Selective Proteolysis of Arrestin by Calpain

MOLECULAR CHARACTERISTICS AND ITS EFFECT ON RHODOPSIN DEPHOSPHORYLATION*

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Sasan M. Azarian‡, Alastair J. King, Mark A. Hallett, and David S. Williams‡

From the ‡School of Optometry, Indiana University, Bloomington, Indiana 47405 and the †Departments of Pharmacology and Neurosciences, University of California San Diego School of Medicine, La Jolla, California 92039

Visual arrestin (48 kDa) plays a role in the deactivation of rhodopsin by binding to the light-activated, phosphorylated form of the receptor. In bovine rod outer segments that were prepared in the presence of protease inhibitors, two faster migrating forms of arrestin, with apparent molecular masses of 46 and 44 kDa, were observed by Western blot analysis. The 46-kDa form was more evident in rod outer segments of eyes kept in the light than those placed in darkness and was found to be identical to that generated by in vitro proteolysis of arrestin by pure retinal calpain II. In vitro analysis showed that arrestin was proteolyzed only when bound to rhodopsin; soluble arrestin was not significantly cleaved by calpain. Proteolysis involves sequential cleavage at two, possibly three sites, resulting in the removal of 27 amino acids from the COOH terminus. The remaining 46-kDa protein was resistant to further proteolysis by calpain. Unlike intact arrestin, the 46-kDa truncated arrestin was not readily released from the receptor after the receptor had lost its chromophore, nor was it released upon the addition of 11-cis-retinal to regenerate the receptor. Truncated arrestin was found to inhibit receptor dephosphorylation to the same extent as intact arrestin. In conclusion, these results provide evidence that a 46-kDa form of arrestin in rod outer segments is a product of selective proteolysis by calpain. Furthermore, they suggest that this proteolysis may provide a mechanism for prolonging the phosphorylated state of the visual receptor.

The Ca2+-activated, neutral cysteine proteases, known as calpains, appear to play roles in a variety of cellular processes (see Murachi (1989), Suzuki (1990), and Suzuki and Ohno (1990) for reviews). Biochemically, two different classes of calpain have been described, based on different concentrations of Ca2+ required for activation in vitro; calpain II requires more Ca2+ than calpain I (Melgren, 1980). Calpains are abundant in neural tissues, where calpain II seems to be the predominant isozyme (Murachi et al., 1981; Nixon et al., 1986; Kawashima et al., 1988). Recently, we demonstrated the presence of calpain II in rat and bovine rod photoreceptor outer segments (Azarian et al., 1993).

The photoreceptor outer segment is an extremely specialized organelle, devoted to the absorption and transduction of light. Phototransduction begins with the absorption of a photon of light by rhodopsin. The chromophore of rhodopsin is isomerized from 11-cis-retinal to all-trans-retinal, inducing a conformational change in the receptor. The photoexcited rhodopsin activates the G protein, transducin, thus triggering an enzymatic cascade that results in the hydrolysis of cGMP and the closing of the cGMP-gated channels (see Hargrave and McDowell (1992) and Lagnado and Baylor (1992) for reviews). Rhodopsin is deactivated by phosphorylation of serine and threonine residues in its carboxyl tail and the subsequent binding of arrestin (also known as S-antigen) (Wilden et al., 1986; Wilden, 1995). The binding of arrestin to rhodopsin inhibits dephosphorylation of the receptor and thus maintains rhodopsin in a deactivated state (Palczewski et al., 1989a). When all-trans-retinal is reduced by retinal dehydrogenase (Ishiguro et al., 1991) and removed from rhodopsin, arrestin is released (Hofmann et al., 1992). Following the release of arrestin, the phospho-opsin can be dephosphorylated by a phosphatase 2A (Palczewski et al., 1989b; Fowles et al., 1989). Regeneration with 11-cis-retinal then returns the dephosphorylated receptor to its dark state, in which it can be activated by the absorption of another photon of light. Any modification of arrestin that affects its ability to be released from rhodopsin should therefore influence how long rhodopsin remains deactivated.

Arrestin contains a PEST sequence (Mangini and Garner, 1991); i.e. a domain rich in proline, glutamate, serine, threonine, and aspartate residues, present in many substrates of calpain (Roger et al., 1986; Wang et al., 1989). Although there is some question about whether such a sequence actually affects substrate susceptibility (Molinari et al., 1995), its presence in arrestin makes this protein a possible candidate for regulation by calpain. In the present study, we noted the presence of two additional, more mobile forms of arrestin in bovine rod outer segment preparations. We found that in vitro proteolysis of arrestin by retinal calpain II generates a product that is identical to one of the additional forms of arrestin detected in rod outer segment preparations. This result suggests that this in vitro event mimics a physiological one. Additional in vitro experiments were performed to characterize calpain proteolysis of arrestin and gain some insight into its potential function.

**EXPERIMENTAL PROCEDURES**

Materials—Polyclonal antibodies against bovine retinal arrestin were generated in Long Evans rats by conventional procedures (Harlow and Lane, 1988). Monoclonal antibodies (mAbs) SC6.47, C10C10, A2G5, S2.4.C5, and A9C6 against visual arrestin (S-antigen) were gifts from 1 The abbreviations used are: mAb, monoclonal antibody; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ROS, rod outer segment; Rh, rhodopsin, unexposed to light; Rh*, light-activated rhodopsin; Rh-P, phosphorylated rhodopsin; Rh*-P, phosphorylated and light-activated rhodopsin; Op, opsin (rhodopsin without chromophore); Op-P, phosphorylated opsin.

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from Dr. Larry Donoso (Thomas Jefferson University). The antibodies recognize the following epitopes on arrestin: 56C.47, residues 42–48; C10C10, residues 288–296; A2G5, residues 360–368; S2.4C5, residues 375–386; and A9C6, residues 375–386 (Donoso et al., 1990; Dua and Donoso, 1993). Hydroxylamine was purchased from Sigma. The sources of other materials are stated below.

Solution—Buffer A, 20 mM Tris-HCl, pH 7.4 at 4°C, 2 mM EGTA, 1 mM MgCl₂, 10 mM d-glucose, 5 mM 2-mercaptoethanol; buffer B, 100 mM NaCl, pH 7.5 at 30°C, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT; buffer C, 10 mM HEPES-NaOH, pH 7.4 at 4°C, 5% glycerol, 0.1 mM EDTA, 1 mM DTT; buffer D, 10 mM HEPES-NaOH, pH 7.5 at 30°C, 100 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT; buffer E, 50 mM HEPES-NaOH, pH 7.5 at 30°C, 0.1 mM EDTA, 1 mM DTT; Buffer F, 20 mM Tris-HCl, pH 8.3 at 4°C, 1 mM EDTA, 1 mM DTT, 40 mM leupeptin, 0.2 mM PMSF, 1 mM benzamidine.

Preparation of Rod Outer Segments—Rod outer segments (ROSs) were purified from fresh bovine retinas on continuous sucrose gradients as described (Azarian et al., 1993), with modifications. Unless otherwise stated, the eyes, which came from light-adapted cattle, were placed in light-sealed plastic containers (PVC Scientific) on ice, immediately after their removal from the animal, and were thus transported to the laboratory (2 h). Subsequent preparation of the ROSs was performed under dim red light. Retinas were removed and vortexed for 30 s with 10 ml/10 retinas of 20% sucrose in buffer A containing 20 mM leupeptin and 0.2 mM PMSF. The homogenate was filtered through a nylon mesh (Sartorius, number 100) and centrifuged among six 23-mL sucrose gradients (25–55% sucrose in buffer A). The gradients were centrifuged for 90 min at 100,000 × g (Beckman SW28), and the ROS band was carefully siphoned off with a Pasteur pipette. The pooled ROSs were diluted with 1 volume of 100 mM KCl in buffer C and centrifuged at 10,000 × g for 5 min (Sorvall SS-34). ROSs were resuspended in buffer B.

To purify ROSs from frozen bovine retinas (J. and A. Lawson, Lincoln, NE) for some of the urea-stripped ROS membrane preparations, the procedure of Wilden and Kühn (1982) was used. With both procedures, purified ROSs contained 0.4–0.5 mg rhodopsin of retinas and had an A₂₅₀/A₅₀₀ ratio of 2.2–2.4 in 3% lauryl-dimethylamine-N-oxide in buffer C. Aliquots of purified ROSs were flushed with argon, covered with aluminum foil, and snap-frozen in liquid nitrogen for storage at −80°C.

To test for the presence of p46 in frozen ROSs, a retinas was removed quickly from an eye less than 5 min post-mortem and placed in ice-cold buffer A, containing 130 mM NaCl, 0.2 mM PMSF, 0.1 mM calpeptin, and 40 μM leupeptin (this procedure was carried out at a small local slaughterhouse). A crude but rapid preparation of ROSs was obtained by vortexing the retina for 20 s and then after 1 min removing the suspended crude ROSs by pipette and placing them directly in sample buffer for SDS-PAGE and Western blotting.

Purification of Proteins—All procedures were performed at 0–4°C unless otherwise stated. Bovine retinal calpain II was purified to homogeneity (−95%) (Azarian et al., 1993). One unit of calpain I is defined as the amount of enzyme required to hydrolyze 1 μg casein/h in 200 μl at 25°C.

Bovine retinal arrestin was purified as described (Buczyiko and Palczewski, 1993), with modifications. Briefly, 40–60 dark-adapted bovine retinas were homogenized with 2 volumes of buffer C containing 2 mM PMSF. The homogenate was centrifuged for 20 min at 10,000 × g (Sorvall SS-34). The supernatant was loaded on a 2.5 × 5-cm heparin-Sepharose column (2.5 × 5 cm) equilibrated in buffer C, and phosphatase 2A activity was eluted with 0.1 M NaCl in buffer C (Erddoi et al., 1992). Arrestin (Buczyiko and Palczewski, 1993) and phosphatase type 1 (Erddoi et al., 1992) remain bound to heparin in the presence of 0.1 M NaCl. The phosphatase activity was concentrated in Centricon-30 cartridges (Amicon) and precipitated in 80% ETOH at room temperature (Brandt et al., 1974). The pellet was redissolved in buffer C and the insoluble fraction was collected from the pooled extracts by centrifugation. The final supernatant was stored in ice and was used as ROS phosphatase.

Isolation of Truncated Arrestin from ROSs—Bovine ROSs, purified as described above, were lysed in 10 volumes of buffer F and centrifuged at 541,000 × g for 15 min (Beckman TLA-100.3). The pellet was washed in 5 volumes of buffer C and the supernatant (containing most of arrestin) was discarded. Truncated arrestins were then eluted by washing the membranes three times with 0.5 M NaCl or KCl in buffer F. The salt extracts were pooled, diluted with buffer F to 50 mM NaCl, and concentrated on Centricon-30 filters. The concentrated extracts were solubilized with Laemmli sample buffer. In some experiments, we used ROS membranes that had been generously provided by Dr. Yee-Kin Ho (University of Illinois at Chicago). The ROSs had been purified from fresh retinas, as described by Ting et al. (1993), except that 10 μM leupeptin was also included in the isolation buffers. They had been lysed and washed once in hypotonic buffer, containing 10 μM leupeptin (to extract phosphocytosolic and other cytosolic proteins), and then washed an additional nine times in hypotonic buffer to extract phosphodiesterase and transducin, as described. We used the remaining ROS membranes to extract truncated arrestins with high salt buffer, as above.

Generation of Different Forms of Rhodopsin—Rhodopsin (Rh), opsin (Op), and their phosphorylated forms (Rh-P, Op-P) were generated together from the same preparation (Kühn et al., 1984), with modifications. Briefly, thawed ROS lysate (0.5 mg/ml rhodopsin in buffer B) was divided into two Lots, and one lot was adjusted to 3 mM ATP (for Rh-P), (g-P²ATP (10 μCi/ml, DuPont NEN), with a specific activity of 10^⁶ dpm/nmol ATP, was used to determine the stoichiometry of phosphorylation or to generate P²-labeled Rh-P. After a 5-min incubation in the dark at 30°C, the lysates were illuminated for 10 min with a 150-watt lamp from a distance of 0.3 m. The lysates were washed to 20 mM EDTA to remove P²-phosphorylated arrestin, and then washed three times with 0.5 μM calpain II. This was complicated with 5 μM urea in 5 mM HEPES, pH 7.5 at 4°C, 2 mM EDTA, and 1 mM DTT (Bennett and Sitaramayya, 1988) and washed three times in buffer D. To generate Rh and Rh-P, the stripped membranes were treated with 11-cis-retinal in ethanol (a gift from the National Eye Institute of the National Institutes of Health) as described (McDowell, 1993). Op and Op-P were obtained by treating regerated Rh and Rh-P, respectively, in stripped membranes, with 2 mM NH₂OH, and by washing three times in buffer D. The stoichiometry of phosphorylation was determined to be 2.3–2.5 mol of P/mol of rhodopsin.

Arrestin Binding Assay—The binding of arrestin to rhodopsin was based on established procedures (Kühn et al., 1984), with modifications provided in the figure legends. Briefly, for each tube, 20 or 200 pmol of arrestin and 200 pmol of different forms of rhodopsin or opsin were incubated in buffer D in the dark for 5 min at 30°C (assay volume, 20 μl/tube). For experiments involving calpain, buffer D was supplemented with 2 mM Ca²⁺; CaCl₂ had no detectable effect on the binding of arrestin to rhodopsin, as determined by SDS-PAGE. Samples to be kept in the dark were covered with aluminum foil. The mixture of rhodopsin and arrestin was illuminated for 5 min and centrifuged through 2–3 volumes of sucrose cushion (0.25 mM sucrose in buffer D) at 460,000 × g for 10 min at 2°C (Beckman TLA-100). Supernatants and pellets were then analyzed by SDS-PAGE (see below). The arrestin concentration in the assays (1–10 μM) was always above the dissociation constant of rhodopsin for arrestin (~50 μM) with 1 mM CaCl₂. Calpain Proteolysis of Arrestin—Arrestin and different forms of rhodopsin or opsin were mixed and illuminated as described above, in the presence of 2 mM Ca²⁺. Retinal calpain II was added (0.05–0.1 unit/μl) and the mixture was incubated at 30°C. Samples were then quenched in sample buffer for SDS-PAGE and Western blot analyses.

P'TA and RTA under for Sequence Analysis—The 46-KDa Arrestin (succession of 46-KDa Arrestin was concentrated in Amicon) and stored at −80°C or in 50% glycerol at −20°C. This preparation of arrestin was homogenously pure as judged by a Coomassie-stained SDS-PAGE and by arrestin binding assays (cf. Fig. 1A, lane 1).

The catalytic subunit of phosphatase 2A was partially purified from bovine ROSs, without contamination by arrestin. The soluble fraction of dark-adapted ROSs (prepared as in previous section) was dialyzed overnight against buffer C and filtered to remove particulate matter. The filtrate was loaded on a heparin-Sepharose column (2.5 × 5 cm)
25% (v/v) glycerol. The dialyzed extract was loaded on to a heparin-Sepharose column (2.5 × 5 cm) which had been equilibrated with 20 mM HEPES-NaOH, pH 7.5 at 0.5 ml/min. Fractions of 8-ml volume were collected and arrestins were eluted with 20 mM HEPES-NaOH, pH 7.5, containing 1 mM phytic acid. Fractions rich in 46-kDa arrestin were pooled and concentrated to 600 μl using Centricon-30 concentrators. The sample was then run down a Superose-6 fast protein liquid chromatography column (20 ml) at 0.3 ml/min in a buffer containing 20 mM Tris-HCl, 140 mM NaCl, pH 7.4, and fractions of 1 ml were collected. Fractions containing 46-kDa arrestin were then pooled and concentrated to 300 μl in a Centricon 30 concentrator. Constituent proteins in this final extract were resolved by SDS-PAGE on two 1.5-mm thick, 10% acrylamide gels. Gels were run under a Superose-6 fast protein liquid chromatography column, parallel to 40 mM COOH-terminal of each p46 species was identified as the peak present in a p46 sample, but not in a 48-kDa arrestin sample. Sequencing and laser desorption mass spectrometry of this peptide was carried out by the Harvard Microchemistry Facility. The remainder of each of the samples was used by this facility in an attempt to obtain NH2-terminal sequence information.

Preparation of Arrestin Cleavage Products for Sequence Analysis—Samples (60 μg) of bovine arrestin were cleaved with purified retinal calpain 11, as above, and then centrifuged through a sucrose cushion (0.25 M in buffer D) at 460,000 × g for 10 min. The supernatants were removed and filtered through Millipore GV13 0.22-μm filters and then passed through a Millipore DeltaP Waters-Millipore DeltaP C18 3.9-mm filters and then reverse-phase HPLC. The peptide corresponding to the COOH-terminal of each p46 species was identified as the peak present in a p46 sample, but not in a 48-kDa arrestin sample. Sequencing and laser desorption mass spectrometry of this peptide was carried out by the Harvard Microchemistry Facility. The remainder of each of the samples was used by this facility in an attempt to obtain NH2-terminal sequence information.

RESULTS

Different Arrestins in ROSs—In addition to native arrestin (48 kDa), two minor forms of arrestin were detected in the membrane fraction of bovine ROSs. They were also detected in crude ROSs, prepared rapidly from retinas that had been removed from eyes less than 5 min post-mortem and placed in ice-cold buffer containing protease inhibitors; thus they are unlikely to have originated from post-mortem proteolysis. Their apparent molecular masses were slightly less than the dominant 48-kDa form: namely, 46 and 44 kDa. These proteins were more easily observed on Western blots of bovine ROS membranes that had been washed several times in low salt buffer, because the more abundant 48-kDa arrestin was more readily eluted from ROS membranes in hypotonic buffer than either p46 or p44. The p44 appeared about five times more abundant than p46. Fig. 1 shows a Western blot, labeled with anti-arrestin (mAb C10C10), of extracts from ROS membranes washed in hypotonic buffer and subsequently several times with buffer containing 0.5 M KCl. Note that only 48-kDa arrestin is evident in the hypotonic wash (lane 1); high salt is required to elute p46 and p44 (lanes 2–4 show sequential high salt washes).

Conditions for Proteolysis of Arrestin by Calpain in Vitro—To help determine whether either p46 or p44 could have arisen from calpain proteolysis of arrestin, we investigated the effects of calpain on arrestin in vitro. Purified arrestin ([32P]-labeled Rh-P, 0.05 unit/μl) was added to 1 mM ATP, 1 μM calpain, and 1 μM p200, and incubated at 20°C for 1 h. The reaction was quenched with 10 μl of 1% BSA and 150 μl of chilled 10% trichloroacetic acid. After centrifugation, the acid-soluble radio label was counted in scintillation fluid. The counts from assay tubes lacking phosphatase were taken as background and were subtracted. Under these conditions, P, release was linear with time up to release of 20% of the total radioactivity into the supernatant. With some preparations of ROS phosphatase, up to 90% of the label became acid-soluble after prolonged incubation with phosphatase. This is consistent with opsin comprising 90% or more of the protein in ROS membranes (e.g. Zimmerman and Godchaux (1982)) and indicates that the acid-soluble radiolabel represents phosphates released from Rh-P.

Determination of Protein Concentration—The concentration of arrestin was determined spectrophotometrically, assuming an extinction coefficient (ε 328 nm) of 9.25 that was derived by dry weight analysis of extinction (Wacker et al., 1977), and an M, of 45,318 (Buczylko and Palczewski, 1993). Based on amino acid analysis, a theoretical extinction coefficient of 6.38 was recently reported (Buczylko and Palczewski, 1993). To convert our values for arrestin concentration to those assuming the more recent extinction coefficient, all figures should be multiplied by a factor of 1.45. Alternatively, the Bradford assay was employed, using the dye reagent from Bio-Rad. Rhodopsin content was measured from the light-sensitive absorbance at 498 nm in 3% lauryldimethylamine N-oxide (De Grip et al., 1980), assuming a mol weight extinction coefficient of 61.600 (Wald and Brown, 1953) and an M, of 40,000.

SDS-PAGE and Immunoblot Analyses—Samples were electrophoresed in 10% SDS-polyacrylamide gels (Laemmli, 1970) using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). The gels were either stained with Coomassie Brilliant Blue R-250 (Harlow and Lane, 1988) or electrophotically transferred onto Immobilon-P membranes (Millipore) at 50 V for 20 min in 0.1% SDS, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA (West and Molloy, 1988). The membranes were immunoblotted with arrestin antibodies as described (Harlow and Lane, 1988). Molecular mass standards were obtained from Sigma. The apparent molecular masses of the truncated forms of arrestin were determined from analysis of digitized images of Coomassie-stained gels with the GelReader software (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign).

PEST Sequence Analysis—The sequences of various arrestin proteins were obtained from the GenBankEMBL, PIR, or SWISS-PROT data bases. To determine the PEST sequences and scores, the arrestin sequences were analyzed with the PEST-FIND program (Rogers et al., 1986), a gift from Dr. Martin Rechsteiner (University of Utah).

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arrestin was analyzed by SDS-PAGE. As shown by others (e.g., Ku¨hn et al., 1984), arrestin bound most markedly to phosphorylated, photoactivated rhodopsin (Fig. 2A, lane 12). In the presence of calpain, proteolysis of arrestin was evident by the appearance of a form with an apparent molecular mass of 46 kDa. Under conditions in which arrestin was bound to rhodopsin, proteolysis of 48-kDa arrestin was practically complete (Fig. 2A, lane 1). The mobility of the 46-kDa truncated arrestin is replaced by the 46-kDa form, which appears to be relatively stable; even addition of fresh calpain after 60 min did not result in further proteolysis in the ensuing 20 min (Fig. 3, lane 8). With light-adapted ROS membranes that had been washed once with hypotonic buffer (and thus contained endogenous bound arrestin) and then incubated with calpain, the final product of arrestin was also found to be the 46-kDa form.

**Immunological Studies of Truncated Arrestins**—Western blots of partially proteolyzed arrestin (as in Fig. 3, lane 2) were labeled with monoclonal antibodies that recognize epitopes defined approximately from peptide competition studies as follows (Donoso et al., 1990; Dua and Donoso, 1993): residues 42–48 (SC6.47), residues 288–296 (C10C10), residues 375–380 (S2.4.C5; although, as shown below, residues 375–377 provide a sufficient epitope), and residues 375–386 (A9C6). Fig. 4A shows that mAb SC6.47 (lane 2) and mAb S2.4.C5 (lane 3) recognized all three species of arrestin after partial calpain proteolysis in vitro: intact arrestin (48 kDa), the 46-kDa intermediate, and the 46-kDa form. However, mAb A9C6 recognized only intact arrestin (lane 4). This result indicates that proteolysis to generate the 46.5-kDa intermediate removes enough of the COOH-terminal to perturb the A9C6 epitope (i.e., removal of at least 19 residues) and that the final 46-kDa product still has the S2.4.C5 epitope preserved.

The same antibodies were used to label Western blots of high salt extracts of bovine ROS membranes that had been prepared in the presence of protease inhibitors and washed with hypotonic buffer. Fig. 4B shows that mAb C10C10 recognized 48-kDa arrestin, plus the two faster migrating forms, p46 and p44 (lane 1). mAb S2.4.C5 recognized p46, but not p44 (lane 2). mAb A9C6 did not recognize either p46 or p44 (lane 3). It is likely that p44 is the variant described in two recent reports (Palczewski et al., 1994; Smith et al., 1994). This variant is formed by alternative mRNA splicing and is identical with 48-kDa arrestin except that the COOH-terminal 35 residues (370–404) are replaced by a single alanine (Palczewski et al., 1994; Smith et al., 1994). On the other hand, p46 possesses the same immunological properties as that generated in vitro by calpain proteolysis, both are recognized by mAb S2.4.C5, but not by mAb A9C6.

The mobilities of p46 and the 46-kDa product of arrestin cleaved by calpain in vitro were compared on Western blots, following SDS-PAGE. Fig. 4C shows a Western blot labeled with mAb C10C10. It contains hypotonically washed ROS membranes (lane 1); as in Fig. 4B, lane 1), 46-kDa truncated arrestin generated from in vitro calpain proteolysis (lane 2; as in Fig. 3, lane 5), and half each of lanes 1 and 2 added together (lane 3). This analysis showed that p46 and the product of calpain-cleaved arrestin in vitro have the same mobility in SDS-PAGE.

**COOH-terminal Sequence Analysis of Truncated Arrestin**—The above results suggest that the p46 arrestin found in rod outer segments could have arisen from proteolysis by calpain and that in vitro proteolysis of purified arrestin with purified calpain might be a good model of that occurring in situ. To test this possibility more precisely, we sought to define the amino acid sequences of the NH2 and COOH termini of p46, obtained directly from ROS membranes and also following in vitro calpain proteolysis of arrestin.

Different samples of p46 were subjected to NH2-terminal sequencing. In three separate attempts, no sequence data could be obtained from either the in vitro or in vivo p46, or from intact arrestin, which was prepared in parallel with the p46 samples. It appears that the NH2 terminus of p46 (from both sources) remains intact and blocked, like that of intact arrestin which is acetylated (Shinohara et al., 1987).

For COOH-terminal analysis, the samples of p46 and samples of intact arrestin were first completely digested with trypsin and the products were separated by HPLC. In comparing the resulting HPLC profiles, there was one peak that was repeatedly present in the p46 samples, but not in the intact arrestin samples, and would immunoreact with mAb S2.4.C5, but not mAb A9C6 (asterisk in Fig. 5A). The sequence and the mass of the peptide from this peak was the same from both sources of p46. The sequence was ESQDENFV, which correspond to residues 368–377 of arrestin (cf. Shinohara et al. (1987)). The masses obtained from laser desorption mass spectrometry of the peptide also indicated truncation at Phe377.
Fig. 2. Conditions for proteolysis of arrestin by calpain. Purified arrestin (20 pmol, as determined by Bradford assay) was incubated without (lanes 1 and 2) or with (lanes 3–14) different forms of rhodopsin or opsin (200 pmol) in stripped ROS membranes for 5 min in the dark at 30 °C, then 5 min in the light (white boxes) or dark (black boxes). Samples were incubated for an additional 40 min without (A) or with (B) 2 units of purified retinal calpain II (final volume of 20 μl) in the dark, then centrifuged through a sucrose cushion. Supernatants (S) and pellets (P) were electrophoresed in a 10% SDS-polyacrylamide gel and visualized with Coomassie Blue. Preparation of the different forms of rhodopsin is described under “Experimental Procedures.” The presence of arrestin in the pellet indicates its binding to rhodopsin. Proteolysis of arrestin is manifest by the presence of a 46-kDa form, which is evident only in the pellet. On the right, the apparent molecular masses are indicated in kDa; the position of the receptor is indicated by rh (Rh, unbleached rhodopsin; Rh-P, phosphorylated rhodopsin; Op, opsins; Op-P, phosphorylated opsins).

Fig. 3. Time course of proteolysis of arrestin by calpain. Arrestin (200 pmol) was incubated with phosphorylated rhodopsin (2 nmol) in stripped ROS membranes for 5 min in the dark at 30 °C. The mixture was illuminated for 5 min and an aliquot (200 pmol of rhodopsin) was transferred to Laemmli sample buffer at t = 0 min (lane 1). Retinal calpain II (18 units, final volume of 180 μl) was then added, and aliquots (200 pmol of rhodopsin) were quenched in sample buffer at the indicated intervals. Protein was separated in a 10% SDS-polyacrylamide gel and visualized with Coomassie Blue. The initial product of proteolysis (46.5 kDa), which is first evident after 2.5 min (lane 2), is slightly less mobile than the final product (46 kDa), which is most evident after 80 min (lane 7). Addition of fresh calpain after 60 min and incubation for 20 min did not result in any further proteolysis (lane 8). The apparent molecular masses (kDa) are indicated on the left.

They were consistently ~25 Da larger than the calculated mass of this peptide (1261 Da), which is probably due to a sodium adduct (Roepstorff, 1994). Truncation after the next residue, which is a glutamic acid in bovine arrestin (Shinohara et al., 1987), would give an extra mass of 129 Da (over the 1261 Da). Amino acid sequences could be obtained from two of the four major peaks isolated by HPLC (Fig. 5B). Both of these sequences corresponded to partial internal sequences of arrestin. One (from peak 1; elution at 12% CH3CN) corresponded to residues 381–385; the other (from peak 2; elution at 19% CH3CN) corresponded to residues 386–404 (Fig. 5C). Calculated and observed masses for the peak 1 peptide were 600 and 601.9 ± 0.6, respectively, for the peak 1 peptide, 2185 and 2186.9 ± 2.2, respectively. Given that a peak containing peptides I and II conjugated together was not obtained, these data suggest that calpain cleaves the COOH terminus of arrestin first between residues 385 (Leu) and 386 (Lys), which is consistent with the immunological results presented in Fig. 4. It appears that there are two more cleavage sites, between residues 380 (Phe) and 381 (Ala) and then between residues 377 (Phe) and 378 (Glu), to generate p46. However, it is possible that only the latter occurs, with residues 378–380 subsequently being lost from the released peptide. We know from the sequence of the COOH terminus of p46 that residues 378–380 are indeed removed, although we did not isolate and obtain the sequence of a released peptide containing these residues. The two peaks from which we were unable to obtain amino acid sequence data might have contained blocked NH2-terminal fragments from other proteins in the ROS membranes or from calpain itself; the NH2 termini of both the large and small subunits of calpain are cleaved by autolysis (Suzuki et al., 1981; Mellgren et al., 1982; Hathaway et al., 1982).

Light Dependence of 46-kDa Arrestin in Situ—The above in vitro studies predict that calpain proteolysis of arrestin to generate p46 should require light, and therefore there should be more p46 in light-adapted retinas. Because bovine eyes are collected from animals that have varying light histories, a well controlled study of the effect of light on the presence of p46 could not be done. Nevertheless, in experiments where only one eye was kept in the light for 30 min, and the other eye from the same animal (less than 5 min post-mortem) was placed in darkness during the same period, p46 was usually more easily detected in crude ROSs of the eye maintained in light than in those of the eye placed in darkness. In the Western blot of Fig. 6, p46 is not detectable at all in the dark samples (lanes 1 and 3), whereas a small amount is evident in the light sample (lane 2). In the light samples, the ratio of p46:p48 appeared by

Fig. 4. Initial Sites of Cleavage of Arrestin by Calpain—The appearance of a 46.5-kDa intermediate during in vitro proteolysis of arrestin by calpain indicates that there is more than one cleavage site in the generation of p46 arrestin. To determine the additional site(s), we analyzed the peptides released during proteolysis.

Peptides released by calpain from stripped ROS membranes, containing arrestin-[32P]phosphorhodopsin complexes, were isolated by HPLC for sequencing and mass spectrometry. After incubation with calpain for 40 min (as above), samples were centrifuged and the supernatants removed. The amount of 32P detected in the supernatant indicated that only 1.3% ± 0.3% S.E.; n = 8) of phosphorylated residues were released into the supernatant. Therefore, although the COOH terminus of rhodopsin is very sensitive to proteolysis by a variety of proteases (Kuhn et al., 1982), calpain cleavage of the phosphorylated COOH terminus of arrestin-bound rhodopsin appears to be negligible. Amino acid sequences could be obtained from two of the four major peaks isolated by HPLC (Fig. 5B). Both of these sequences corresponded to partial internal sequences of arrestin. One (from peak 1; elution at 12% CH3CN) corresponded to residues 381–385; the other (from peak 2; elution at 19% CH3CN) corresponded to residues 386–404 (Fig. 5C). Calculated and observed masses for the peak 1 peptide were 600 and 601.9 ± 0.6, respectively, for the peak 1 peptide, 2185 and 2186.9 ± 2.2, respectively. Given that a peak containing peptides I and II conjugated together was not obtained, these data suggest that calpain cleaves the COOH terminus of arrestin first between residues 385 (Leu) and 386 (Lys), which is consistent with the immunological results presented in Fig. 4. It appears that there are two more cleavage sites, between residues 380 (Phe) and 381 (Ala) and then between residues 377 (Phe) and 378 (Glu), to generate p46. However, it is possible that only the latter occurs, with residues 378–380 subsequently being lost from the released peptide. We know from the sequence of the COOH terminus of p46 that residues 378–380 are indeed removed, although we did not isolate and obtain the sequence of a released peptide containing these residues. The two peaks from which we were unable to obtain amino acid sequence data might have contained blocked NH2-terminal fragments from other proteins in the ROS membranes or from calpain itself; the NH2 termini of both the large and small subunits of calpain are cleaved by autolysis (Suzuki et al., 1981; Mellgren et al., 1982; Hathaway et al., 1982).

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was partially proteolyzed (2.5 min) with calpain II. lane 1
ROS membranes, 46-kDa mAb S2.4.C5 (lane 3) recognized only 48-kDa arrestin (lane 2).
mAb A9C6 (lane 4) and monoclonal antibodies that recognize epitopes of residues 42–48 (polyclonal antibody (pAb) and mAbs S2.4.C5, and 375–386 (d, A9C6). The apparent molecular masses (kDa) of arrestin and its truncated products are indicated on the left. A, arrestin was partially proteolyzed (2.5 min) with calpain II in vitro (as in Fig. 3, lane 2). mAb A9C6 (d) recognized only nonproteolyzed arrestin (lane 4). The polyclonal antibody (pAb) and mAbs S2.4.C5 (a) and S2.4.C5 (c) recognized arrestin and both of its truncated products (46.5 and 46 kDa) (lanes 1–3). B, high salt extracts of ROS membranes (as in Fig. 3, lane 2). mAb C10C10 (b) recognized 48-kDa arrestin and both p46 and p44 (lane 1). mAb S2.4.C5 (c) recognized only 48-kDa arrestin and p46 (lane 2). mAb A9C6 (d) recognized only 48-kDa arrestin (lane 3). C, high salt extract of ROS membranes (lane 1), 46-kDa in vitro proteolytic product (lane 2), and half each of lanes 1 and 2 added together (lane 3), labeled with mAb C10C10 (b). D, diagram of arrestin, indicating its PEST sequence (PEST) and the epitopes recognized by the mAbs used in A–C (from Donoso et al., 1990) and Dua and Donoso (1993)). Using the PEST-FIND program (Rogers et al., 1986), the PEST score for the sequence shown was determined to be ±13. PEST scores may range from −45 to +50; scores of ≥5 or greater indicate potential PEST sequences (Rogers et al., 1986). Bold italic letters in the PEST sequence represent the PEST amino acids. Numbers indicate the residues of each site. Domains I and II refer to the calpain generated fragments isolated by HPLC and sequenced in Fig. 5.

Immunostaining to be in the range of 1:20 to 1:50. Note, however, that 1) these experiments probably did not employ the optimal lighting conditions to generate maximal p46 (see Discussion), and 2) in an illuminated intact eye, only a small percentage of the arrestin is bound to rhodopsin; most of the p48 arrestin in the light sample as well as the dark samples of Fig. 6 is cytosolic and therefore could not be proteolyzed by calpain.

Release of Arrestin from Rhodopsin—Intact arrestin is released from the phosphorylated receptor after the photosensitized chromophore has been removed (Hofmann et al., 1992). Under physiological conditions, reduction of the chromophore by retinol dehydrogenase (Ishiguro et al., 1991) effects its removal. Treatment with NH$_2$OH converts all-trans-retinal to all-trans-retinol oxide, which, like all-trans-retinal, can no longer reside in the receptor. Fig. 7 (lanes 3 and 4) shows that exposure of a sample, containing intact arrestin bound to phosphorhodopsin, to 2 mM NH$_2$OH resulted in the release of most of the arrestin into the supernatant (as shown by Hofmann et al., 1992). However, under the same conditions, if the arrestin had been previously cleaved by calpain, most of the p46 arrestin remained in the pellet (Fig. 7, lanes 7 and 8). Densitometric scanning to determine the relative amounts of intact arrestin and p46 in the supernatants and pellets following NH$_2$OH treatment (e.g. lanes 3 and 4 and lanes 7 and 8), indicated that p46 has a 40-fold greater affinity for phospho-opsin. In the experiment shown in Fig. 7, arrestin was only partially proteolyzed, leaving some of the 46.5-kDa truncated arrestin. In contrast to p46, this intermediate behaved more like intact arrestin and was mostly released into the supernatant (lane 7). Therefore, this experiment shows that removal of the chromophore promotes significant release of intact arrestin and the 46.5-kDa intermediate from the receptor, but most of the p46 remains bound to phospho-opsin.

To test if the p46 would be released from opsin upon its regeneration, we added excess 11-morphine to previously stripped ROS membranes, containing phospho-opsin bound to arrestin that had been proteolyzed by calpain in vitro. Aliquots were removed after various intervals, centrifuged, and the supernatants and pellets were analyzed by SDS-PAGE. The arrestin was not detectable in the supernatant, even after 4 h of incubation. In another experiment, hypotonically washed ROS membranes, containing p44 and p46, were incubated with excess 11-cis-retinal. In this case, neither truncated arrestin was detected in the supernatant.

**Arrestin Inhibition of Receptor Dephosphorylation—Dephosphorylation of rhodopsin by ROS phosphatase is inhibited by bound arrestin (Palczewski et al., 1989a).** Because 46-kDa truncated arrestin binds more tightly to phospho-opsin than intact arrestin does, it might be expected to inhibit receptor dephosphorylation even more effectively. On the other hand, it is possible that truncation of arrestin results in a loss of this inhibitory effect. Thus, we determined the effectiveness of 46-kDa truncated arrestin at inhibiting the dephosphorylation of phospho-opsin (Fig. 8).

In this experiment, sample 1 contained phosphorhodopsin, and samples 2, 3, and 4 contained phosphorhodopsin with arrestin bound. Sample 4 was then treated with calpain to cleave the arrestin, and samples 1, 2, and 4 were incubated with 2 mM NH$_2$OH for 10 min. The samples were then incubated with ROS phosphatase. During this incubation, as shown in the inset of Fig. 8, which is an SDS-PAGE analysis of the arrestin after centrifugation: sample 1 contained phospho-opsin only; sample 2 contained phospho-opsin with intact arrestin, most of which was soluble; sample 3 contained phospho-opsin with intact arrestin bound; and sample 4 contained phospho-opsin with p46 arrestin bound (no intact arrestin was detectable).

Sample 1 was used as a control standard; addition of the NH$_2$OH to phosphorhodopsin in this sample had no effect on receptor dephosphorylation. Samples 3 and 4, containing bound intact and truncated arrestin, respectively, inhibited dephosphorylation of the receptor to the same extent (60–70% inhibition, compared with the control), when dephosphorylation was linear with time (R $\approx$ 0.95 for all curves) (Fig. 8). This level of inhibition of dephosphorylation is comparable with that described in a previous report (Palczewski et al., 1989a). In sample 2, release of most of the intact arrestin into...
the supernatant by \( \text{NH}_2\text{OH} \) permitted dephosphorylation that was only 20–30% (range) less than that of the control (Fig. 8).

**DISCUSSION**

We have shown that purified bovine ROSs contain two truncated arrestins, p46 and p44, in addition to 48-kDa arrestin. Both forms were found to be bound tightly to ROS membranes (requiring high salt to be eluted), making it unlikely that they represent soluble contaminants released from other cells when the retina was disrupted to release the ROSs. During the course of the present study, p44 has been shown to be an alternatively spliced variant of arrestin (Palczewski et al., 1994; Smith et al., 1994), and the presence of p46 has been reported in human and rabbit retinas, as well as bovine retinas (Smith, 1995). Our studies indicate that p46 is the same as that generated by calpain proteolysis of arrestin in vitro, providing strong evidence that p46 is formed by calpain proteolysis of arrestin and that in vitro analysis of the proteolysis is pertinent.

In vitro analysis showed that arrestin is selectively proteolyzed by retinal calpain II when arrestin is bound to rhodopsin. Proteolysis of arrestin occurs at the COOH terminus. First, a 19-amino acid peptide is removed to give rise to a 46-kDa intermediate. This final product is resistant to further proteolysis by calpain. The consequence of arrestin proteolysis is that the remaining product maintains a high binding affinity for the receptor after the removal of all-trans-retinal (Hofmann et al., 1992). The presence of truncated arrestin on opsin inhibits its dephosphorylation. It is unclear how p46 arrestin might be released from phospho-opsin; incubation of the complex with 11-cis-retinal did not elicit release.

**Selective Proteolysis**—The proteolytic truncation of the carboxyl tail of arrestin by calpain, only when arrestin is bound to...
rhodopsin, indicates that arrestin undergoes a conformational change that exposes its calpain-sensitive domain when it binds to rhodopsin. The PEST sequence of arrestin (Fig. 4D) is likely to be included in the domain that becomes exposed (cf. Rogers et al., 1986 and Wang et al., 1989)). Proteolysis by the less selective protease, trypsin, also indicates that arrestin undergoes a conformational change upon binding to rhodopsin. This protease cleaves arrestin at many sites, but a major effect is to remove its carboxyl tail (Palczewski et al., 1991b). Trypsin cleaves the carboxyl tail from both soluble and rhodopsin-bound arrestin, but proteolysis of the latter is more rapid, and the sizes of the resulting products from the two conditions differ (Palczewski et al., 1991a).

An alternative possibility to explain the selective proteolysis of arrestin could be that calpain might be only active at the ROS disk membrane. Consistent with this suggestion, the active form of calpain I has been localized at the membrane in situ (Saido et al., 1993), and certain phospholipids have been shown to lower its Ca\(^{2+}\) requirement for activity (Saido et al., 1992). However, the conditions of the in vitro assays used in the present study included sufficient Ca\(^{2+}\) for maximal proteolysis of a soluble substrate, casein (cf. Azarian et al. (1993)). Indeed, casein was proteolyzed by calpain in the presence of soluble arrestin, which resisted proteolysis.

Consequence of Proteolysis—The consequence of calpain proteolysis of arrestin is that it inhibits the release of arrestin from the receptor under conditions which promote the release of intact arrestin. This finding is consistent with studies showing that truncation of arrestin at its COOH terminus (albeit not at the same sites as calpain), either by tryptic digestion (Palczewski et al., 1991b) or expression of deletion mutants (Gurevich and Benovic, 1992, 1993), results in enhanced binding to phosphorylated rhodopsin that has been either kept in the dark or exposed to light. Gurevich and Benovic (1993) also showed that arrestin that was missing 39 amino acids from its COOH terminus binds to phospho-opsin and phosphorylated, light-activated rhodopsin to the same extent. Palczewski et al. (1994) found that p44 bound tightly to nonphosphorylated, photoactivated rhodopsin, as well as phosphorylated, photocatalyzed rhodopsin. A major difference between p44 and p46 is that the latter would not be generated until phosphorylated rhodopsin is present. In contrast to alternative mRNA splicing, calpain proteolysis of arrestin would provide a rapid means of generating truncated arrestin in response to rhodopsin phosphorylation.

The binding of arrestin to rhodopsin has two functions. First, it enhances the decoupling of phosphorylated rhodopsin from transducin (Wilden et al., 1986; Bennett and Sitaramaya, 1988). Second, it inhibits the dephosphorylation of rhodopsin by a phosphatase 2A (Palczewski et al., 1989a). We are a long way from understanding the physiological function of arrestin.
Ca²⁺tein activators (Pontremoli et al., 1990). A similar consideration stems from studies showing that some proteins have particularly high Ca²⁺ levels in ROSs of bovine eyes kept in the light than in ROSs of eyes placed in darkness. Using toad retinas, which are more amenable than bovine retinas for in situ studies and for controlling light exposure, Mangini and co-workers (Mangini and Pepperberg, 1988) have found a faster migrating form of arrestin present in light-adapted retinas but not in dark-adapted retinas. This faster migrating form is less abundant when the retinas are incubated in the presence of the general cysteine protease inhibitor, E-64d, suggesting that this form of arrestin could be generated by calpain proteolysis of arrestin.

It is not clear what lighting conditions might be optimal for promoting arrestin degradation. Investigation of this question will require experiments with retinas that are better suited than bovine retinas for physiological studies. It is noteworthy that when frog ROSs are exposed to dim conditioning illumination prior to a bright flash of light, the subsequent receptor dephosphorylation is inhibited (Biernbaum et al., 1991); inhibition of receptor dephosphorylation is the predicted consequence of arrestin degradation.

An apparent inconsistency with calpain proteolysis of arrestin occurring in the light is that cytosolic Ca²⁺ levels of ROSs have been reported to decrease upon illumination (Ratto et al., 1988; Gray-Keller and Detwiler, 1994). However, as noted above, arrestin cannot be proteolysed until it binds rhodopsin, so that the light dependence of its proteolysis is determined by its own conformation. Moreover, especially in subsaturating light, the ROS Ca²⁺ concentration in the light is reported to be still rather high (no less than 325 nM in light sufficient to induce a photoreceptor that is 70% of maximal; Gray-Keller and Detwiler, 1994) in comparison with that in other cell types. Nevertheless, how does ROS calpain II function in a submicro-molar concentration of Ca²⁺, when, like other calpains, it requires hundreds of μM Ca²⁺ for half-maximal casodectytic activity in vitro (Azarian et al., 1993)? This question, as it applies to calpain I generally, has been discussed extensively elsewhere (e.g., Croall and DeMartino, 1991). The most important consideration stems from studies showing that some protein activators (Pontremoli et al., 1988, 1990) and lipids (Codican and Hathaway, 1984; Saito et al., 1992) lower the Ca²⁺ requirement for in vitro calpain activity. In the cell, the concentration of lipid and protein activators are orders of magnitude higher than they are in in vitro studies. Because such activators increase the affinity of calpain for Ca²⁺, in vitro Ca²⁺ requirements do not reflect Ca²⁺ requirements in the cell. For example, another Ca²⁺-regulated enzyme, protein kinase C, is not dependent on any Ca²⁺ for activity in the presence of sufficiently high concentrations of lipid (Mosior and E pand, 1994).

Arrestin as a Calpain Substrate—The list of putative calpain substrates is a long one. Most, however, have been identified as substrates only from in vitro experiments, and in only a few cases (all from in vitro experiments) has the nature of the cleaved peptide bond been determined. In these few studies that have defined the site of cleavage, it is nevertheless evident that calpain proteolysis occurs at specific and unique sites (Takahashi, 1990). In the present study, we determined the sites of truncation of arrestin both in vitro and in vivo, and we present the finding that an in vivo form of arrestin is identical to the final product of in vitro proteolysis as evidence for calpain proteolysis of arrestin in vivo.

Of the many proteins considered as putative calpain substrates, most are cytoskeletal proteins, enzymes, or membrane proteins (Takahashi, 1990; Saito et al., 1994). Interestingly, arrestin is none of these, suggesting that the role of calpain might be broader than previously anticipated. Given the similarity in structure and function among the arrestin family (Lohse et al., 1992), it is plausible that non-visual arrestins might be also substrates of calpain. Consistent with this notion, we have found, using the PEST-FIND program of Rogers et al., 1986, that most arrestins have PEST sequences. Indeed, some forms of β-arrestin have particularly strong PEST sequences. Human β-arrestin-1 (Parruti et al., 1993), bovine β-arrestin (Lohse et al., 1990), and rat β-arrestin-1 (Attramadal et al., 1992) have PEST scores of +11 for sequences near their carboxyl tail; the score for the bovine visual arrestin PEST sequence is +13.

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