Arrestin with a Single Amino Acid Substitution Quenches Light-Activated Rhodopsin in a Phosphorylation-Independent Fashion†

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ABSTRACT: Arrestins are members of a superfamily of regulatory proteins that participate in the termination of G protein-mediated signal transduction. In the phototransduction cascade of vertebrate rods, which serves as a prototypical G protein-mediated signaling pathway, the binding of visual arrestin is stimulated by phosphorylation of the C-terminus of photoactivated rhodopsin (Rh*). Arrestin is very selective toward light-activated phosphorhodopsin (P-Rh*). Previously we reported that a single amino acid substitution in arrestin, Arg175Gln, results in a dramatic increase in arrestin binding to Rh* [Gurevich, V. V., & Benovic, J. L. (1995) *J. Biol. Chem. 270*, 6010−6016]. Here we demonstrate that a similar mutant, arrestin(R175E), binds to light-activated rhodopsin independent of phosphorylation. Arrestin(R175E) binds with high affinity not only to P-Rh* and Rh* but also to light-activated truncated rhodopsin in which the C-terminus phosphorylation sites have been proteolytically removed. In an *in vitro* assay that monitored rhodopsin-dependent activation of cGMP phosphodiesterase (PDE), wild type arrestin quenched PDE response only when ATP was present to support rhodopsin phosphorylation. In contrast, as little as 30 nM arrestin(R175E) effectively quenched PDE activation in the absence of ATP. Arrestin(R175E) had no effect when the lifetime of Rh* no longer contributed to the time course of PDE activity, suggesting that it disrupts signal transduction at the level of rhodopsin−transducin interaction.

In vertebrate retinal rods light initiates the transduction process by converting dark rhodopsin (Rh)[1] to metarhodopsin II (Rh*), an excited intermediate which stimulates cGMP phosphodiesterase (PDE) by catalytically activating transducin (T), a photoreceptor-specific G protein. G protein-coupled receptors are generally thought to be inactivated by a common mechanism. First, receptors are phosphorylated by a member of a family of G protein-coupled receptor kinases. Phosphorylated activated receptor then binds a member of the arrestin family of regulatory proteins, which shuts off further signaling (Sterne-Marr & Benovic, 1995). This process thus controls the gain of the first stage of signal transduction and also sets a limit on the temporal resolution of the signaling pathway.

Arrestin binds preferentially to the phosphorylated form of light-activated rhodopsin (P-Rh*), but it also shows significant binding to phosphorylated dark rhodopsin (P-Rh) as well as to unphosphorylated Rh*. These observations are consistent with arrestin having distinct binding sites that independently recognize the activation and phosphorylation state of rhodopsin and promote a conformational change that increases the binding affinity when both sites are simultaneously occupied by light-activated phosphorylated rhodopsin (Schleicher et al., 1989; Gurevich & Benovic, 1992, 1993, 1995; Gurevich et al., 1994). Recently we found that three positively charged residues, Arg171, Arg175, and Lys176, play a major role in arrestin interaction with phosphate moieties on the C-terminus of phosphorhodopsin and that Arg175 might function as a phosphorylation-sensitive "trigger" (Gurevich & Benovic, 1995). To further investigate the functional role of Arg175 we studied the effects of replacing Arg175 with negatively charged glutamic acid on arrestin binding to various functional forms of rhodopsin and on its ability to quench rhodopsin activation of PDE.

EXPERIMENTAL PROCEDURES

Mutagenesis. Bovine visual arrestin cDNA (Shinohara et al., 1987) was subcloned under control of SP6 promoter into pARR-VSP plasmid (Gurevich & Benovic, 1995). The antisense primer 5′-cat atc ccg cgg cgc atg ctg tac ctt **ctc** gat cag cag acg-3′ (anticodons 171−184, anticodon 175 underlined) was used with the sense primer 5′-ag agc ctg atc aag aag ctg-3′ (codons 105−111) to generate a 239 bp fragment by PCR. The fragment was purified, digested with *Sal*I and *Sac*II, and subcloned into *Sal*I/*Sac*II-digested pARR-VSP. The sequence of the PCR-generated portion was confirmed by the dideoxy method.

Arrestin Expression

In Vitro Translation. pARR-VSP and pARR-VSP(R175E) were linearized with *Hind*III. *In vitro* transcription and

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[1] Abbreviations: Rh, dark rhodopsin; Rh*, light-activated rhodopsin; P-Rh, dark phosphorylated rhodopsin; P-Rh*, light-activated phosphorylated rhodopsin; ^{329}G-Rh*, light-activated rhodopsin, from which the C-terminus beginning with residue 330 was proteolytically removed; T, transducin; PDE, cGMP phosphodiesterase; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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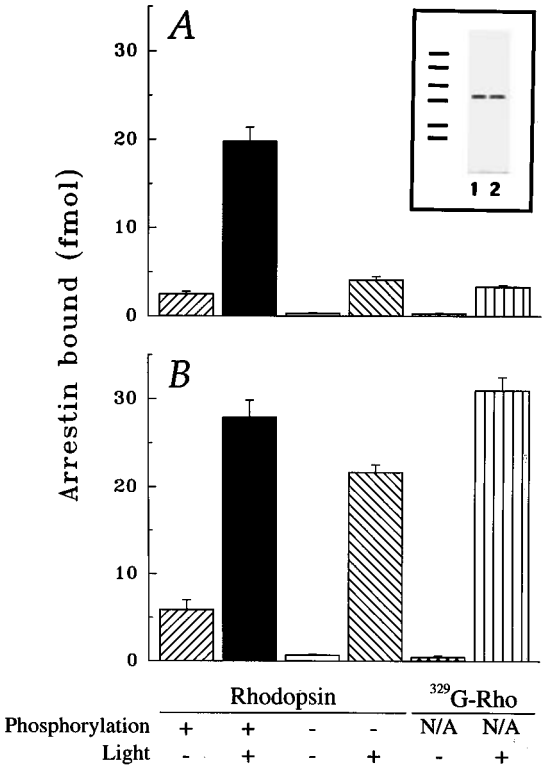

FIGURE 1: Binding selectivity of wild type arrestin (A) and mutant arrestin(R175E) (B). The binding of tritiated arrestins (100 fmol, specific activities 947 and 963 dpm/fmol, respectively) to 0.3 μg of the indicated functional form of rhodopsin is shown. Means ± SD of three experiments performed in duplicate are presented. Inset: 2 μL (about 100 fmol) of *in vitro* translated wild type arrestin (lane 1) and arrestin(R175E) (lane 2) were subjected to SDS−PAGE. The gel was soaked in 20% diphenyloxazole in glacial acetic acid. The gel was then soaked in water (to precipitate the fluorochrome), dried, and exposed for 24 h to X-ray film (Fuji). The positions of prestained protein markers (Sigma) with apparent molecular weights (in kDa) of 123, 89, 67, 49, 37.5, and 34 are shown.

translation were performed as described (Gurevich & Benovic, 1995). Both arrestin proteins were labeled by incorporation of [^{3}H]leucine with specific activity 12−14 Ci/mmol, resulting in the specific activities of arrestin proteins of 400−450 Ci/mmol (900−1000 dpm/fmol). The expression of wild type and mutant arrestins in the *in vitro* translation system yielded single-labeled protein bands of the expected mobility on SDS−PAGE (Figure 1A, inset). Previously (Gurevich & Benovic, 1992) we have demonstrated that partially purified *in vitro* translated wild type arrestin has the same ability to bind to P-Rh* as purified bovine retinal arrestin. In order to estimate the percentage of functional proteins expressed for these experiments, we varied the amount of P-Rh* in the binding assay (see below) from 0.075 to 2.4 μg/assay. We found that up to 85−90% of both wild type and mutant arrestins are capable of specific (light-dependent) binding to P-Rh*, suggesting that >85% of the radiolabeled arrestins are fully functional.

Expression in Escherichia coli. Wild type arrestin and the R175E mutant were expressed in *E. coli* BL21 cells using vector pTrcHis-B (Invitrogen) and purified by sequential heparin-Sepharose and Q-Sepharose chromatography, as described (Goodman et al., 1996). Briefly, BL21 cells transformed with the appropriate plasmid were grown for 7−8 h in LB with 100 μg/mL ampicillin at 30 °C, then IPTG was added to 20−30 μM, and the incubation was continued for 12 h. Pelleted cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM DTT, 2 mM benzamidine, 1 mM PMSF, 10 μM leupeptin, 0.7 μg/mL pepstatin A, 10 μM chymostatin). Lysis was accomplished by a brief freezing at −80 °C followed by 15 min incubation on ice with 0.07 mg/mL lysozyme and sonication (5 × 1 min). $MgCl_2$ and DNase I were added to final concentrations of 7 mM and 0.02 mg/mL, respectively. Cell debris was pelleted at 20000*g*, 30 min. Protein was precipitated by ammonium sulfate (0.6 g/mL) and pelleted by centrifugation. The protein pellet was dissolved in 100 volumes of column buffer (CB) (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, and the same cocktail of protease inhibitors as in lysis buffer), filtered (0.8 μm membrane), and applied to a 20 mL column of heparin-Sepharose, equilibrated with CB/0.15 M NaCl, at 2 mL/min. The column was washed with 200 mL of CB/0.15 M NaCl. Arrestin was eluted with the linear gradient CB/0.15 M NaCl → CB/0.4 M NaCl (200 + 200 mL). Arrestin-containing fractions were concentrated (Amicon, membrane YM-30), diluted with CB, and applied to a 10 mL Q-Sepharose column, equilibrated with CB at 1 mL/min. The column was washed with 50 mL of CB, and arrestin was eluted with the linear gradient CB → CB/0.1 M NaCl (200 + 200 mL). Peak arrestin-containing fractions were pooled, concentrated by ultrafiltration, and stored at −80 °C. Both arrestin proteins were >95% pure (Figure 2B, inset). In order to ascertain that these proteins are functional, we compared the inhibition of 1 nM [^{3}H]-arrestin binding to 50 nM P-Rh* in our standard assay caused by 1−500 nM concentrations of recombinant wild type arrestin, arrestin(R175E), and purified bovine retinal arrestin. Under these conditions 50% inhibition of specific binding was achieved at 86.9 ± 7.3, 33.5 ± 2.6, and 81.8 ± 5.2 nM, respectively.

These data suggest that the activity of purified recombinant wild type arrestin is comparable to that of bovine retinal arrestin. The somewhat higher potency of arrestin(R175E) likely reflects its higher affinity for P-Rh*.

Arrestin Binding. Tritiated arrestin (100 fmol, 2 nM final concentration) was incubated with 0.3 μg (150 nM) of different functional forms of rhodopsin at 37 °C for 5 min in 50 μL of 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol in the dark or in room light. The samples were cooled on ice and loaded onto 2 mL Sepharose 2B columns equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA at 4 °C under dim red light. Bound arrestin eluting with ROS membranes in the void volume (0.5−1.1 mL) was collected and quantitated in a liquid scintillation counter. Nonspecific binding was determined in the presence of 0.3 μg of liposomes and subtracted. For binding experiments rhodopsin was phosphorylated by recombinant rhodopsin kinase to the stoichiometry of 1.4 mol of phosphate/1 mol of rhodopsin and regenerated with 11-*cis*-retinal. Truncated ^{329}G-Rh was prepared as described (Palczewski et al., 1991).

PDE Assay. cGMP hydrolysis was monitored as described (Yee & Liebman, 1978). A pH electrode (Orion model 8103) was positioned in a rapidly stirred 500 μL cuvette containing ROS homogenate in a buffer made up of (mM): 5 cGMP, 0.5 GTP, 13 NaCl, 14 KCl, 120 KAsp, 5 Hepes (pH 8.0 with KOH), and either 3.4 or 1.45 $MgSO_4$ with or without 2 mM ATP, respectively. Free Ca was adjusted to 600 nM with the addition of 1 mM EGTA and 0.714 mM

Ca (in the presence of ATP) or 0.986 mM Ca (in the absence of ATP). The system was calibrated with 5 μL additions of 0.01 N HCl. The electrode had a response time of $t = 1.49 \pm 0.09$ s. Delivery of the light flash and digitization of pH meter output was controlled by a computer operating FASTLAB45 data acquisition software (Indec systems). Inactivation kinetics were approximated as a single-exponential time course using SigmaPlot software (Jandel Scientific). Experiments were performed at room temperature (22 °C). ROS homogenate contained 4 μM rhodopsin, 37 ± 1.1 nM endogenous wild type arrestin, and 22.1 ± 2.3 nM endogenous truncated arrestin splice variant p44 (13) as determined by quantitative immunoblotting using monoclonal anti-arrestin antibodies F4C1 (kindly provided by Dr. L. A. Donoso), peroxidase-conjugated secondary goat anti-mouse antibodies (Boehringer), and SuperSignal enhanced chemiluminescense system (Pierce).

Quantification of endogenous arrestins was performed as follows. In preliminary experiments with purified arrestin we found that the response is linear in the range of 2 ng to 10 ng per lane. Five standard samples of purified arrestin (2−10 ng/lane) and five different dilutions of ROS homogenate were loaded onto each gel. After transfer onto PVDP membrane and immunovisualization gray values of corresponding bands were determined using imaging software. In each experiment the intensities of at least three bands of arrestin and three bands of p44 were within linear range of the calibration curve derived from five standard samples run on the same gel. These were used for the quantification of endogenous arrestin and p44. Experiments were performed twice; obtained mean values ± SD are presented above.

RESULTS

Arrestin(R175E) was functionally expressed in an *in vitro* translation system (Gurevich & Benovic, 1992, 1993, 1995), and its binding characteristics were compared with those of wild type arrestin. Arrestin(R175E) showed strong phosphorylation-independent binding to all forms of light-activated rhodopsin including P-Rh*, Rh*, and even ^{329}G-Rh*, a truncated form of rhodopsin lacking C-terminal phosphorylation sites. In all cases the binding required light-activation of rhodopsin, with levels as high as or higher than the binding of wild type arrestin to P-Rh* (Figure 1).

To test if arrestin(R175E) can quench the phototransduction cascade in a phosphorylation-independent manner, wild type and R175E mutant arrestins were functionally expressed in *E. coli* and then purified proteins were tested in a PDE assay. The time course of the cGMP hydrolysis evoked by a brief subsaturating light flash in a bovine rod outer segment (ROS) homogenate is shown in Figure 2A with no additions (control) or with the addition of 10 μM of either wild type arrestin or arrestin(R175E) or 2 mM ATP. The recovery of light-stimulated PDE activity to its basal dark level is seen more clearly in Figure 2B, where the rates of cGMP hydrolysis are plotted as a function of time. Inactivation rate was not affected by wild type arrestin in the absence of ATP but was dramatically accelerated (about 10-fold) by the addition of arrestin(R175E) or ATP. Neither arrestin(R175E) nor ATP affected the initial activation kinetics, while both agents accelerated inactivation, decreasing the amplitude and time-to-peak of the response. The concentration dependence of the effect of arrestin(R175E) on the peak amplitude, time-to-peak, and inactivation rate constant of the flash-evoked cGMP hydrolysis is plotted in Figure 3. In the absence of ATP inactivation was facilitated to the same degree by 100 nM arrestin(R175E) and 10 μM wild type arrestin. A 2-fold increase in inactivation rate constant was observed with about 300 nM arrestin(R175E). This concentration of mutant arrestin also caused about a 10% decrease in both amplitude and time-to-peak of light-activated cGMP hydrolysis (Figure 3). Maximum (10-fold) increase in inactivation rate as well as about 2-fold decrease in peak amplitude and time-to-peak were observed with 10 μM arrestin(R175E). The same concentration of wild type arrestin had no appreciable effect on the latter two parameters of the response.

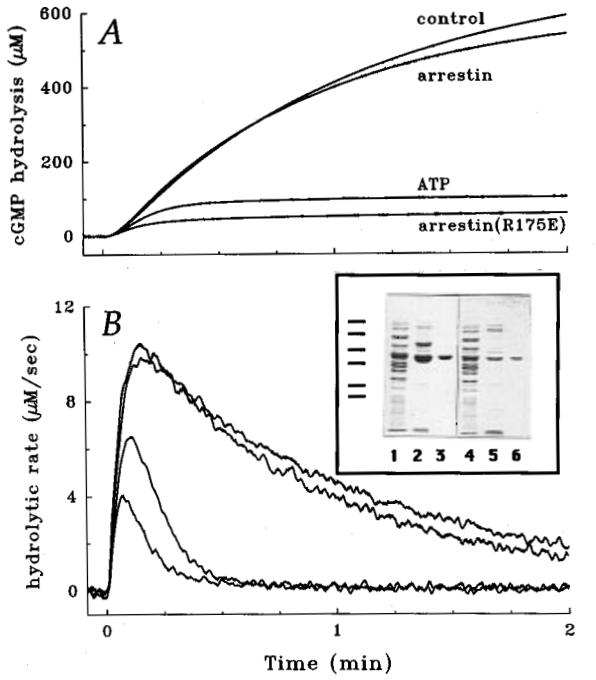

FIGURE 2: Mutant arrestin(R175E) quenches light-activated phosphodiesterase without rhodopsin phosphorylation. A 200 ms subsaturating light flash, causing a fractional bleach of 2.6×10^{-5}, was delivered at time zero to stimulate cGMP hydrolysis in bovine ROS homogenate containing 4 μM rhodopsin, 37 nM endogenous wild type arrestin, and 19 nM p44 (as determined by quantitative Western blotting), 0.5 mM GTP (to support G protein activation) in the absence or presence of 10 μM exogenous arrestin (wild type or R175E), or 2 mM ATP. Each trace shows the average of three to six trials. The amount of cGMP hydrolyzed in response to the light flash is plotted in panel A. Panel B shows the rate of hydrolysis (the first derivative of the traces in A). Average basal rate of cGMP hydrolysis in darkness (for control, or in the presence of 10 μM wild type arrestin, 10 μM R175E mutant, or 2 mM ATP it was 1.5 ± 0.3, 1.2 ± 0.1, 0.22 ± 0.02, and 0.32 ± 0.06 μM/s, respectively) was subtracted from the traces in A. Inactivation rates for control or in the presence of 10 μM wild type arrestin, 10 μM R175E mutant, or 2 mM ATP were (s^{-1}) 0.0158 ± 0.0009 ($n = 5$), 0.019 ± 0.002 ($n = 3$), 0.155 ± 0.009 ($n = 6$), and 0.15 ± 0.01 ($n = 6$), respectively. The peak rate of cGMP hydrolysis evoked by a saturating light step was ~50 μM/s in all four conditions. Inset: purification of wild type arrestin (lanes 1−3) and arrestin(R175E) (lanes 4−6) expressed in BL21 cells. Aliquots of protein before (lanes 1 and 4) and after (lanes 2 and 5) heparin-Sepharose chromatography and of Q-Sepharose-purified protein (lanes 3 and 6) were subjected to SDS−PAGE. The gels were stained with Coomassie R-250, destained, and photographed. The positions of the same protein markers as in Figure 1 are indicated.

To explore the possibility that mutant arrestin accelerated inactivation by acting on elements "downstream" of Rh* (e.g., activated T or PDE), a stable Rh*−transducin complex was first formed by bleaching ROS membranes in the

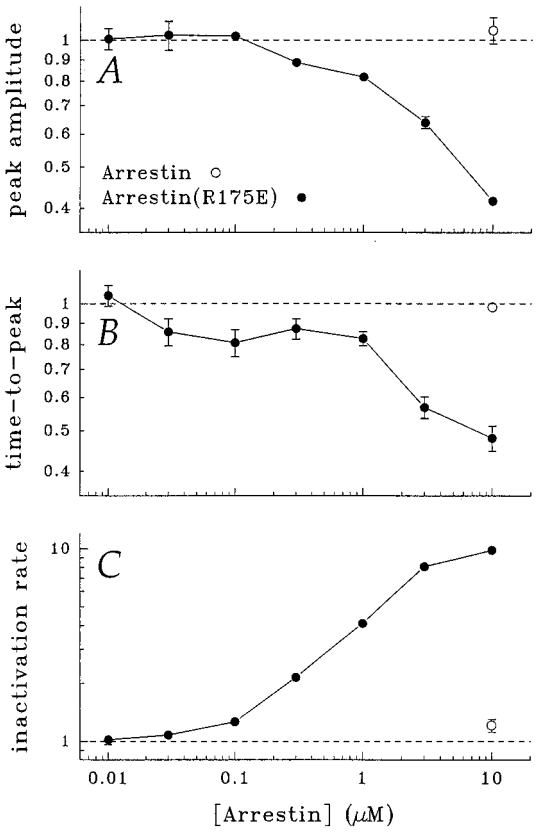

FIGURE 3: Concentration dependence of arrestin(R175E)-induced quench of light-stimulated PDE activity. The concentration of arrestin(R175E) (●) was varied from 10 nM to 10 μM in the absence of ATP using the same experimental conditions as in Figure 2. The peak rate (A), time-to-peak (B), and inactivation rate constant (C) of light-evoked cGMP hydrolysis are plotted relative to the average response observed in the absence of exogenous arrestin which had values of 9.9 ± 0.5 μM/s, 9.0 ± 0.5 s, and 0.0158 ± 0.0009 s^{-1} (t = 64 ± 3 s), respectively. Each data point is the mean of three to six separate determinations and shows the standard error of the mean except when smaller than the plotted symbol. Open circles show the measurement in the presence of 10 μM wild type arrestin.

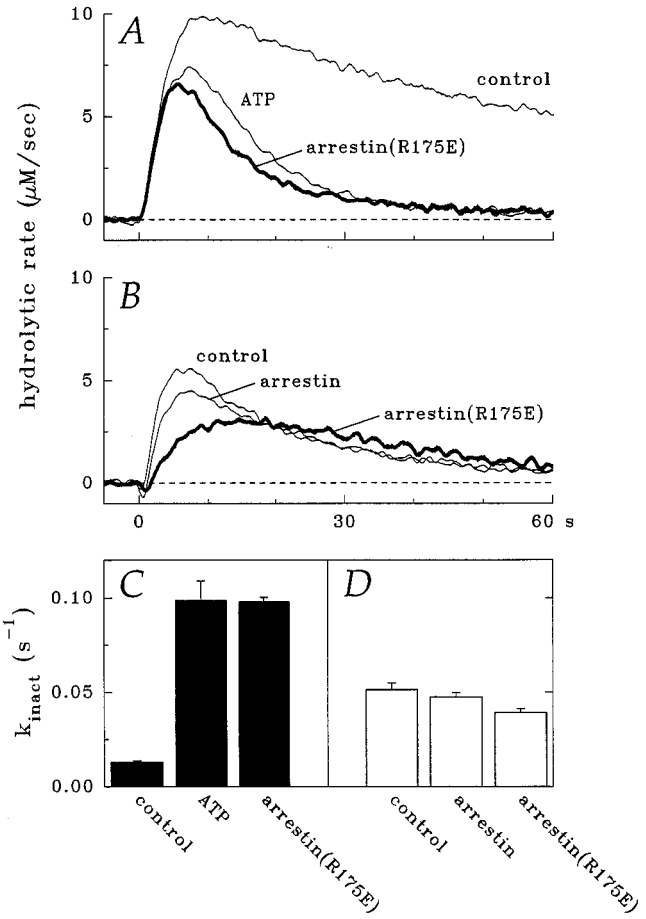

FIGURE 4: Mutant arrestin(R175E) does not accelerate inactivation kinetics under "single-turnover" conditions. Phosphodiesterase activity was stimulated either with a brief light flash (same intensity as in Figure 2) delivered to dark-adapted ROS (A) or by adding 50 nM GTP to ROS previously exposed to room light for 3 min in the absence of GTP (B). To compare the effect of arrestin under these two conditions, homogenates (same concentrations as in Figure 2) were supplemented with either 3 μM R175E (bold traces) or wild type (middle trace in B) arrestin or 2 mM ATP (middle trace in A). Control (top traces in A and B) homogenates contained no exogenous arrestin or ATP. Panels C and D plot the inactivation rate constants determined for the responses shown in panels A and B, respectively.

absence of GTP. The reaction was then initiated by adding an amount of GTP that was substoichiometric to the amount of Rh*−transducin present. Under these conditions there is only enough GTP to permit one round of transducin activation making the quenching of the resulting increase in cGMP hydrolysis independent of Rh* lifetime (Arshavsky et al., 1991). The effect of arrestin(R175E) on the change in hydrolytic rate caused by either a brief flash of light (panels A and C) or a single injection of 50 nM GTP (panels B and D) is compared in Figure 4. Mutant arrestin suppressed the GTP-induced activation of PDE but did not affect the "single-turnover" (Arshavsky et al., 1991) inactivation rate. The reason for its minor effect on activation is not known, but the changes in the response are consistent with it lowering the effective concentration of GTP either by hindering its access to the nucleotide binding site in the Rh*−T complex or by actually lowering GTP concentration due to traces of ATPase or GTPase activity in the preparation, which can affect activation at very low GTP concentrations employed in these experiments. The data in Figure 4 also introduce a paradox: the rates of inactivation observed with light activation are different than those observed in the "single-turnover" experiments. The quenching of the cascade following light activation is 2 times faster than that observed in the "single-turnover" experiment (Figure 4C,D). This is surprising because one would expect that the rate of inactivation of the entire cascade could not be faster than the inactivation of the slowest step, which the data from the "single-turnover" experiment would indicate is downstream of Rh*. To summarize, the results of "single-turnover" experiments indicate that arrestin(R175E) does not facilitate shutoff of activated T or PDE and support the conclusion that it facilitates inactivation of light-activated PDE by quenching Rh*.

DISCUSSION

The finding that a single-point mutation in the previously identified (Gurevich & Benovic, 1992, 1993, 1995) phosphorylation recognition region of visual arrestin yields a mutant that binds to light-activated rhodopsin independent of its phosphorylation has several implications. Previous mutagenesis studies of visual arrestin (Gurevich & Benovic, 1992, 1993, 1995) indicate that it has multiple functional domains. These include a "phosphorylation recognition" region which interacts with the phosphorylated C-terminus

of rhodopsin and an "activation recognition" region that interacts with those regions of rhodopsin that change conformation upon light activation. We hypothesized (Gurevich & Benovic, 1993, 1995; Gurevich et al., 1994) that simultaneous engagement of both these primary binding regions by P-Rh* induces a further change in arrestin that involves a mobilization of an additional binding site and gives rise to the high-affinity binding conformation. The mobilization of this "secondary" binding site was tentatively identified (Gurevich & Benovic, 1993) as the conformational rearrangement (Schleicher et al., 1989) in arrestin that occurs upon binding to P-Rh*. This model implies that arrestin is normally constrained to a low-affinity binding conformation by intramolecular interactions that are controlled by specific "trigger" residues in the two recognition regions (Gurevich & Benovic, 1993, 1995). The constraints controlled by both triggers must be relieved by the interaction with light-activated phosphorylated rhodopsin in order for arrestin to be fully activated for binding. An interesting corollary of this hypothesis is that either of these "triggers" can be artificially activated by mutagenesis yielding activation or phosphorylation-independent arrestin binding to rhodopsin.

The most likely scenario in the case of phosphorylation recognition is that in the basal conformation Arg175 interacts with an unidentified negatively charged residue in arrestin. Interaction of Arg175 with a phosphate on phosphorhodopsin neutralizes its charge, thus disrupting this constraining interaction. The finding that the addition of a fully phosphorylated C-terminal peptide of rhodopsin promotes arrestin binding to Rh* supports this hypothesis (Puig et al., 1995). The deletion of negatively charged arrestin C-terminus by mutagenesis (Gurevich & Benovic, 1992, 1993) or alternative splicing (Smith et al., 1994) yields proteins with enhanced ability to bind to Rh*, suggesting that the partner of Arg175 may be localized there. Our finding that C-terminally truncated arrestin(1−365) and arrestin(R175E)(1−365) in contrast to full-length proteins demonstrate virtually identical selectivity (data not shown) supports this conclusion, although the specific residue(s) involved in interaction with Arg175 remains to be identified. In general, our results indicate Arg175 is a phosphorylation-sensitive trigger. Its replacement by negatively charged Glu is the most effective way of inducing high-affinity arrestin binding to Rh* reported thus far.

The availability of a phosphorylation-independent arrestin mutant opens several prospects. Excessive signaling by rhodopsin mutants with constitutive activity (Rao et al., 1994) or those lacking C-terminal rhodopsin kinase phosphorylation sites (Horn et al., 1992; Apfelstedt-Sylla et al., 1993; Kim et al., 1993; Restagno et al., 1993) necessary for normal shutoff (Chen et al., 1995) has been recently implicated in congenital night blindness and certain cases of autosomal dominant retinitis pigmentosa. Expression of an arrestin mutant that quenches the photoresponse in a phosphorylation-independent fashion and does not require the rhodopsin C-terminus for tight binding appears to be a promising approach for gene therapy of these disorders. Excessive signaling by other G protein-coupled receptors brought about by mutations in receptors or their expression in "wrong" cell types has been reported to induce uncontrolled cell proliferation (Allen et al., 1991) and Jansen-type metaphyseal chondrodysplasia (Schipani et al., 1995). Conceivably, similar phosphorylation-independent mutants of nonvisual arrestins that attenuate or block this faulty signaling can be used for gene therapy in these situations.

A two-step quenching mechanism, receptor phosphorylation followed by arrestin binding, is employed in phototransduction and other G protein-mediated signaling pathways in vertebrates. A phosphorylation-independent arrestin(R175E) with a single-point mutation can quench the response in one step with high efficiency (Figure 2). Arguably, such a mutation could have occurred many times during evolution of the visual system in vertebrates. Apparently, it was selected against due to certain advantages of a more complex two-step mechanism. A normal two-step mechanism can be substituted by one-step quenching in retinal rods by means of expression of the arrestin(R175E) mutant in recently created transgenic mice expressing truncated rhodopsin (Chen et al., 1995) and arrestin "knock-out" mice (Dodd et al., 1996). This will improve our understanding of the biological significance of a two-step shutoff mechanism and lay the groundwork for future use of arrestin(R175E) in gene therapy.

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