# Rhodopsin's Carboxyl-Terminal Threonines Are Required for Wild-Type Arrestin-Mediated Quench of Transducin Activation in Vitro†

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ABSTRACT: Many recent reports have demonstrated that rhodopsin's carboxyl-terminal serine residues are the main targets for phosphorylation by rhodopsin kinase. Phosphorylation at the serines would therefore be expected to promote high-affinity arrestin binding. We have examined the roles of the carboxyl serine and threonine residues during arrestin-mediated deactivation of rhodopsin using an in vitro transducin activation assay. Mutations were introduced into a synthetic bovine rhodopsin gene and expressed in COS-7 cells. Individual serine and threonine residues were substituted with neutral amino acids. The ability of the mutants to act as substrates for rhodopsin kinase was analyzed. The effect of arrestin on the activities of the phosphorylated mutant rhodopsins was measured in a GTP$\gamma$S binding assay involving purified bovine arrestin, rhodopsin kinase, and transducin. A rhodopsin mutant lacking the carboxyl serine and threonine residues was not phosphorylated by rhodopsin kinase, demonstrating that phosphorylation is restricted to the seven putative phosphorylation sites. A rhodopsin mutant possessing a single phosphorylatable serine at 338 demonstrated no phosphorylation-dependent quench by arrestin. These results suggest that singly phosphorylated rhodopsin is deactivated through a mechanism that does not involve arrestin. Analysis of additional mutants revealed that the presence of threonine in the carboxyl tail of rhodopsin provides for greater arrestin-mediated quench than does serine. These results suggest that phosphorylation site selection could serve as a mechanism to modulate the ability of arrestin to quench rhodopsin.

The amplitude of a rod photoresponse is determined, in part, by the active lifetime of the light receptor molecule rhodopsin. Factors that regulate this lifetime not only control response deactivation but also contribute to the phenomenon of photoreceptor adaptation. The deactivation of rhodopsin is generally viewed as a two-step process, involving phosphorylation by rhodopsin kinase followed by the binding of the inhibitory protein arrestin or its splice variant p44 (*1*−*3*). Extensive investigation over the past 3 decades has yielded a broad qualitative understanding of these systems. It is believed that most of the principal components have been identified, but an explicit understanding of their functions remains in doubt, particularly in regard to how the roles may change during light adaptation.

The role of rhodopsin phosphorylation is particularly intriguing. The consensus of several studies, including the present one, is that phosphorylation of rhodopsin by rhodopsin kinase is limited to the three serine and four threonine residues of the carboxyl terminus of rhodopsin (*4*−*6*). There is also overwhelming agreement that, in in vitro bovine preparations, the initial site of phosphorylation is at one of the three serines (*3, 7*). McDowell et al. (*8*) reported that mono- and diphosphorylated forms were a mixture of rhodopsins phosphorylated at S338 and S343, while triphosphorylated forms contained an additional phosphorylation at one of the four threonines. Ohguro et al. (*9*) found that S338 was the primary site of initial phosphorylation, followed by S343 or T336. Papac and colleagues concluded that S338 and S343 were the major sites of phosphorylation (*10*). In live mice, S334 and S338 were found to be equally preferred substrates with S343 less preferred (*11*).

The number of phosphates incorporated into a rhodopsin molecule is still open to debate. Estimates greater than 7 P/R[1] have been reported (*12*−*14*), yet the maximum number is probably 7. High levels of rhodopsin phosphorylation have been difficult to achieve in vitro and difficult to detect in vivo; the possibility has been raised that phosphorylation beyond ∼3 P/R may be an artifact of in vitro phosphorylation conditions (*15*). Typical levels of phosphorylation range from 1 to 3 P/R (*8*−*10*) for in vitro preparations. In live mice, Ohguro et al. (*11*) found that phosphorylation was limited to only a single P/R, although the presence of multiply phosphorylated species may have been below the detection limit of the assay. However, Hurley et al. (*3*) detected as many as 3 P/R under conditions of intense illumination. How the extent of phosphorylation or the identity of the phosphorylation sites depends on the adaptational state of the cell is a question only recently addressed (*3, 11*).

High-affinity arrestin binding is thought to involve both a phosphorylation recognition component and a component that is specific for light-activated (Meta II) rhodopsin (*16*). The

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[1] Abbreviations: BTP, bis-tris propane; $G_t$, rod-specific G-protein transducin; P/R, molar ratio of phosphates to rhodopsin; R*, light-activated rhodopsin; Rho, rhodopsin; ROS, rod outer segment; RK, rhodopsin kinase.

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phosphorylation recognition domain in bovine arrestin has been localized to amino acid positions 163−176, containing 6 positively charged residues. Mutagenic analysis of these residues demonstrates that they are involved in binding to phosphorylated rhodopsin. Strikingly, substitution of R175 with a neutral or negatively charged residue results in phosphorylation-independent arrestin binding to light-activated rhodopsin (*17*, *18*). The amount of P/R required for an arrestin-mediated quench of rhodopsin in vivo is still in question. Observations of arrestin binding based on measurements of average P/R incorporation must be interpreted with caution because of the cooperative and heterogeneous nature of in vitro phosphorylation reactions (*19*). Bennett and Sitaramayya reported an arrestin effect at an estimated average phosphate incorporation of 1 P/R (*14*). Wilden, however, purified rhodopsin on the basis of P/R and was also able to demonstrate a partial arrestin quench on species containing as few as 1 P/R in a phosphodiesterase assay (*20*). However, this effect was dependent on constructing the assay such that the $G_t$ recycled multiple times. Arrestin had no detectable effect on monophosphorylated rhodopsin when the assay conditions were such that the recycling of $G_t$ was prevented. Interestingly, Sagoo and Lagnado have recently provided evidence that recycling of $G_t$ does not occur endogenously during the response to a flash of light (*21*).

If, indeed, S338 is the preferred residue for phosphorylation, and arrestin binds to singly phosphorylated rhodopsin, a logical expectation is that S338 would be necessary or at least sufficient for the arrestin-mediated deactivation of rhodopsin. This question has been addressed by Zhang et al. (*6*), who found that neither S338 nor S343 is necessary for arrestin binding in vitro. These results are supported by Mendez et al. (*22*), who report that transgenic mice expressing mutations in these residues have normal response sensitivity and shut off kinetics.

In the present study, we extend these findings by examining the ability of rhodopsin phosphorylation site mutants to be quenched by arrestin at near-physiological concentrations of arrestin. We find that the serine residues in the carboxyl tail of rhodopsin are neither necessary nor sufficient for strong quench of rhodopsin activity by arrestin and that the threonine residues contribute more to this interaction than do the serines.

## MATERIALS AND METHODS

Mutations were created in a synthetic bovine rhodopsin gene (*23*) using either the Transformer Mutagenesis system (Clontech) or a "cassette" mutagenesis strategy (see Table 1 for a list of mutants used in this study). The mutants were sequenced using Sequenase (Amersham Corp.) according to the manufacturer's instructions. Glutamine was used as the phosphorylation site replacement amino acid for the majority of experiments. One study (*24*) found that a region spanning positions 332−338 in bovine rhodopsin may be involved in a $\beta$-sheet structure. This small region contains a majority of rhodopsin's potential phosphorylation sites. Although we have not attempted a comprehensive analysis of the effects of different amino acid substitutions at the various positions, glutamine may be more likely to preserve a $\beta$-sheet structure than alanine (*25*) which is commonly used as a neutral substitution.

Table 1: Bovine Rhodopsin Phosphorylation Site Mutants[a]

| | 334 338 343 | Arrestin effect | Phosphorylation |
|---|---|---|---|
| Wild type | **STT**V**S**K**T**E**TS**QVAPA | 89 ±2 | 1.00 ±0.05 |
| S338/Atail | AAA-S-A-AA----- | 8 ±9 | 0.35 ±0.03 |
| S334/Atail | SAA-A-A-AA---- | 8 (n=1) | + |
| all-Ala | AAA-A-A-AA----- | N/D | 0.007 |
| all-Ser | SSS-S-S-SS----- | 70 ±1 | 0.72 ±0.06 |
| all-Thr | TTT-T-T-TT----- | 87 ±3 | 0.80 ±0.06 |
| T⇨Q | SQQ-S-Q-QS----- | 38 ±4 | 0.52 ±0.06 |
| T⇨Q,S⇨T | TQQ-T-Q-QT----- | 68 ±6 | 0.43 ±0.14 |
| S⇨Q | QTT-Q-T-TQ----- | 75 ±6 | 0.53 ±0.06 |
| S⇨Q,T⇨S | QSS-Q-S-SQ----- | 38 ±8 | 0.51 ±0.20 |
| Q335 | SQT-S-T-TS----- | 70 ±3 | + |
| QT335 | STQ-S-Q-QS----- | 44 ±6 | + |
| Q336 | STQ-S-T-TS----- | 85 ±1 | + |
| QT336 | SQT-S-Q-QS----- | 60 ±6 | + |
| Q340 | STT-S-Q-TS----- | 81 ±1 | + |
| QT340 | SQQ-S-T-QS----- | 80 ±2 | + |
| Q342 | STT-S-T-QS----- | 87 ±3 | + |
| QT342 | SQQ-S-Q-TS----- | 57 ±3 | + |

[a] Carboxyl-terminal residues are shown. Mutations were constructed in the expression vector PMT4. Potential phosphorylation sites in wild type are in boldface. Dashes indicate residues identical to wild type. Arrestin effect is the percent suppression of rate in the presence of 1.1 $\mu$M arrestin. The extent of phosphorylation is quantitatively expressed as a fraction of wild type; + indicates that the mutant was qualitatively determined to be phosphorylated. N/D = not done. Errors are SD.

Opsins were expressed in COS-7 cells using Lipofectamine (Life Technology). COS cell membranes containing expressed opsins were harvested according to Weiss et al. (*26*). Rhodopsin kinase (RK) was expressed in High Five cells (Invitrogen) and partially purified according to Ohguro et al. (*7*) with modification (see below). Transducin ($G_t$) was purified from bovine retina as described (*27*). Arrestin was purified from bovine retina as described (*28*), dialyzed against 100 mM NaCl, 10 mM Hepes, and concentrated (Centricon 30, Amicon).

*Rhodopsin Quantitation.* Urea-washed bovine ROS were prepared and rhodopsin quantitated spectrophotometrically as described (*29*). ROS rhodopsin was used as a standard in Western blots of COS cell-expressed opsins. ROS and COS cell membranes were solubilized in 1% SDS by vortexing 30 min at 4 °C. SDS−PAGE and blotting were performed using established procedures except that electrophoresis was carried out with the apparatus packed in ice to minimize rhodopsin multimerization. Blots were probed with mAb 4D2 (gift of R. Molday) which recognizes the N-terminus of rhodopsin. An $^{35}$S-labeled goat anti-mouse-IgG antibody (Amersham) was used as the secondary antibody. Blots were quantitated using a Molecular Dynamics PhosphorImager with ImageQuant v4.2 software.

*Rhodopsin Kinase Preparation.* A baculovirus stock containing a bovine rhodopsin kinase cDNA was the generous gift of K. Palczewski. This stock was used to purify plaques in Sf9 cells, and several clones were amplified. Suspension cultures of High Five cells ($10^6$ cells/mL) in Excell 405 media (JRH Biosciences) were infected at MOI = 5 and allowed to grow an additional 72 h. Cells were harvested in 50 mL aliquots and pelleted at 1000*g* for 10 min. Pellets can be stored at −80 °C for greater than 6 months, although freezing reduces the RK activity by ∼50%. RK was prepared from a single pellet by dounce homogenization (glass/glass) 5× in 3 mL of 0.4% Tween 80, 10 mM BTP, pH 7.5. The homogenate was mixed with 2.5 g of DE52 cellulose. The DE52 cellulose had been equilibrated in 30 mL of homogenization buffer, degassed, and allowed to settle, and then 25 mL of buffer was removed. The mixture of homogenate and DE52 cellulose was poured into a 1 × 10 cm glass column and washed with 30 mL of buffer at a

flow rate of 25 mL/h. RK was eluted in 100 mM NaCl, 0.4% Tween 80, 10 mM BTP, pH 7.5, at a flow rate of 12 mL/h; 1.5 mL fractions were collected; peak activity was usually found in fraction 5.

The activities of the RK fractions were determined as described (*30*) with modification. Twenty microliters of each fraction was incubated in a volume of 60 $\mu$L containing 20 $\mu$M urea-washed bovine rhodopsin, 100 $\mu$M [$\gamma$-$^{32}$P]ATP (100−1000 cpm/pmol), 2 mM $MgCl_2$, 20 mM BTP, pH 7.5, at 30 °C for 10 min. Reactions were quenched on ice, and then 40 $\mu$L was washed through nitrocellulose filters using a Millipore collection manifold as described below. Filters were counted in a scintillation counter, and the amount of ATP incorporated into rhodopsin was calculated. Activities ranged from 1 to 10 pmol of phosphate incorporated per microliter of kinase.

*$G_t$ Activity Assay.* A 15−30 pmol sample of opsin was regenerated in the dark at 4 °C in the presence of 360 $\mu$M 11-*cis*-retinal in a volume of 50 $\mu$L for 2−3 h on a vertically rotating platform at 30 rpm. Twelve microliters of regenerated opsin was then incubated in a volume of 35 $\mu$L which contained 3−8 $\mu$L (see below) of kinase (Figure 1a: $V_2$ and $V_3$) or kinase elution buffer ($V_1$) for 15 min at 30 °C under bright illumination in a buffer containing 30 mM BTP, pH 7.5, 3 mM $MgCl_2$, 0.5 mM ATP, and 1 mM DTT. Fifteen microliters of arrestin ($V_3$) or buffer ($V_2$ and $V_1$) was added to achieve 60% of the final indicated concentrations and incubated an additional 15 min. Arrestin or buffer was again added to achieve the final indicated concentrations, and the reaction volumes were increased to 90 $\mu$L and made 2 $\mu$M transducin, 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. The assay was initiated by the addition of 3 $\mu$M [$^{35}$S]GTP$\gamma$S. Total reaction volumes were 100 $\mu$L. Ten microliter aliquots were taken at 30 s intervals and washed with 15 mL of reaction buffer through nitrocellulose filters (Schleicher & Schuell) using a Millipore collection manifold. Filters were added to 5 mL of scintillation fluid (Amersham, BCS) and allowed to shake vigorously ≥30 min before counting. Reaction rates were expressed as picomoles of GTP$\gamma$S bound per minute; regressions were calculated using Sigma Plot software. At least three separate experiments were performed on at least two different transfections of each mutant, with the exception of the single threonine substitution mutants in which only one preparation of each was tested.

*Rhodopsin Phosphorylation Assays.* Phosphorylation of COS cell-expressed rhodopsin was carried out exactly as in the $G_t$ activity assay as described above with the following exceptions: the reactions were scaled up 5-fold and contained 0.1 mM [$\gamma$-$^{32}$P]ATP (360 Ci/mol). Arrestin was not added. After a 30 min incubation with bright illumination, reactions were terminated with 1 mL of 10 mM EDTA and vortexed for 10 min at room temperature followed by a 15 min centrifugation at 14 000 rpm. Supernatants were removed and pellets washed in 1 mL of 10 mM EDTA by vortexing 5 min followed by 15 min centrifugation. Supernatants were removed and pellets were solubilized in 20 $\mu$L of 1% SDS, 10 mM bis-tris propane, pH 7.5, and 10 $\mu$L of 3× loading dye. Pellets were vortexed for 30 min at 4 °C and then loaded onto 12% SDS−PAGE. Electrophoresis was carried out with the apparatus packed in ice. Gels were fixed by soaking in 15% MeOH, 5% acetic acid for 15 min and dried in a vacuum gel dryer. A radiolabeled ATP standard curve was constructed by spotting 5 $\mu$L dilutions of ATP on Whatman filter paper. The ATP standards and dried gels were analyzed with a Phosphorimager. The extent of phosphorylation was found to saturate at ~8 min of illumination (data not shown). The average phosphate incorporation per mole of wild-type

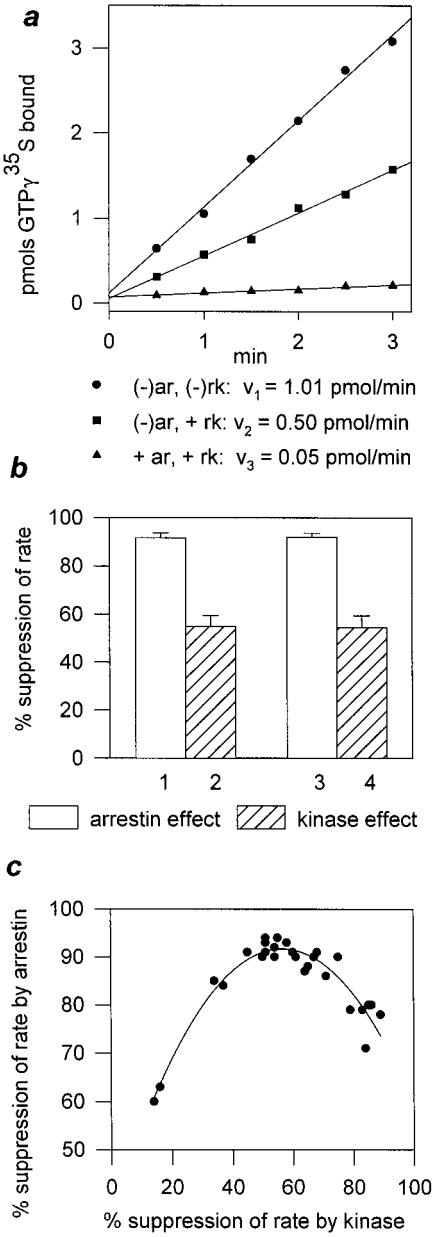


FIGURE 1: Arrestin assay. (*a*) Data from a single experiment with wild-type rhodopsin. The rhodopsin-catalyzed binding of GTP$\gamma$S to transducin is plotted: $V_1$ = rate (pmol/min) of the reaction in the absence of kinase and arrestin, $V_2$ = rate with kinase, $V_3$ = rate with kinase and 1.1 $\mu$M arrestin. (*b*) Effect of 1.1 $\mu$M arrestin is expressed as the percent suppression of the rate caused by the addition of arrestin: $(1 - V_3/V_2) \times 100$. The kinase effect is expressed as $(1 - V_2/V_1) \times 100$. Reactions in experiments 1 and 2 contained 25 nM rhodopsin and 1.5 $\mu$L of kinase; reactions in experiments 3 and 4 contained 50 nM rhodopsin and 3.0 $\mu$L of kinase. The arrestin effect is independent of the rhodopsin concentration as long the amount of kinase used is adjusted to produce a kinase effect of ~50%. (*c*) Dependence of the arrestin effect on the kinase effect. Data are from 27 experiments. The inhibitory effect of arrestin is greatest and consistent when an amount of kinase is used which depresses the activity of rhodopsin by 40−65%. Error = SD.

rhodopsin was found to be 1.2 ± 0.6 SE, $n = 11$; however, this is probably a low estimate (see Results and Discussion). All of the mutants possessing at least one phosphorylation site were found to be phosphorylated. We chose several of the mutants for a quantitative measure of phosphorylation. At least four separate determinations of the rhodopsin concentration and the extent of phosphorylation were made for each mutant. Phosphorylation data are expressed as the mean phosphate incorporation plus standard deviation divided by the mean of the measured rhodopsin concentration. Data were normalized with respect to wild-type.

## RESULTS

Rhodopsin's carboxyl serines are the preferred sites of phosphorylation by rhodopsin kinase. During the deactivation of rhodopsin, the serines are therefore expected to promote high-affinity arrestin binding. To assess the importance of the serines and to look for positional information within the seven potential phosphorylation sites, phosphorylation site mutants of rhodopsin were analyzed for the ability to be phosphorylated by rhodopsin kinase (RK) and to be quenched by arrestin in an in vitro $G_t$ activity assay.

The rate at which light-activated rhodopsin (R*) catalyzed the binding of GTP$\gamma$S to $G_t$ was measured under each of three conditions (Figure 1a): in the absence of RK and arrestin ($V_1$), with RK and no arrestin ($V_2$), and with both RK and arrestin ($V_3$). The effect of RK is expressed as the percent difference between $V_1$ and $V_2$. The effect of arrestin as the percent difference between $V_2$ and $V_3$. Because the rate of GTP$\gamma$S binding is dependent on the rhodopsin concentration, a chief concern is that error would be introduced when making comparisons between different rhodopsin preparations. The final rhodopsin concentration in the assays ranged from ~30 to 100 nM. Figure 1b demonstrates that the effect of 1.1 $\mu$M arrestin is independent of the rhodopsin concentration within this range as long as the amount of kinase is adjusted to produce a suppression of about 50%.

The activity of RK was found to decay rapidly, with a half-life of ~12 h. Consequently, RK was freshly prepared for each experiment. The amount of RK used in the arrestin assay was determined empirically for each preparation. $G_t$ activity assays were performed on wild-type rhodopsin to determine the amount of RK required to quench the activity of rhodopsin ~50%. The effect of arrestin on wild-type was then confirmed by demonstrating that 1.1 $\mu$M arrestin could suppress the rate an additional ~90% (Figure 1b). The results of 27 sets of assays are plotted in Figure 1c, demonstrating that the effect of arrestin is maximal and consistent within the range of a 40−65% kinase effect. We speculate that the effect of RK on rhodopsin activity is the sum of the effects of rhodopsin phosphorylation and of competition between RK and $G_t$. We have measured the extent of phosphorylation of wild-type rhodopsin to be 1.2 ± 0.6 P/R SE in our assays. This is probably a minimum estimate. Perhaps as much as 40% of rhodopsin molecules remain unphosphorylated under a variety of conditions (*3*, *7*, *11*). The fraction of denatured rhodopsin in our preparations is unknown; because our quantitation method would include these denatured opsins, our measurements of P/R are probably low, and may differ between membrane preparations. Thus, although we have measured the extent of phosphorylation of some of the more important mutants, the phosphorylation data should not be used to exclude the possibility that small changes in the extent of phosphorylation could account for the observed differences in arrestin binding between mutants.

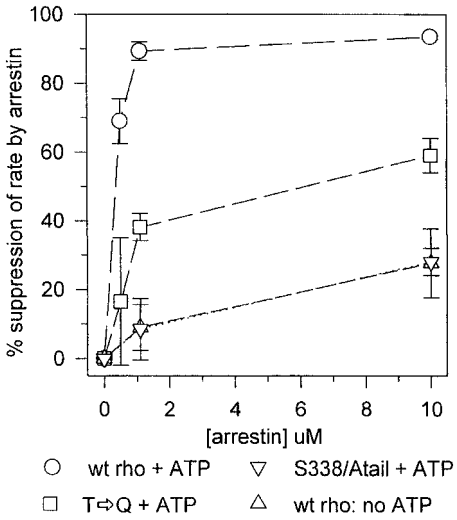


FIGURE 2: Rhodopsin's carboxyl serines are not sufficient for wild-type quench by arrestin. The effect of arrestin at concentrations of 0.0, 0.5, 1.1, and 10 $\mu$M is plotted. Note that the data for mutant S338/Atail (in the presence of ATP) overlap those with wild type in the absence of ATP, indicating that a single serine is not sufficient for phosphorylation-dependent quench by arrestin. The presence of all three carboxyl serines in the T→Q mutant promoted relatively poor quench by arrestin, even at 10 $\mu$M arrestin. 1.1 $\mu$M arrestin was used in subsequent experiments because it was sufficient to provide strong deactivation of wild type. Error = SD.

*Rhodopsin's Serines Cannot Support Wild-Type Quench by Arrestin.* The effects of different concentrations of arrestin on wild type and mutants S338/Atail and T→Q are plotted in Figure 2 (see Table 1 for amino acid sequences of the mutants). S338/Atail is a mutant in which only the preferred phosphorylation site, S338, is retained. The data for S338/Atail with ATP overlap those of wild type without ATP, indicating that S338/Atail exhibits no discernible phosphorylation-dependent arrestin quench, even at a near-physiological level of arrestin (10 $\mu$M). S338/Atail is a good substrate for RK, however (Figure 3b), being phosphorylated to $>1/3$rd the level of wild type while possessing only $1/7$th the number of potential phosphorylation sites. Identical results were obtained with a mutant S334/Atail (data not shown). The presence of all three serines in the T→Q mutant provides for only a small phosphorylation-dependent quench (Figure 2). These results led us to hypothesize that rhodopsin's threonine residues may be more important than previously assumed.

*The Function of Serine in Rhodopsin Quench Can Be Performed by Threonine yet the Reverse Is Not True.* Figure 3a demonstrates that replacing rhodopsin's serines with threonines (all-Thr mutant) results in wild-type quench by arrestin. The reverse mutations (all-Ser), however, result in a 20% reduction in quench compared to wild type. Note that these mutants do not differ significantly in their ability to become phosphorylated (Figure 3b). The mutant S→Q, which has only four threonine residues, is quenched to the same extent (Figure 4a) as is the all-Ser mutant with seven potential phosphorylation sites (Figure 3a), even though S→Q is a

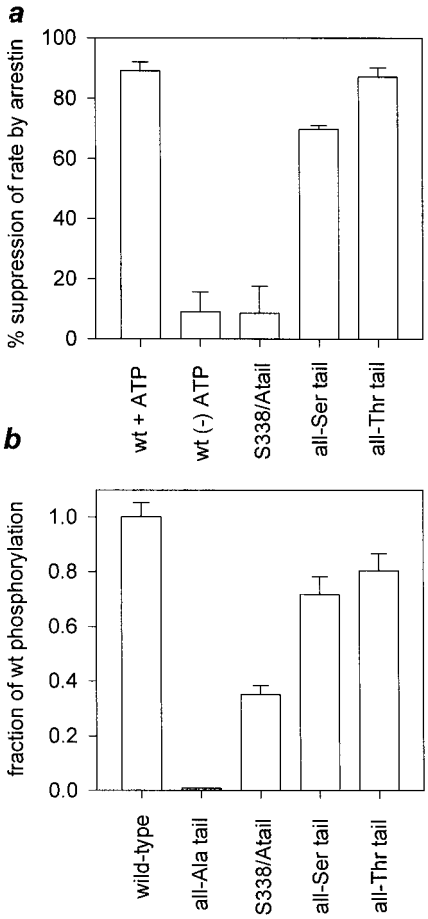


FIGURE 3: (*a*) Arrestin inhibition of all-serine and all-threonine tail mutants compared with inhibition of wild type and with mutant S338/Atail, which demonstrates no phosphorylation-dependent deactivation by arrestin. Arrestin concentration is 1.1 $\mu$M. The effect of arrestin on the all-Thr mutant is identical to wild type, indicating that phosphothreonine can promote wild-type arrestin binding. However, phosphoserines in the all-serine mutant were not sufficient for wild-type quench by arrestin. (*b*) Phosphorylation assay of clones in (*a*) plus the all-alanine mutant. Data are normalized to the mean level of wild-type phosphorylation. The single serine of S338/Atail is a good substrate for RK ($\geq$0.5 P/R; see Results and Discussion sections for analysis of the extent of phosphorylation). No significant differences were detected in the extent of phosphorylation of the all-threonine and all-serine tail mutants, indicating that threonine is a good substrate for RK under the conditions of the assay. Lack of phosphorylation of the all-alanine mutant demonstrates that phosphorylation is limited to the seven putative sites. Error = SD.

much poorer substrate for the kinase (Figure 4b) than all-Ser (Figure 3b). Importantly, the results with the all-Thr mutant demonstrate that threonine is an adequate substrate for RK under the conditions of the assay and also that phosphothreonine can mediate wild-type quench by arrestin.

*Threonine Promotes Greater Rhodopsin Quench than Does Serine.* Replacing the existing serines in the T→Q mutant with threonines (T→Q,S→T) dramatically increases the ability to be quenched by arrestin (Figure 4a). The reverse is not true, however. S→Q is a good substrate for arrestin, but replacing the existing threonines with serines (S→Q,T→S) dramatically reduces arrestin binding. Significantly, the differences in the abilities of serine and threonine to promote quench by arrestin correlate not with the positions of these amino acids in the peptide chain but with the identity of the side chains. These results clearly demonstrate that the

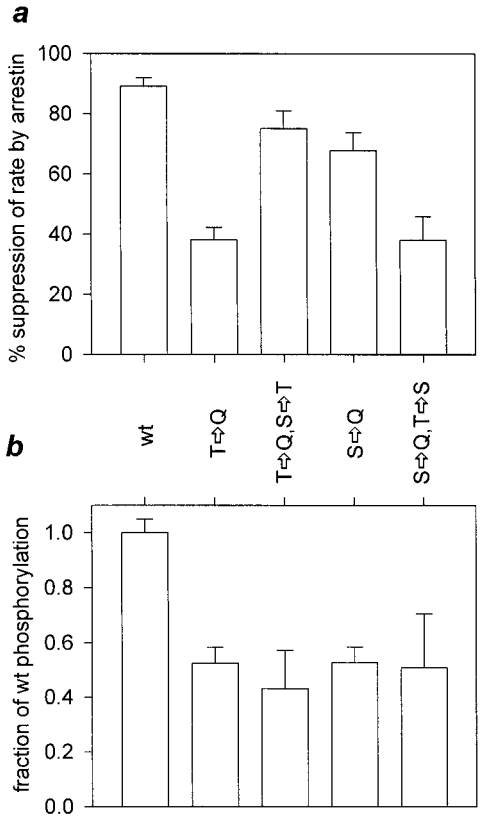


FIGURE 4: (*a*) A mutant having the carboxyl threonines replaced with glutamine (T→Q) is a poor substrate for arrestin. Mutation of the remaining serines to threonines (T→Q,S→T) dramatically improves quench. A mutant lacking the endogenous serines (S→Q), however, is a good substrate for arrestin. Replacing the remaining endogenous threonines with serines (S→Q,T→S) diminishes binding. Note that threonine promotes greater quench by arrestin regardless of its position: compare S→Q and T→Q,S→T. Conversely, serine promotes poor quench by arrestin regardless of position: compare T→Q and S→Q,T→S. (*b*) Phosphorylation assay of clones in (*a*); mutants do not differ significantly in their ability to be phosphorylated, indicating that differences in quench by arrestin are unlikely to be due to the extent of phosphorylation.

threonine residues contribute more to the rhodopsin/arrestin interaction than do the serines.

To determine which threonines may be most important, a series of mutants were constructed where a single threonine was replaced by glutamine (Q mutants) and another series where all the threonines except one were replaced by glutamine (QT mutants). The results depicted in Figure 5 indicate that any of the threonines can be deleted with only a small effect on rhodopsin quench, but that T340 alone seems to contribute the most to the rhodopsin/arrestin interaction. Two other mutants, Q335,336 and Q340,342, were also tested, and both were found to have an arrestin effect of approximately 60−70% (data not shown). These results strongly suggest that the positions of the threonines are not critical to rhodopsin quench by arrestin. Interestingly, however, the structure of QT340 provides an excellent substrate for arrestin.

## DISCUSSION

The physiological consequence of arrestin quench in vivo is still not completely understood. Recent studies in transgenic mice suggest that the amplitude of a photoresponse is determined by rhodopsin kinase (*31*), while the rate of return

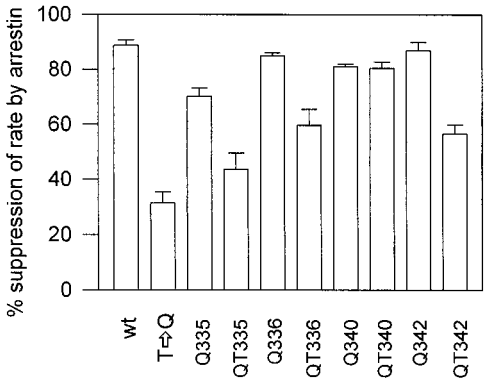


FIGURE 5: Analysis of individual threonines: an individual threonine either is substituted with glutamine (Q mutants) or is the only threonine remaining (QT mutants). Substitution of any one of the threonines produces only a modest decrease in quench by arrestin: Q335 is 21% reduced compared to wild type, and Q342 is essentially unchanged. By itself, T340 promotes the greatest quench by arrestin (QT340).

to base line may be determined by arrestin or its splice variant p44 (*32*). Rhodopsin's carboxyl serine residues are reported to be the preferential sites of phosphorylation by RK and are therefore thought to mediate arrestin binding.

We have developed a quantitative method for assessing arrestin function in vitro. This system has several advantages compared to other reported assays. It measures arrestin function, not simply arrestin binding, and it employs a direct measure of function at the first step in the signal transduction cascade. The central findings of our study are that the serines are not sufficient to provide for wild-type quench by arrestin, and that the threonines contribute more to the arrestin/rhodopsin interaction than do the serines. Based on these results, we propose that the affinity of rhodopsin for arrestin can be modulated by controlling not only the number of phosphorylation events but also the positions of the phosphorylated residues.

A preliminary study of transgenic mice (*22*) found that knockout of both S338 and S343 produced no defects in photoresponse sensitivity or shut off. However, the remaining serine at 334 could potentially mediate deactivation. S334 and S338 have been shown to be equally likely targets for phosphorylation in live mice (*11*). That any one of the serines is necessary for arrestin binding has been ruled out by an in vitro study of rhodopsin mutants (*6*). Whether or not the serines were sufficient for arrestin binding was also addressed; it was found that a mutant possessing only S338 and S343 bound arrestin to only ~10% that of wild type. Interestingly, a mutant possessing only S343 and T340 was able to bind to ~20% that of wild type. It is unclear if the reduced binding capacity of these mutants would affect their ability to be quenched by the high arrestin concentrations found in vivo. We have found that mutants possessing only a single phosphorylatable serine are virtually incapable of being quenched at near-physiological concentrations of arrestin (Figure 2).

The arrestin concentration in a dark-adapted ROS is estimated at 10−15 $\mu$M (*1*). In an assay that measured the heat evolved from cGMP hydrolysis in a highly concentrated bovine ROS homogenate, Langlois et al. found that the effect of arrestin began to saturate at 5−10 $\mu$M arrestin (*1*). We obtained comparable results (Figure 2) with saturation beginning at 1−5 $\mu$M arrestin. Thus, it is unlikely that our mutants which possess only a single phosphorylatable serine would be quenched in vivo by arrestin.

It has been well established that in vitro phosphorylation reactions are cooperative (*12*, *13*, *33*). In phosphorylation preparations averaging 2 P/R, the predominate species are 0 P/R and 3 P/R (*12*). The fact that the activity of wild-type rhodopsin in our assays is suppressed ~85% (Figure 2) demonstrates that the maximum amount of active unphosphorylated rhodopsin is ≤15%. The actual number may be even less if arrestin cannot deactivate singly phosphorylated rhodopsin. A level of 15% unphosphorylated rhodopsin would correspond to an average of 3−4 P/R in the experiments reported by Wilden (*12*). How the cooperativity of the reaction may change under different conditions is unknown.

The actual sites of phosphorylation by RK under our conditions are also unknown. Using truncated rhodopsins, several groups have demonstrated that rhodopsin phosphorylation is confined to the C-terminus of rhodopsin. Comparison of mutants all-Ala and S338/Atail (Figure 3b) demonstrates that, with a full-length carboxyl tail, phosphorylation is also limited to the C-terminal. The robust phosphorylation of S338/Atail (35% of wild type) emphasizes the relatively lax substrate specificity of RK and is consistent with the conclusions of Palczewski et al. (*5*) that RK recognition of rhodopsin is mediated primarily by interaction with rhodopsin's cytoplasmic loops. No discernible RK substrate consensus sequence or motif has been identified, although there is some observable pattern in the hierarchy of site preference (*34*).

Although the overwhelming consensus of many reports is that the serine residues are the preferred substrates for phosphorylation, we find that mutants possessing only threonines are phosphorylated to the same extent as those with serines. The extent of phosphorylation correlates more with the number of available sites and not with their identity. The all-Ser and all-Thr mutants have seven potential phosphorylatable sites and do not differ significantly from each other, and only slightly from wild type, in their ability to be phosphorylated (Figure 3b). Similarly, loss of either the serines (S→Q) or the threonines (T→Q) results in an equivalent reduction in the extent of phosphorylation (Figure 4b). Interestingly, interchanging the identities of the residues in the deletion mutants (T→Q,S→T and S→Q,T→S) has no discernible effect on phosphorylation (Figure 4b). Adamus et al. (*19*) demonstrated that phosphorylation at certain residues promoted faster rates of subsequent phosphorylation. The above data suggest that the identity of the first phosphorylated residue may not be critical to the extent of the reaction, however.

The finding that rhodopsin's C-terminal serine residues did not support wild-type quench by arrestin (Figure 2) was surprising and led us to examine the role of the threonines. The all-Thr mutant is capable of wild-type quench by arrestin, and the all-Ser mutant is not (Figure 3a). These data demonstrate conclusively that phosphothreonine can mediate wild-type quench by arrestin and that phosphoserine is not sufficient for wild-type efficacy of quench. The possibility remains, however, that the strict requirement for threonine exists in a function other than phosphorylation. This idea was addressed by Zhang et al. (*6*), who found that T340 was required for wild-type arrestin binding but did not signifi-

cantly affect the extent of phosphorylation. Perhaps the specificity of the arrestin/rhodopsin interaction is maintained by the unphosphorylated side chain of T340. Puig et al. (*35*) found that a hepta-phosphorylated peptide corresponding to the C-terminal of rhodopsin induced a conformational change in arrestin and promoted the binding of arrestin to unphosphorylated rhodopsin. Palczewski et al. (*36*) found that heparin induced a conformational change in arrestin but did not induce binding. These results suggest that the phosphorylated C-terminal domain of rhodopsin provides more to the interaction than negative charge. However, because the hepta-phosphorylated peptide contained phosphorylated T340, it is doubtful that threonine could provide its presumed specificity function in both the phosphorylated and unphosphorylated states. The data in Figure 4a also argue against the idea of threonine providing a specific structural role because the results clearly indicate that threonine, not the position of threonine, is critical to higher affinity arrestin quench.

Zhang et al. (*6*) found that substitution of T340 with either alanine or glutamic acid produced similar decreases in arrestin binding, to about 50% that of wild type. Interestingly, they did find that a mutation of S343 to glutamic acid bound arrestin better than that with alanine. This increase in arrestin binding reflected the dramatically enhanced phosphorylation of the mutant S343E. Rhodopsin kinase prefers acidic residues on the carboxyl side of the phosphorylation site (*37*); thus, the increased arrestin binding could be due to enhanced phosphorylation of T342 in the S343E mutant. These data suggest that arrestin binding, itself, may be little affected by the differences between alanine and glutamic acid. Our results with glutamine substitution of T340 were not as dramatic (Figure 5), reducing arrestin quench by only 8% compared to wild type. The effect of single substitution at any of the threonine sites ranged from 0 to 21% (Figure 5). This effect was approximately doubled by knocking out both T335 and T336 or both T340 and T342 (data not shown). Again, these results emphasize that the positions of the threonines are not critical to arrestin quench. Interestingly, however, T340 was found to promote arrestin quench significantly better than the other threonines when only one threonine was present (mutant QT340, Figure 5). Inspection of the data in Figure 5 of the QT mutants suggests that position 340 is in some way optimal, because the closer the remaining threonine is to 340 the more efficient the quench is.

The results of this study indicate that rhodopsin's carboxyl threonines, known to be phosphorylated last, make a greater contribution to the efficacy of quench than do the serines, which are phosphorylated initially. Interestingly, however, mutants possessing only a single phosphorylated serine demonstrate no phosphorylation-dependent quench by arrestin. Langlois et al. provided strong evidence that, under conditions of dim illumination, rhodopsin deactivation is performed by p44 and not arrestin (*1*). p44 is thought to have a relaxed energetic barrier toward binding R* relative to arrestin (*38*). Perhaps a single phosphoserine is sufficient to promote p44-mediated quench of R*.

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