Activation of Arrestin: Requirement of Phosphorylation as the Negative Charge on Residues in Synthetic Peptides from the Carboxyl-Terminal Region of Rhodopsin

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PURPOSE. To determine whether substitution of the potential phosphorylation sites of bovine rhodopsin’s carboxyterminal region with the acidic residues aspartic acid, glutamic acid, or cysteic acid promotes the activation of arrestin.

METHODS. Three peptide analogues of the 19-residue carboxyterminal region of rhodopsin (330-348) were synthesized: the fully phosphorylated peptide (7P-peptide), the peptide with all potential phosphorylation sites substituted with glutamic acid (7E-peptide), and the peptide with the phosphorylation sites substituted with cysteic acid (7Cya-peptide). The peptides were tested in assays in which the 7P-peptide had previously been shown to have an effect. Rhodopsin with glutamic acid (Etail) or aspartic acid (Dtail) substituted for the phosphorylation sites in rhodopsin were constructed and expressed in COS-7 cells and tested in an in vitro assay.

RESULTS. Earlier work has demonstrated that the 7P-peptide activates arrestin, showing induction of arrestin binding to light-activated unphosphorylated rhodopsin, inhibition of the light-induced phosphodiesterase (PDE) activity in rod outer segments (ROS) with excess arrestin, increase in the initial rapid proteolysis of arrestin by trypsin, and enhanced reactivity of one of arrestin’s sulfhydryl groups with inhibition of the reactivity of another. None of these effects was observed in the presence of 7E-peptide or 7Cya-peptide. The 7Cya-peptide inhibited the PDE activity in ROS, but the same effect was observed both in the presence and the absence of excess arrestin. Because none of the other effects was observed with the 7Cya-peptide, the authors conclude that the 7Cya-peptide does not activate arrestin, but acts, probably nonspecifically, through some other part of the transduction system. Considerable arrestin-mediated rhodopsin inactivation was observed with both the Etail and the Dtail mutant, although these substitutions did not yield rhodopsins that were equivalent to phosphorylated rhodopsin.

CONCLUSIONS. These results, taken together, suggest that the negative charge due to phosphates in the carboxyterminal region of rhodopsin are required for the full activation of arrestin and that acidic amino acids (carboxyl and sulfonic) do not mimic the negative charge of phosphorylated residues. (Invest Ophthalmol Vis Sci. 2001;42:1439–1443)

Visual arrestin inactivates the light-activated visual G-protein receptor rhodopsin by binding to phosphorylated rhodopsin, preventing further activation of the G-protein, transducin (reviewed in Ref. 1). Phosphorylation of rhodopsin is required for arrestin to bind to light-activated rhodopsin. Earlier work has suggested that phosphorylation of rhodopsin is necessary, primarily to change arrestin’s conformation to an active form that can bind to and quench rhodopsin. A synthetic peptide that is identical with the fully phosphorylated carboxyterminal region of rhodopsin (330-348 of bovine rhodopsin), 7P-peptide, activates arrestin. The 7P-peptide has been shown to affect the conformation of arrestin by three additional assays. In the presence of 7P-peptide (1) the rate of initial tryptic proteolysis of arrestin is enhanced, and further cleavage is suppressed (heparin has a similar effect); (2) arrestin inhibits light-induced phosphodiesterase (PDE) activity bypassing the need for phosphorylated rhodopsin; and (3) the reactivity of one of the sulfhydryls of arrestin is greatly enhanced, whereas the reactivity of another is reduced.

In the current work, we asked whether other negatively charged residues (glutamic acid or cysteic acid) in the synthetic peptide would yield similar results. In addition, to test whether negatively charged amino acid residues could substitute for the phosphorylated residues in vitro, we replaced the seven potentially phosphorylated residues with glutamic acid and aspartic acid residues and measured the ability of arrestin to inhibit the activation of transducin by these mutant rhodopsins.

MATERIALS AND METHODS

Peptide 330-348 from the carboxyterminal region of bovine rhodopsin containing four phosphothreonine (PThr) and three phosphoserine (Pser) residues was previously synthesized. Two new analogues of this peptide were synthesized for the present study. In one peptide, cysteic acid was substituted for each of the phosphorylated residues: DDEAXXXVXXXXQVAPA, where X is cysteic acid (7Cya-peptide). In the other peptide, glutamic acid was substituted for each of the phosphorylated residues: DDEAEVEKEEEQVAPA. This peptide (7E-peptide) was made on an automated peptide synthesizer (model 431A; Perkin Elmer-Applied Biosystems, Foster City, CA) on resin (FmocAla-Pam; Bachem, Torrence, CA). The peptide was purified by reversed-phase HPLC (2.5 × 25-cm Partisil-10 ODS-3 column; Whatman, Clifton, NJ) with a linear gradient from 100% A (0.1% acetic acid in water) to 30% B (0.1% acetic acid in acetonitrile) in 40 minutes at 10 ml/min, with the eluent monitored at 230 nm. The peptide displayed the correct mass spectrum and amino acid composition and was essentially homogeneous by HPLC. The effect of the peptides on the light-acti-
vated PDE activity in the presence of excess arrestin was determined as described earlier.3

The ability of the peptides to induce arrestin binding to light-activated, unphosphorylated rhodopsin was tested using a centrifuge assay. Disks from rod outer segments (ROS) were prepared according to the method of Smith et al.8 Arrestin was prepared by the method of Buczylko and Palczewski9 with modifications as described earlier.2 The reaction mixture contained 22 \( \mu M \) rhodopsin in disks, 18 \( \mu M \) arrestin, and approximately 0.5 mM of the peptide in 0.1 M NaPO\(_4\) buffer prepared by mixing 0.1 M NaH\(_2\)PO\(_4\) and 0.1 M Na\(_2\)HPO\(_4\) to pH 7.0 and then adjusting to a concentration of 0.1 M NaCl. Aliquots (200 \( \mu l \)) were prepared and either bleached for 2 minutes on a light box or kept in the dark. The samples were centrifuged at 39,000 g for 20 minutes at 4°C. The pellets were washed once with 0.1 M NaPO\(_4\)-0.1 M NaCl buffer. The pellets were then dissolved in a 2× SDS-PAGE sample buffer. SDS-PAGE was performed under reducing conditions, according to the method of Laemmli.10

The effect of the peptides on the reactivity of the sulphydryl groups of arrestin was determined as described earlier.3 Limited proteolysis using trypsin in the presence of the peptides was performed as described earlier.2

Rhoiodopsins with the seven serine and threonine residues in the carboxy-terminal region replaced with glutamic acid (Etail) or aspartic acid (Dtail) were prepared by a method described earlier.11 The arrestin-mediated quenching of these mutant rhodopsins was measured as previously described.11

**RESULTS**

**Effect of Peptide Analogues on PDE Activity**

Light-induced PDE activity in ROS preparations in the presence of cGMP was measured as a decrease in pH (Figs. 1A, 1B, 1C; trace a). In the presence of arrestin and adenosine triphosphate (ATP), this activity was markedly reduced (Figs. 1A, 1B, 1C; trace b). The reduction in PDE activity was due to the phosphorylation of the bleached rhodopsin by endogenous rhodopsin kinase followed by arrestin binding. These reactions blocked the binding and activation of transducin. Addition of 7P-peptide without added arrestin had little effect on PDE activity (Fig. 1A, trace c). However, in the presence of both 7P-peptide and arrestin, PDE activity was reduced (Fig. 1A, trace d) almost to the same extent as with ATP (trace b). Note that there should be no phosphorylation of rhodopsin under conditions depicted in traces c and d, because there was no ATP present in these samples. The 7Cya-peptide reduced the PDE activity, both in the presence (Fig. 1B, trace f) and the absence (Fig. 1B, trace e) of added arrestin. This inhibition in the absence of added arrestin suggests that the 7Cya-peptide did not act in the same manner as the 7P-peptide. In contrast, the 7E-peptide had only a slight effect on PDE activity in the

**FIGURE 1.** PDE activity in an ROS preparation in the presence of negatively charged peptides. PDE activity was determined in the presence of excess arrestin, as described earlier.3 PDE activity cleaved cGMP, resulting in a release of H\(^+\), shown as an upward deflection in the curves. Trace a and b, displayed in all three panels, show the PDE activity of the preparation in the absence (trace a) and presence (trace b) of ATP. (A) Trace c: PDE activity in the presence of 7P-peptide (190 \( \mu M \)), but with no added arrestin or ATP. Trace d: PDE activity in the presence of added arrestin and 7P-peptide, but no ATP. (B) Trace e: PDE activity in the presence of 7Cya-peptide (318 \( \mu M \)), but with no added arrestin or ATP. Trace f: PDE activity in the presence of added arrestin and 7Cya-peptide, but no ATP. (C) Trace g: PDE activity in the presence of 7E-peptide (200 \( \mu M \)), but with no added arrestin or ATP. Trace h: PDE activity in the presence of added arrestin and 7E-peptide, but no ATP.
presence of added arrestin (Fig. 1C, trace h) and almost no effect in the absence of added arrestin (Fig. 1C, trace g).

**Effect of Peptide Analogues on Binding to Bleached, Unphosphorylated Rhodopsin**

The peptide analogues were tested for their ability to induce arrestin binding to bleached, unphosphorylated rhodopsin in a centrifuge binding assay (Fig. 2). All samples have disc membranes and added arrestin with no added ATP. The odd-numbered samples were kept in the dark, and the even-numbered samples were bleached. The ratio of the arrestin band to the rhodopsin monomer band was determined by gel scans (NIH Image 1.62; National Institutes of Health, Bethesda, MD; available in the public domain at http://www.nih.gov/od/oba) and is shown in the bar graph under the photograph of the gel.

With no peptide present (Fig. 2, lanes 1, 2), there was a small increase in arrestin binding to the disc membranes after bleaching. There was considerably more light-induced arrestin binding in the presence of the 7P-peptide (lane 4). However, neither the 7Cya-peptide (lane 6) nor the 7E-peptide (lane 8) showed any additional light-induced binding when compared with conditions with no peptide present. These results indicate that only phosphorylated residues in the rhodopsin carboxyl-terminal peptide serve to activate arrestin, allowing it to bind to bleached, but unphosphorylated, rhodopsin.

**Effect of Peptide Analogues on the Sulphydryl Reactivity of Arrestin**

The negatively charged peptide analogues were tested for their ability to enhance the rate of reactivity of one sulphydryl group of arrestin and reduce the reactivity of another. In Figure 3, trace a shows the reaction of the sulphydryl groups of arrestin with 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) with no peptide additions and shows the relatively slow reaction of arrestin’s sulphydryl groups with DTNB. Neither 7Cya-peptide (trace b) nor 7E-peptide (trace c) had much effect on the sulphydryl reactivity. Only 7P-peptide (trace d) enhanced the reactivity of one sulphydryl group and inhibited another.

**Effect of Peptides on Limited Proteolysis of Arrestin by Trypsin**

Arrestin was submitted to limited proteolysis by trypsin in the presence of the synthetic peptides (Fig. 4). Only the 7P-peptide (lane 3) and bleached, phosphorylated rhodopsin (lane 6) affected the limited proteolysis, by enhancing an initial cleavage followed by inhibition of further digestion. Digestion in the presence of 7Cya-peptide (lane 4) and 7E-peptide (lane 5) was essentially identical with the digestion with no additions (lane 2). This shows that both 7P-peptide and bleached, phosphorylated rhodopsin affect the conformation of arrestin. However, no conformational changes were observed in the presence of the other peptide analogues.

**Effect of Aspartic Acid and Glutamic Acid Mutants of the Rhodopsin Carboxyl-Terminal Region**

Rhodopsin mutants were prepared by substituting acidic residues for the phosphorylation sites in the carboxyl-terminal region of rhodopsin. Two mutants were prepared: one with glutamic acid substituted for each of the three serine and four threonine residues and the other with aspartic acid substitutions. The ability of arrestin to quench the activation of transducin by these mutant rhodopsins is shown in Figure 5. For both mutants, considerable quenching of the activation was observed, but only approximately 50% to 60% of the quenching in the wild type.

**DISCUSSION**

A 19-amino-acid peptide that is identical with the completely phosphorylated carboxyl terminus of bovine rhodopsin was shown to activate arrestin, allowing it to bind to bleached, unphosphorylated rhodopsin and to quench transducin activation. Several effects of this peptide on arrestin conformation were observed. The same peptide in which glutamic acid residues were substituted for the phosphorylated residues failed to show any of these effects. Substituting cysteic acid for the phosphorylated residues produced a peptide that inhibited...
light-induced PDE activity, but this occurred both in the presence and absence of added arrestin. Because none of the other effects on the conformation of arrestin was observed in the presence of this peptide, we conclude that the cysteic acid peptide inhibits the PDE through some other mechanism, not by activating arrestin. Similarly, the 7 glutamic acid peptide also failed to promote any apparent conformational change in arrestin. This suggests that the function of phosphorylation is more than to add negative charges to the carboxyl region of rhodopsin.

The number of phosphorylated residues required to allow arrestin to fully quench the rhodopsin activation has been the target of several studies. In one study, only one or two sites were observed to be phosphorylated (serine 338 and serine 334). However, if only serine 338 or only serine 338 and 334 were available, little quenching was observed, suggesting that multiple phosphorylation is required for full quenching of activated rhodopsin. Replacing one site, serine 343, with glutamic acid yields a rhodopsin equivalent to wild type in its ability to bind arrestin, whereas replacing threonine 340 with glutamic acid results in reduced suppression of activated rhodopsin by arrestin. Replacing all four threonines with alanines in the carboxyl-terminal tail also results in reduced suppression of activated rhodopsin by arrestin.

In the present study, both of the mutants that contain all the potential phosphorylation sites converted to negatively charged residues did not quench as effectively as when native rhodopsin, kinase, and ATP were present. This indicates that these negatively charged residues are not equivalent to phosphoserine or phosphothreonine in their ability to promote arrestin quenching of transducin activation.

Although the charged residues were clearly not equivalent to phosphorylated residues, this finding suggests that other negatively charged residues can at least partially substitute for phosphorylated residues in rhodopsin.
CONCLUSIONS

These results, taken together, suggest that negative charge alone in the carboxyl-terminal region of rhodopsin is insufficient for the maximum activation of arrestin, giving rise to its quenching of transducin activation. Full effectiveness is achieved only with phosphate. Carboxylic and sulfonic acids are less effective.

References