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Sites of arrestin action during the quench phenomenon in retinal rods

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The target proteins for arrestin (48 kDa protein) action during the quench of cGMP phosphodiesterase (PDE) activation in retinal rod disk membranes were identified by the use of a cross-linking reagent. A heterobifunctional, cleavable, photoactivatable cross-linker (sulfo-SADP) was coupled to purified arrestin. Under precise weak visible light bleach and nucleotide conditions of quench, the cross-linker was UV flash-activated at a time when quench was well established. The target proteins covalently linked to arrestin by cross-linker activation were identified by immunoblotting. In the presence of ATP arrestin cross-linked to both PDE and rhodopsin during the quench phenomenon. Removal of ATP from the reaction mixture essentially abolished the cross-link with PDE, just as ATP omission abolishes quench, but significantly increased the cross-link to rhodopsin. The absence of a cross-link to the plentiful β-subunit of transductin, as well as the results of competition studies employing arrestin without attached cross-linker, suggest that the observed cross-links are specific and reflect true binding interactions of arrestin during quench. The data are consistent with a model of quench in which photolyzed rhodopsin (R*) catalyzes the formation of an activated form of arrestin, which dissociates from R* in the presence of ATP, and binds to PDEs, thereby deactivating them.

Photoreceptor; Phosphodiesterase; Arrestin; Quench

1. INTRODUCTION

The light-induced activation of the cGMP phosphodiesterase (PDE) by transducin is terminated by the action of rhodopsin kinase and arrestin (48 kDa protein). Rhodopsin kinase phosphorylates rhodopsin in the presence of ATP or GTP, thereby reducing rhodopsin's ability to activate transducins [1–3]. Arrestin in the presence of ATP dramatically shortens the time required for activation to return to its preactivation level [3,4]. The mechanisms by which arrestin down regulates the PDE are not completely resolved. Wilden et al. [4] have suggested that arrestin achieves turnoff by binding to bleached, phosphorylated rhodopsin, thereby deactivating it, allowing quench to proceed passively through the decay of activated transducins. We alternatively have provided kinetic evidence of arrestin's ability to act directly on PDE, inhibiting its activation by activated transducins [3].

Here, we have employed a heterobifunctional, cleavable, photoactivatable cross-linker attached to purified arrestin to identify the target proteins for arrestin action during quench, and thus obtain direct, visible evidence of the sites of arrestin action. The cross-linker was UV flash-activated at the time of quench, thus covalently linking arrestin to nearby proteins within the span of the cross-

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Abbreviations: R*, photolyzed rhodopsin; PDE, cGMP phosphodiesterase; cGMP, cyclic guanosine 3′:5′-monophosphate; sulfo-SADP, sulfosuccinimidyl (4-azidophenyldithio)-propionate; DTT, dithiothreitol; transducin, G-protein of retinal rods; arrestin, 48 kDa protein, S-antigen of retinal rods; RDMs, retinal rod disk membranes; Mops, 3-(*N*-morpholino)-propanesulfonic acid

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linker. The component proteins in the cross-linked product were subsequently identified by immunoblotting. This procedure, then, provides a powerful tool for 'freezing' protein interactions in time.

We report that arrestin cross-links to both PDE and rhodopsin during the quench of cGMP PDE activation in such experiments. Moreover, we provide evidence that the cross-link with PDE requires ATP, as does the quench of PDE activation, while the cross-link with rhodopsin does not. We also present evidence that the cross-links are not nonspecific, but rather represent true binding phenomena.

2. MATERIALS AND METHODS

2.1. *Materials*

Fresh bovine eyes were purchased from Mopac (Souderton, PA). cGMP, Mops and salts were purchased from Sigma. ATP, GTP and DTT were obtained from Boehringer Mannheim. Affigel-10 was purchased from Biorad, sulfosuccinimidyl (4-azidophenyldithio)proprionate (sulfo-SADP) from Pierce and 3,3′-diaminobenzidine tetrahydrochloride from Polysciences.

2.2. *Preparation of rod disk membranes*

RDMs were prepared by a standard method [5], with all procedures carried out under infrared illumination with the aid of image converters.

2.3. *Purified proteins*

Arrestin was purified as we previously described [3]. Transducin and phosphodiesterase were purified according to [6].

2.4. *Coupling of SADP to arrestin, and arrestin-SADP to Affigel*

Purified arrestin was incubated with 4 mM sulfo-SADP in Mops buffer at pH 8.0 for 30 min in the dark. The arrestin-SADP was separated from excess reagent by gel-filtration chromatography in the dark using a short G-25 column. The UV absorbance of the arrestin-SADP reveals that each arrestin molecule has one to two cross-linkers attached via an amino group. Washed Affigel-10 beads (2 ml gel slurry) were suspended in 100 mM Mops (pH 7.5) and 150 μg arrestin-SADP added. The arrestin-SADP and Affigel beads were incubated in the dark for 1.5 h at room temperature, and then for 1.5 h at 4°C. Washed arrestin-SADP coupled to beads were incubated with 1 M ethanolamine. The arrestin-SADP coupled to beads was further washed with Mops buffer and protected from visible light.

2.5. *PDE assay*

PDE velocity was monitored by a real-time pH assay using a modification of the method of Liebman and Evanczuk [7], as described [3].

2.6. *Immunoblotting*

Immunoblotting was performed according to [8]. Monoclonal and polyclonal antibodies were visualized with peroxidase-conjugated secondary antibodies (Cappel and Pelfreez), and the blots developed in 3,3′-diaminobenzidine tetrahydrochloride. Quantification of proteins on immunoblots by reflection densitometry was performed as in [9].

2.7. *Cross-linking experiments*

Arrestin-SADP, coupled to beads, was incubated with RDMs and nucleotides, in the indicated amounts, in a well-stirred quartz cuvette. The RDMs were activated by a weak visible light flash bleach (spectrally shaped by a Wratten 57 filter), and at the indicated time (45 s after visible light activation), the cross-linker was UV flash-activated at full power by a 1 m flash from a Vivitar photographic strobe. The reaction mixture was added in the dark to 12 ml Mops buffer containing 0.5% Triton to solubilize membranes. The beads and reaction mixture were spun twice in the dark at 480 × g for 20 min per spin. The supernatant was removed and the beads washed in the light with Triton-free Mops buffer or Mops with 1% SDS when indicated, and spun at 4000 × g for 10 min. The beads were incubated with 100 mM DTT for 30 min, the beads spun, and the supernatant removed and Laemmli sample buffer added to the supernatant. The supernatant was run on polyacrylamide mini slab gels [6], transferred to nitrocellulose and immunoblotted.

3. RESULTS

Fig.1 demonstrates that coupling sulfo-SADP to arrestin does not significantly impair arrestin functionally. The effect of arrestin-SADP, coupled to beads, on turning off of flash-activated PDE velocity is comparable to that shown for purified arrestin (inset).

Fig.2 shows the results of a typical cross-linking experiment performed in the presence of 75 μM ATP. Reduction of the internal disulfide bonds in the cross-linker, as described in section 2, cleaves the cross-linker allowing the proteins to which arrestin is cross-linked to be separated from arrestin which remains coupled to the beads. The supernatant containing the target proteins for arrestin action are run on the immunoblot. Cross-reactivity of the proteins in the supernatant with appropriate antibodies is also shown in fig.2. Lane A shows positive cross-reactivity (45.4 ng, by reflection densitometry) of the sample with an anti-PDE polyclonal antibody, identifying PDE as a target protein cross-linked to arrestin during the quench of cGMP PDE activation. In lane D we observe cross-reactivity (47.2 ng) of the sample with anti-rhodopsin. Therefore, rhodopsin is also cross-linked to arrestin during quench. To determine

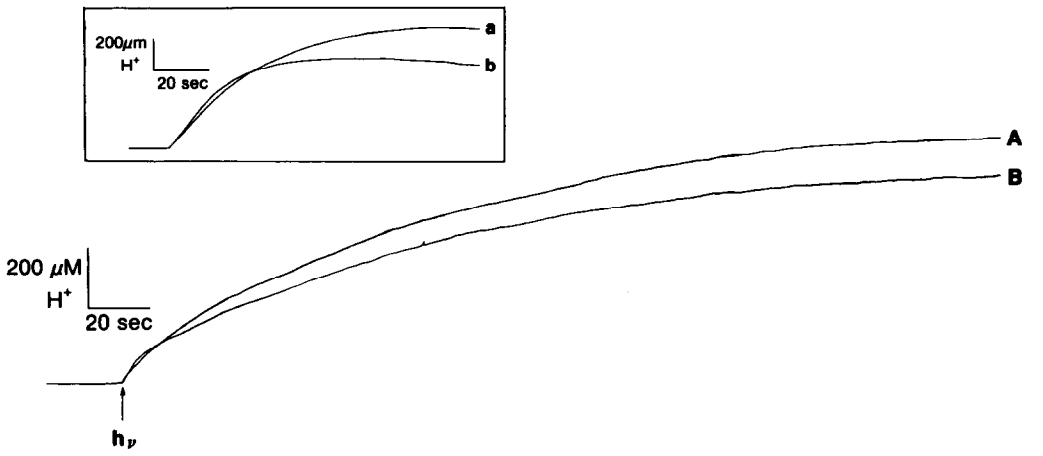

Fig.1. Coupling SADP to arrestin does not impair arrestin functionally. Stoichiometric proton release with cGMP hydrolysis following a flash ($h\nu$) of 1 ms duration that bleached a 6×10^{-4} fraction of the rhodopsin in the presence of 250 μM GTP and 250 μM ATP. Curve B: sample contained 0.27 μM arrestin-SADP coupled to beads. Both traces from separate disk membrane samples of the same preparation contained a 4 μM rhodopsin, 5 mM cGMP, 2 mM $MgCl_2$, 100 mM KCl, 20 mM Mops (pH 8.0), with $T = 30°C$ and reaction volume = 0.75 ml. Linear dark rates have been subtracted from the records. (Inset) Comparable effect of purified arrestin on turning off of PDE activation (curve b).

that the cross-links observed were not nonspecific we looked for the presence of cross-links with the plentiful β-subunit of transducin. In lane E, no cross-reactivity of the sample is observed at the level of the transducin band, although some nonspecific cross-reactivity is observed at higher molecular masses. In addition, silver-stained gels of the supernatants (not shown) revealed a total of four proteins cross-linked. The significance of cross-links to the two proteins other than PDE and rhodopsin is at present unknown.

As a further test of the specificity of the cross-links, we explored the effect of omitting ATP from the reaction mixture. ATP is an obligatory component required for rapid quench in RDMs [10]. As expected of specific cross-linking, we observed marked effects on omission of ATP from the reaction mixture. The effects of ATP removal are shown in fig.3. In lane E omitting ATP from the reaction mixture practically abolishes the cross-link with PDE (11.2 ng), just as its omission abolishes quench in the pH cuvette. Lane D shows

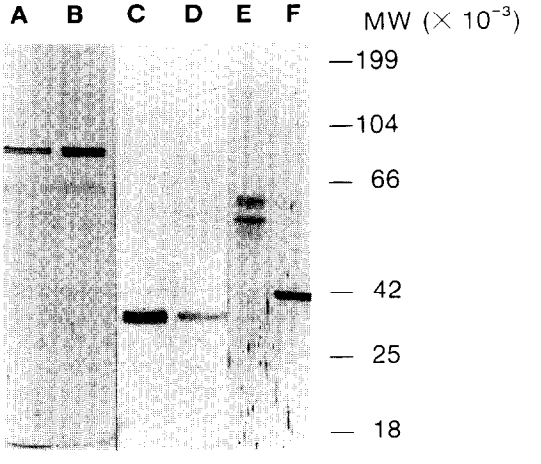

Fig.2. Arrestin-SADP forms cross-links with PDE and rhodopsin under quench conditions. (Lane A) Immunoblot of cross-linked proteins (see section 2) from reaction mixture containing RDM equivalent to 20 μM rhodopsin, 75 μM ATP, 1.32 μM arrestin-SADP, activated by a visible light flash that bleached a 1×10^{-3} fraction of the rhodopsin. SADP was UV flash-activated at 45 s after visible light activation of the preparation. Immunoblot stained with anti-PDE at 1:400. (Lane B) Sample contained 100 ng purified PDE run as a control and stained with anti-PDE (1:400). (Lane C) Sample contained RDM equivalent to 250 ng rhodopsin, run as a control, and stained with monoclonal anti-rhodopsin (1:2500). (Lane D) Sample identical to that of lane A stained with monoclonal anti-rhodopsin (1:2500). (Lane E) Sample identical to lane A stained with anti-transducin β (1:300). (Lane F) 250 ng purified transducin, run as a control, and stained with anti-transducin β (1:300). All lanes developed identically in diaminobenzidine.

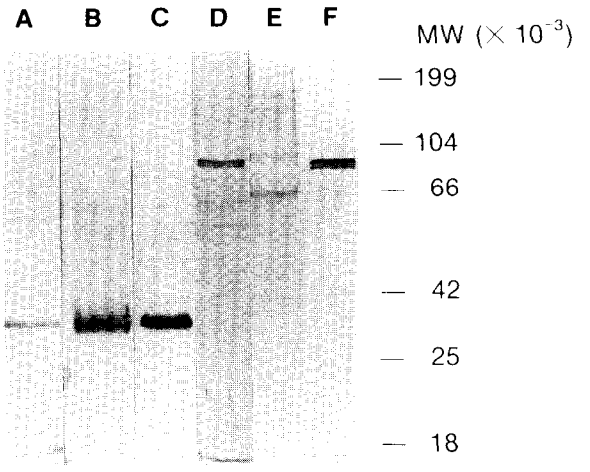

Fig.3. Omission of ATP abolishes cross-linking of arrestin-SADP with PDE, but significantly enhances that with rhodopsin. Immunoblot of cross-linked proteins (see section 2). Rhodopsin, arrestin-SADP, and ATP concentrations, as well as bleach and UV activation, were identical to those in fig.2. Lanes: (A) immunoblot stained with monoclonal anti-rhodopsin (1:2500); (B) identical to (A), except no ATP, stained with anti-rhodopsin (1:2500); (C) RDM equivalent to 250 ng rhodopsin, run as a control, and stained with anti-rhodospin (1:2500); (D) identical to (A), with ATP, stained with anti-PDE (1:400); (E) identical to (B), no ATP, stained with anti-PDE (1:400); (F) 100 ng purified PDE, run as a control, and stained with anti-PDE (1:400).

the cross-link between arrestin and PDE in the presence of ATP for comparison. Reflection densitometry of the immunoblots reveals that the amount of PDE cross-linked in the presence of ATP is 405% of that in its absence. In lane B, omitting ATP from the reaction mixture interestingly results in considerably increased cross-linking between arrestin and rhodopsin. Compared to the cross-linking between arrestin and rhodopsin in the presence of ATP (lane A), removing ATP from the reaction mixture results in a 441% increase in cross-linking to rhodopsin (208 ng). The data, then, suggest that ATP dissociates arrestin from bleached rhodopsin and results in its binding to PDE.

As a more classical test of the observed cross-linking representing true binding interactions, we performed competition experiments employing arrestin without attached cross-linker. If the cross-links represent binding interactions, we should expect arrestin without cross-linker to compete for the same sites as arrestin-SADP and so diminish the extent of cross-linking. This is precisely what is observed in fig.4, in which a 5-fold excess of arrestin without cross-linker diminishes both the cross-links with PDE (lane B) and those with

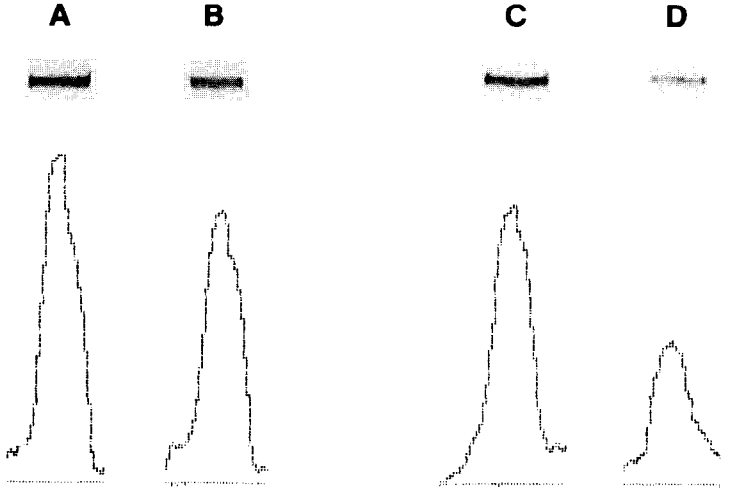

Fig.4. Arrestin without attached cross-linker competitively inhibits cross-links with PDE and rhodopsin. (Upper) Immunoblots of proteins; (lower) reflection densitometry of immunoblots. Conditions identical to those in figs 2,3. Lanes: (A) immunoblot stained with anti-PDE (1:400); (B) identical to (A), with the addition of 6.60 μM arrestin, and stained with anti-PDE (1:400); (C) identical to (A), but stained with monoclonal anti-rhodopsin (1:2500); (D) identical to (A), with addition of 6.60 μM purified arrestin, and stained with monoclonal anti-rhodopsin (1:2500). Immunoblots developed identically in diaminobenzidene. Beads with coupled arrestin and covalently linked proteins received an additional wash in Mops buffer with 1% SDS to remove noncovalently bound proteins. Affigel beads with arrestin were dried and weighed for quantification of results for these and previous immunoblots.

rhodopsin (lane D). Integration of the reflection densitometry of the immunoblots reveals that a 5-fold excess of arrestin without cross-linker reduces cross-linking of PDE by 20%, and that of rhodopsin by 53%. Cross-linking with both PDE and rhodopsin was observed in the presence of ATP, under quench conditions. The competition studies therefore provide further evidence that the observed cross-links of arrestin during quench represent binding phenomena.

4. DISCUSSION

The sites of arrestin action during quench have not been resolved at present. Wilden et al. [4] have suggested that arrestin interacts solely with bleached, phosphorylated rhodopsin during quench. We [3] alternatively have proposed that arrestin interacts with R*; but in the presence of ATP, dissociates from R* and ultimately binds to PDEs, thereby directly deactivating them.

In the present report we have employed a photoactivatable cross-linker attached to purified arrestin to reveal the target proteins for arrestin action during quench. Fig.1 demonstrates that the cross-linker does not functionally impair arrestin. The present approach further has the advantage of allowing the protein interactions of arrestin to be determined under the precise bleach and nucleotide conditions of quench, essentially identical to those shown to result in potent turning off of PDE activation by arrestin in the pH cuvette (fig.1). UV flash activation of the cross-linker at a time when quench is well established results in the formation of nitrene free radicals whose lifetime is equivalent to the flash duration (1 ms) [11]. The arrestins are thus covalently linked to nearby proteins within the latency of further PDE activation. The experiments demonstrate that in the presence of ATP arrestin interacts with both PDE and rhodopsin (fig.2).

Evidence that the observed cross-links represent binding interactions of arrestin is necessary. By limiting activated cross-linker lifetime to 1 ms, nonspecific protein interactions should be significantly reduced. Similarly, evidence of specificity of the observed cross-links includes the absence of cross-linking to the β-subunit of transducin, which is found in approx. 6-fold greater concentration than PDE. In addition, the cross-linking was found to be profoundly affected by the presence or absence of ATP in the reaction mixture, just as quench is ATP-dependent. Removing ATP (fig.3) almost completely abolished the cross-link between arrestin and PDE, while significantly increasing the extent of the cross-link between arrestin and rhodopsin. Moreover, competition studies (fig.4) revealed that relatively modest amounts of arrestin without cross-linker (5-fold excess) were able to reduce significantly the extents of the cross-links between arrestin and both PDE and rhodopsin, observed in the presence of ATP (quench conditions). This suggests not only that the cross-links to PDE and rhodopsin are specific, but also that the binding interactions between arrestin and these molecules are relatively 'tight'.

The results of ATP omission from the reaction mixture may provide mechanistic insight into the quench reaction. We previously proposed [3] that it is ATP that dissociates arrestin from R*, forming activated arrestins, which subsequently bind to and deactivate PDEs. The present observations of the effects of ATP removal are consistent with our hypothesis. Our findings, however, contrast sharply with the binding centrifugation experiments of Kuhn et al. [12]. However, the present experiments are the first to be conducted on the time scale and under the weak bleach conditions of quench, whereas the binding centrifugation experiments had a time resolution of 10 min, and employed strong bleaches which would preclude quench.

The present evidence of the direct interaction of arrestin with PDE has important implications for rod function. The binding of arrestin to PDEs might not only account for the quench or turning off of PDE activation, and thus the falling phase of the rod photocurrent, but also affect rod sensitivity to subsequent flash bleaches. As such arrestin might play a role in light adaptation. Whether arrestin's inhibitory effect on PDE is used for this purpose remains to be seen.

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REFERENCES

[1] Sitaramayya, A. (1986) Biochemistry 25, 5460–5468.
[2] Shichi, H., Yamamoto, K. and Somers, R.L. (1984) Vision Res. 24, 1523–1531.
[3] Zuckerman, R. and Cheasty, J.E. (1986) FEBS Lett. 207, 35–41.
[4] Wilden, U., Hall, S.W. and Kuhn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174–1178.
[5] Sitaramayya, A. and Liebman, P.A. (1983) J. Biol. Chem. 258, 12106–12109.
[6] Baehr, W., Morita, E.A., Swanson, R.J. and Applebury, M.L. (1982) J. Biol. Chem. 257, 6452–6460.
[7] Liebman, P.A. and Evanczuk, A.T. (1982) Methods Enzymol. 81, 532–542.
[8] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 72, 4483–4486.
[9] Jahn, R., Schiebler, W. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 1684–1687.
[10] Liebman, P.A. and Pugh, E.N. jr (1980) Nature 287, 734–736.
[11] Ji, T.H. (1979) Biochim. Biophys. Acta 559, 39–69.
[12] Kuhn, H., Hall, S.W. and Wilden, U. (1984) FEBS Lett. 176, 473–478.