high affinity interaction with P-Rh* (e.g. the basic and hydrophobic stretches localized in the booster site of arrestin and/or the phosphorylation-recognition site of arrestin). The ability of the first and third cytoplasmic loop peptides to disrupt arrestin binding to Rh* and 329G-Rh* suggests that the corresponding arrestin sites for interaction with these cytoplasmic loops of rhodopsin are accessible in both the low affinity and high affinity arrestin binding states. The predominant interaction in arrestin binding to Rh* still appears to be the third cytoplasmic loop as demonstrated by the more potent effect of the third loop peptide on ARR1-191/Rh* interaction (IC50 values of ~1000 μM and ~50 μM for the first and third loop peptides, respectively). Thus, the third and, to a lesser extent, the first cytoplasmic loops of rhodopsin may be the counterparts for interaction with the activation-recognition site(s) of arrestin and are therefore critical for the ability of arrestin to interact with all light-activated forms of rhodopsin. Since the third cytoplasmic loop is also critical for the light-dependent interaction of rhodopsin with transducin and rhodopsin kinase, it is likely that this loop is the major domain of rhodopsin that changes conformation upon activation.

The comparable IC50 values of the first and third cytoplasmic loop peptides for inhibiting arrestin binding to both P-Rh* and Rh* suggests that the mechanism of inhibition of these peptides may be the direct blockade of the interaction of the activation-recognition site(s) of arrestin with the corresponding loops of rhodopsin. The comparable peptide potencies for inhibition of arrestin interaction with both Rh* and 329G-Rh* (Fig. 5A) also demonstrates that the non-phosphorylated C terminus of rhodopsin does not alter the activation-recognition function of arrestin (i.e. does not alter arrestin interaction with the cytoplasmic loops of light-activated rhodopsin), consistent with the comparable affinity of arrestin for both Rh* and 329G-Rh* (32). Consistent with the notion that the first and third loop peptides interact specifically with the activation-recognition site(s) of arrestin is the finding that these peptides do not disrupt arrestin binding to phosphorylated dark rhodopsin (Fig. 5A) in an interaction that does not involve the activation-recognition site. Moreover, it appears that these peptides may modestly enhance arrestin binding to P-Rh. This could be explained by the ability of the first and third loop peptides to partially mimic rhodopsin regions that display its activation and thus interact with the activation-recognition region(s) of arrestin (unlike the more conformationally restrained corresponding loops of dark P-Rh). Thus, in the absence of these peptides, low affinity arrestin binding occurs through interaction of arrestin's phosphorylation-recognition region with the phosphorylated C-terminal tail of rhodopsin, whereas, in the presence of these peptides arrestin may bind to P-Rh with a higher affinity due to a partial conformational change induced by the occupation of both the phosphorylation-recognition and, to a lesser extent, activation-recognition region(s) of arrestin.

We have also used the rhodopsin peptides to investigate the corresponding sites in arrestin that interact with the cytoplasmic loops of rhodopsin. Previous studies have shown that an arrestin mutant containing only the N-terminal half of the molecule, ARR[1-191], still partially retains its ability to recognize the phosphorylation and, to a lesser extent, activate two of the characteristic states of rhodopsin, although it exhibits a marked reduction in affinity and selectivity for P-Rh* (31). The first and third cytoplasmic loop peptides were also found to disrupt ARR1-191/ P-Rh* interaction (Table I), suggesting that the amino half of arrestin contains most of the sites for interaction with these cytoplasmic loops of rhodopsin. However, the third cytoplasmic loop peptide had a decreased ability to inhibit ARR1-191 binding to P-Rh* as compared to ARR1-404 binding. This may suggest that a portion of the corresponding arrestin site for interaction with the third cytoplasmic loop is located in the C-terminal half of arrestin, consistent with the observation that this deletion impairs the activation-recognition function of arrestin. Alternatively, the difference in peptide potency may reflect the difference in its mechanism of inhibition of arrestin binding. With respect to the ARR1-404/P-Rh* interaction, the potency of the third cytoplasmic loop peptide may reflect not only disruption of the direct interaction with the third cytoplasmic loop but also the conformational change of arrestin necessary for its high affinity binding. However, with respect to the ARR1-191/P-Rh* interaction, the potency of this peptide may reflect only disruption of the direct interaction with the third cytoplasmic loop since this arrestin mutant lacks the ability to undergo a conformational change. The previous identification of key rhodopsin recognition sites in the N-terminal half of arrestin (31,32) is consistent with our present localization of the corresponding arrestin sites for interaction with the cyto-
plasmic loops of rhodopsin to the N-terminal half of arrestin. ARR(Δ[2–16]) lacking the N-terminal residues 2–16, demonstrated poor affinity and selectivity for P-Rh* (32). We have studied the ability of the first and third cytoplasmic loop peptides to inhibit ARR(Δ[2–16]) binding to P-Rh* in order to assess whether the N terminus directly participates in its interaction with the cytoplasmic loops of rhodopsin. These peptides were able to inhibit ARR(Δ[2–16]-404)/P-Rh* interaction with potencies comparable to their inhibition of ARR(1–404)/P-Rh* interaction (Table I). These results suggest that the N-terminal residues 2–16 do not directly participate in arrestin interaction with the cytoplasmic loops of rhodopsin. The impairment of arrestin function by deletion of these residues may have been a result of disruption of the proper conformation required for the transition from its low affinity to high affinity binding state. We cannot, however, exclude the possibility that the N-terminal residues 2–16 interact directly with the phosphorylated C terminus of rhodopsin. Overall, these results suggest that the major region of arrestin (not including the hydrophobic booster site) involved in rhodopsin interaction is contained within residues 17–191.

The use of synthetic peptides to probe protein/protein interactions provides an important tool for investigating the molecular nature of these interactions. Peptides can be used to uncover large domains or regions critical to these interactions and provide a focus for more detailed mutagenesis studies. Indeed, peptides have been very instrumental to the investigation of protein-mediated signal transduction can be achieved through multi-site interaction between arrestin and rhodopsin. More recently, peptides from the various receptor/AR protein (13, 14, 20, 48, 49) and receptor-receptor interactions (27, 28) are likely to compete with each other for receptor interaction. Investigation of this aspect of G protein-mediated signal transduction can be achieved through protein/protein binding competition studies. The use of the cell-free expression system and direct binding assays will undoubtedly aid in these investigations.

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REFERENCES