ATP-Dependent Calcium Uptake Activity Associated with a Disk Membrane Fraction Isolated from Bovine Retinal Rod Outer Segments†

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ABSTRACT: Ca^{2+} sequestration and release from disks of rod outer segments may play a critical role in visual transduction. An ATP-dependent Ca^{2+} uptake activity has been identified in association with purified disks of bovine rod outer segments. A crude preparation of osmotically active disks was obtained from rod outer segments by hypoosmotic shock and subsequent flotation on a 5% Ficoll 400 solution. These "crude" disks were further purified by separation into two distinct components by centrifugation in a linear Ficoll gradient. Disks comprised the major component; at least 60% of the protein was rhodopsin. This fraction also contained a Ca^{2+} uptake activity stimulated ~4-fold by ATP. The initial rate was ~0.21 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}. Most of the ATP-dependent accumulation of $^{45}Ca^{2+}$ was released by the calcium ionophore A23187. The uptake activity was sensitive to vanadate ($K_i \sim 20\ \mu M$) and insensitive to the mitochondrial Ca^{2+} uptake inhibitor ruthenium red (10 μM). The ATP-dependent Ca^{2+} uptake exhibited Michaelis–Menten activation kinetics with respect to $[Ca^{2+}]$ ($K_m \sim 6\ \mu M$). The osmotic properties of the sealed disk membranes were exploited to determine whether the association of Ca^{2+} transport activity with the disks was merely coincidental. The sedimentation properties of these disks, upon centrifugation on a second Ficoll linear density gradient, varied with the osmolarity of the gradient solution. In several separate gradient solutions of differing osmotic and ionic strengths, the Ca^{2+} uptake activity always comigrated with the disks. These results indicate that the ATP-dependent Ca^{2+} uptake activity was physically associated with sealed native disk membranes. The characteristics of the Ca^{2+} uptake activity suggest that it may play a major role in the regulation of cytosolic Ca^{2+} levels in rod cells in vivo.

In 1971 Yoshikami and Hagins proposed that calcium may act as the intracellular transmitter in the visual transduction process [presented in detail by Hagins (1972)]. According to their hypothesis, calcium stored within the rod outer segment (ROS)[1] disks is released upon illumination. The cytoplasmic-free Ca^{2+} concentration rises, which in turn causes a decrease in the Na^{2+} conductance of the plasma membrane. The total Ca^{2+} content in bovine ROS has been estimated to be 3–10 mM or one to three Ca^{2+} per rhodopsin molecule (Kaupp & Schnetkamp, 1982; Schnetkamp, 1979; Schröder & Fain, 1984). Although it is not clear where the calcium is stored in the ROS, there is indirect evidence suggesting that the cytoplasmic-free Ca^{2+} levels are low, i.e., $\sim 10^{-7}$–10^{-6} M (Wormington & Cone, 1978; Hagins et al., 1974). A likely sequestration site for Ca^{2+} is within the lumen of the disks. If indeed Ca^{2+} is sequestered within disks and is released upon illumination, it is essential that a mechanism exist to resequester the Ca^{2+} into the disk lumen. An ATP-dependent Ca^{2+} uptake system in the disk membrane could, in principle, serve this function.

Several investigators have studied light-induced Ca^{2+} release and Ca^{2+} reuptake into various preparations of rod cells. Light-induced Ca^{2+} release of an appropriate magnitude and time course has been reported for intact rod cells still attached to the retina (Gold & Korenbrot, 1980; Yoshikami et al., 1980). In these studies Ca^{2+} efflux into the extracellular space was measured in response to light stimulation. Light-induced Ca^{2+} release has also been reported in sonicated bovine ROS (Smith, 1977), reconstituted rhodopsin vesicles (O'Brien, 1979), and permeabilized ROS (George & Hagins, 1983). To date, however, only the intact rod cell studies (Gold & Korenbrot, 1980; Yoshikami et al., 1980) have demonstrated both the magnitude and time course of Ca^{2+} release approximating that predicted for the Ca^{2+} hypothesis.

There have been reports of ATP-dependent Ca^{2+} binding activity in homogenized bovine retinas (Neufeld et al., 1972) and of ATP-dependent Ca^{2+} uptake in sonicated frog ROS (Mason et al., 1974) and homogenized frog ROS (Miki et al., 1980). A report of ATP-dependent Ca^{2+} uptake into a preparation of frog ROS (Bownds et al., 1971) was subsequently attributed by the authors to mitochondrial contamination [cited in Szuts (1980)]. No ATP-dependent Ca^{2+} uptake activity has yet been reported for isolated intact disks, which makes it possible that the previous reports could be ascribed to Ca^{2+} uptake by rod cell components other than disk membranes.

In addition to these studies on Ca^{2+} uptake and release, light-regulated ATPase activities have also been reported. A Ca^{2+}-dependent ATPase activity is associated with bovine ROS (Sack, 1977). Thacher (1978) reported a Mg^{2+}-dependent ATPase in toad ROS that increased approximately 2-fold in response to light stimulation under optimal conditions. Berman et al. (1977), upon careful fractionation, localized a Ca^{2+}/Mg^{2+}-ATPase activity to the rod inner segment (RIS) and not ROS.

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[1] Abbreviations: ROS, rod outer segment; RIS, rod inner segment; EDTA, ethylenediaminetetraacetic acid; AMP-PNP, adenylyl imidodiphosphate; TCA, trichloroacetic acid; βME, 2-mercaptoethanol; BSA, bovine serum albumin; STX, saxitoxin; SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATPase, adenosinetriphosphatase; Tris, tris(hydroxymethyl)aminomethane.

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Ca^{2+}-ATPase and ATP-dependent Ca^{2+} uptake activities have been identified in plasma membranes from various cell types (Schatzman, 1981; Michaelis et al., 1983; Papazian et al., 1984), sequestration organelles (deMeis & Inesi, 1982; Chan et al., 1984), and mitochondria (Crompton & Carafoli, 1979). A systematic purification and characterization of the membrane subfractions containing these activities has been instrumental in determining their functional significance. We have applied such an approach to retinal rod cells and report here the subfractionation of crude bovine ROS disks into two components, one of which constitutes highly purified disks. We further report the identification of an ATP-dependent Ca^{2+} uptake activity in this preparation and evidence for its physical association with the purified disk membranes.

Experimental Procedures

All KH_2PO_4 solutions were titrated with NaOH to the indicated pH. When employed, the antioxidant 2-mercaptoethanol (βME) was added immediately prior to use. All procedures were performed in room light at 0–4 °C unless otherwise stated.

Isolation of Disks from Frozen Retina. Osmotically active disks were obtained from bovine retinal rod outer segments by hypoosmotic shock and subsequent flotation on a 5% w/v Ficoll 400 solution using slight modifications of the procedures of Smith (1975) and McCaslin (1981). Briefly, 50 frozen bovine retinas (Hormel) were defrosted and suspended in 45% sucrose w/v in buffer A (0.1 M KH_2PO_4, 60 mM NaOH, and 5 mM βME, pH 7.0). The retina suspension was forced through a disposable (50 mL) plastic syringe (without needle), divided equally, and centrifuged in an SW-27 rotor for 1 h at 25 000 rpm. The floated crude ROS were collected, washed in buffer A, and pelleted. Each pellet was resuspended in 40% sucrose (w/v) in buffer A, shaken, and centrifuged for 1 h at 25 000 rpm. The ROS float was again collected, diluted ~8-fold with 5 mM βME, and centrifuged at 17 500 rpm for 30 min in a 50-mL Sorvall centrifuge tube. The pellet was then resuspended in 17 mL of 5 mM βME and immediately further diluted to a final volume of 34 mL with 10% w/v Ficoll 400 in 5 mM βME. After 2 h the osmotically lysed ROS suspension was transfered to an SW-27 centrifuge tube and centrifuged at 25 000 rpm for 2 h. Osmotically intact disks were collected from the surface of the 5% Ficoll solution and washed twice in 5% (w/v) sucrose in buffer A. The final pellet was resuspended in an equal volume of this 5% sucrose solution and stored in the dark. The entire isolation procedure was performed under dim red light (Kodak no. 1 safelight filter, 15 W).

Isolation of Disks from Fresh Retinas. Twenty to twenty-five fresh bovine eyes were hemisected under dim red light. The retinas were carefully dissected away from the eyecup and placed in a 45% (w/v) sucrose solution in buffer A (60 mL final volume). Fresh retinas were mixed by using a Vortex Genie to release the ROS, in lieu of being forced through a plastic syringe. All subsequent steps for fresh retinal disk isolation were identical with the disk isolation for frozen retinas described above. The yield for fresh retinas was $\sim 0.19 \pm 0.03$ mg of protein/retina ($n = 2$). The yield for frozen retinas was $\sim 0.60 \pm 0.05$ mg of protein/retina ($n = 6$).

Fractionation of Crude Disks on Linear Ficoll Gradients. Linear density gradients were formed from 18 mL of 20% w/v Ficoll 400 in buffer A (heavy phase) and 16 mL of 5% w/v Ficoll in buffer A (light phase). The disk preparation (~1.5 mL) was mixed with an equal volume of buffer A and carefully loaded onto the gradients with a peristaltic pump. Gradients were centrifuged in a Beckman VTi 50 rotor for 140 min at 50 000 rpm, and fractions were collected. When small gradients (~5 mL total volume) were used, the conditions were the same as described above except 2.3 mL of 20% w/v Ficoll in buffer A and 2.1 mL of 5% w/v Ficoll in buffer A were used, ~200 μL of the preparation was loaded onto each gradient, and the gradients were centrifuged in a Beckman VTi65 rotor, 60 000 rpm for 45 min. When rhodopsin quantification was to be performed, the loading and collecting of the gradients was done under dim red light. The conditions of the centrifugation were such that the membranes did not reach their equilibrium buoyant density. We were unable to reach equilibrium density on these Ficoll gradients, perhaps due to osmotic effects on the membranes of the gradient itself.

Ca^{2+} Transport Assays. $^{45}Ca^{2+}$ transport assays were performed by the method of Papazian et al. (1979), with slight modifications. Two-milliliter disposable columns of Dowex 50W-X2-200 resin (Tris form) were equilibrated with 0.1 M KH_2PO_4–60 mM NaOH–10 mM Tris, pH 7.0, and washed with 2 mL of bovine serum albumin (10 mg/mL) in the same buffer. Aliquots (50 μL) of the membrane preparation were incubated at 24 °C (unless otherwise stated) in 5.5 mM $MgCl_2$ and 0.275 mM $^{45}CaCl_2$ (150 000 cpm/nmol) with or without 2 mM Mg^{2+}ATP. Forty-five microliters of the incubation mixture was then loaded onto the column and washed with 2 mL of 0.1 M KH_2PO_4–60 mM NaOH–10 mM Tris, pH 7.0, buffer at an elution rate of approximately 2 mL/min. Sixteen milliliters of Hydrofluor (National Diagnostics) was added to each sample for scintillation counting. Disks were stored in the dark; under these conditions the ATP-dependent Ca^{2+} uptake activity was stable (>90% active after 7 days).

When the effect of various agents on Ca^{2+} uptake activity was determined, the Ca^{2+} transport assays were the same as described above except 5 μL of a concentrated stock solution of each was added to 50 μL of the membrane solution and then 5 μL of the concentrated $^{45}Ca^{2+}$ solution with or without Mg^{2+}ATP was added. Fifty microliters of this incubation mixture was then assayed.

Specific activity of ATP-dependent Ca^{2+} uptake [nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}] was determined by extrapolation from 5–10-min incubation points.

After centrifugation of the crude disk preparation on the 5–20% Ficoll gradient, each gradient was fractionated and assayed for the following: (1) ATP-dependent Ca^{2+} transport activity, (2) rhodopsin content (by bleaching and regeneration), and (3) total protein.

The Ca^{2+} uptake activity of each gradient fraction was determined by one of the following methods as specified in the text: (1) The crude disks were preincubated with 0.308 mM $^{45}CaCl_2$ (2.6×10^4 cpm/nmol) and 4.84 mM $MgCl_2$ with or without 1.76 mM Mg^{2+}ATP for 1 h (24 °C) prior to gradient centrifugation (see Figure 4). (2) The crude disks were preincubated with unlabeled Ca^{2+} with or without Mg^{2+}ATP for 1 h, and gradient fractions were subsequently assayed for $^{45}Ca^{2+}$ uptake. (3) No preincubation was performed on the crude disks, and fractions were assayed for $^{45}Ca^{2+}$ transport after centrifugation as described above.

Ca^{2+} Affinity Determination. The affinity of the Ca^{2+} uptake activity for Ca^{2+} was determined by using an EDTA/Ca^{2+} buffering system. Incubation conditions were the same as described above except 3 mM EDTA was present and total unlabeled Ca^{2+} was varied from 0 to 2 mM. The amount of $^{45}CaCl_2$ was 2.28×10^6 cpm/assay tube. Separation of disk associated from external $^{45}Ca^{2+}$ was performed by suction filtration on 0.45-μm pore size Millipore HA filters. The filters were presoaked in 3 mM $MgCl_2$ in buffer A, placed

in the suction filtration unit, the rinsed with 1 mL of 1% BSA in buffer A. Aliquots (50 μL) of the incubation mixture were removed following the 5.5-min incubation, placed in 455 μL of the ice-cold buffer A, vortexed, and added to the filter dropwise. This step was followed by three consecutive 8-mL washes of 3 mM $MgCl_2$ in buffer A. Five milliliters of Hydrofluor was added to the filter, and the samples were agitated on a Thomas rotator for $\geq$20 min before scintillation counting.

The free calcium concentration was determined by two independent methods. First a Ca^{2+} selective electrode (Philips IS 561) was used to directly measure free [Ca^{2+}] under the specific assay conditions (Simon & Carafoli, 1979). Second, the theoretical free [Ca^{2+}] was calculated by using the known affinity constants of the calcium chelating components in the incubation mixture (Graf & Penniston, 1981).

Washing of the "R-Band". The pooled rhodopsin-containing fraction ($\sim$7 mL), termed the R-band (see Results), was placed in an SS-34 centrifuge tube and diluted with buffer A to a final volume of 40 mL. The sample was then pelleted at 17 500 rpm for 30 min. After the supernatant was discarded, the pellet was resuspended in $\sim$1 mL of buffer A and divided into three equal portions. The three portions were each then diluted to 40 mL final volume with one of the following solutions: (1) 0.1 M KH_2PO_4, 60 mM NaOH, and 5 mM βME, pH 7.0; (2) 5 mM KH_2PO_4, 3 mM NaOH, and 5 mM βME, pH 7.0; (3) 220 mM mannitol, 5 mM KH_2PO_4, 3 mM NaOH, and 5 mM βME, pH 7.0.

The wash procedure, as described above, was repeated twice. Equal volumes of the respective buffer solutions were added to resuspend the final pellet ($\sim$300 μL/pellet). An aliquot (150 μL) of each washed rhodopsin band pellet was then layered with a peristaltic pump, under dim red light, onto its respective gradient and centrifuged at 50 000 rpm for 65 min in a Beckman VTi65 rotor.

Rhodopsin Assay: Bleaching and Regeneration. Aliquots of assay material were added to 1% w/v digitonin in 67 mM KH_2PO_4, pH 6.4, vortexed, and incubated for various times (1–24 h) in the dark. [For preparation of digitonin solution, see Hubbard et al. (1971).] Following incubation the samples were centrifuged for 10 min in a Beckman microfuge B centrifuge (maximum setting). The supernatant was aspirated and saved for rhodopsin determination. When necessary the pellet was reextracted with 1% digitonin solution and centrifuged, and the supernatant was assayed.

For rhodopsin assays performed directly on Ficoll gradient fractions (i.e., both the 5–20% and the 2–15% Ficoll gradients), 50-μL aliquots of each fraction were added to 260 μL of the 1% digitonin solution and incubated for 2 h. When necessary the pellets were reextracted with 260 μL of 1% digitonin, and the assay was repeated. The rhodopsin concentration of the supernatants was 0.2–3 μM for each assay.

A series of five spectrophotometric scans (650–250 nm) on each sample were performed at room temperature as follows: (1) in the dark, (2) after bleaching by 2-min exposure to fluorescent laboratory light, (3) after regeneration by 5-min incubation with 75 μM 11-*cis*-retinal (a 5-min incubation with 11-*cis*-retinal was shown to produce a maximal steady-state level of regeneration of bleached rhodopsin), (4) after addition of 32 mM NH_2OH [NH_2OH is used to shift the photolysis product of rhodopsin from 500 to 398 nm (Hubbard et al., 1971)], and (5) after bleaching by 2-min exposure to fluorescent laboratory light.

The difference in A_{500} between the first scan and the fifth scan was used to calculate the rhodopsin concentration in each sample [ϵ_{500} = 40 500 L/(mol·cm)]. A concomitant increase in A_{380} was also ascertained during the bleaching process. The percent of rhodopsin regeneration was calculated by taking the difference at A_{500} between the fourth scan (regeneration) and the fifth scan (second bleach) and comparing that to the original amount of rhodopsin that was present. It was necessary to scan from 650 to 250 nm, rather than to simply read absorbance at 500 nm, to correct for any base-line changes.

Rhodopsin content of the crude disks, washed R-band, and washed W-band was also determined. The rhodopsin concentration for each of these samples was 187 $\pm$ 14 (n = 15), 200 $\pm$ 29 (n = 6), and 10.1 $\pm$ 3 μM (n = 5), respectively. The 1% digitonin solution was used for all regeneration studies, and samples were extracted to completion, requiring one to nine extractions per sample. The amount of regeneration of rhodopsin in each preparation was as follows: (1) 60–100% for the Ficoll gradient fractions, (2) 83 $\pm$ 4%, range 79–91% (n = 3) for the crude disks, (3) 81 $\pm$ 3%, range 68–94% (n = 8) for the R-band, (4) 80 $\pm$ 5%, range 65–100% (n = 6) for the W-band.

For a more efficient extraction of the rhodopsin from the various membrane preparations, 3% w/v Ammonyx-LO in 67 mM KH_2PO_4, pH 6.4 (DeGrip et al., 1980), was sometimes employed. Although regeneration was not possible in this detergent, the total rhodopsin extracted was not significantly different from the 1% digitonin extractions, and only one to two incubations with detergent per sample were required for complete extraction.

Linear Density Gradients of Various Ionic and Osmotic Strengths. Linear density gradients were formed by using 2.3 mL of 15% w/v Ficoll 400 (heavy phase) and 2.1 mL of 2% w/v Ficoll 400 (light phase). The above Ficoll 400 solutions were made up in each of the following buffers: (1) 0.1 M KH_2PO_4, 60 mM NaOH, and 5 mM βME, pH 7.0 (high phosphate); (2) 5 mM KH_2PO_4, 3 mM NaOH, and 5 mM βME, pH 7.0 (low phosphate); (3) 220 mM mannitol, 5 mM KH_2PO_4, 3 mM NaOH, and 5 mM βME, pH 7.0 (mannitol/phosphate).

The high phosphate buffer and mannitol/phosphate buffer were calculated to be of the same osmotic strength ($\sim$240 mOsm). The mannitol/phosphate gradient centrifugation was performed in parallel with both the high and low phosphate gradients to allow direct comparison.

Protein Determination. Protein determination was performed via the modified Lowry protein assay, with TCA precipitation in the presence of deoxycholate (Bensadoun & Weinstein, 1976).

SDS/Laemmli Gel Electrophoresis. Electrophoresis in SDS slab gels (1.5 mm thick slabs) was performed by the method of Laemmli (Laemmli, 1970). The membrane samples were prepared as described previously (Papazian et al., 1979).

Electron Micrographs of R-Band and W-Band Membranes. The membrane sample was diluted 1:1 with 0.2 M sodium cacodylate, pH 7.0, and centrifuged for 5 min in a Beckman microfuge tube. The supernatant was discarded and replaced with cold fixative (1% w/v paraformaldehyde, 1% w/v gluteraldehyde, 3% w/v sucrose, 0.012% w/v $CaCl_2$, in 0.1 M sodium cacodylate, pH 7.3–7.4). The pellets were resuspended and fixed 30 min at room temperature. The sample was then centrifuged, fixative was removed, and cold 0.1 M sodium cacodylate was added to resuspend. The samples were left overnight and then washed twice with 0.1 M sodium cacodylate as described above. Samples were postfixed for 1 h at room temperature in 1% v/v OsO_4. After removal of the OsO_4, the samples were washed 3 times in 0.1 M sodium cacodylate and once in H_2O. After addition of 1% w/v aqueous uranyl acetate

for 1 h (room temperature), the samples were washed twice with H_2O. Dehydration was done in the following percent (v/v) ethanol solutions for 10 min in each solution: 25, 50, 75, 95, 100 and 100. The samples were then immersed for 10 min in propylene oxide and infiltrated with Epon–propylene oxide solutions in the following ratios for 30 min in each solution: 1:1, 2:1, 3:1, and pure Epon. The samples were embedded for 24 h at 60 °C in fresh Epon. Sections were cut and stained for 2 min in 2% uranyl acetate, rinsed with H_2O, and immersed in Reynolds lead citrate stain for 1 min.

Saxitoxin Binding Assay. [^{3}H]Saxitoxin (STX) was prepared and binding assays performed as described (Rhoden & Goldin, 1979) with the following modifications. Nine hundred microliter Dowex columns, 50W-X2-200 (Tris form), were washed with 1 mL of 20 mM Tris, pH 7.5, and 1 mL of 10 mM Tris–10 mg/mL BSA, pH 7.5. Aliquots (20 μL) of the membrane preparation were diluted to a final volume of 70 μL with 10 mM Tris, pH 7.5, and followed by the addition of 10 μL of 0.6 μM [^{3}H]STX (~2050 cpm/pmol). Sixty microliters of this mixture was layered onto the Dowex columns and eluted under pressure with 0.8 mL of 10 mM Tris, pH 7.5. For nonspecific binding determination, 5 μL of 10^{-4} M unlabeled STX was added prior to addition of [^{3}H]STX. Eluate was counted after addition of 10 mL of Hydrofluor.

To unmask possible STX binding sites in inside-out vesicular structures, the STX binding assay above was performed in the presence and absence of detergent. Membranes were diluted 1:1 in 0.2% Tween 80–20 mM Tris, pH 7.5, or diluted with 20 mM Tris, pH 7.5, alone and assayed as above.

Preparation of Synaptosomal Membranes. Synaptic plasma membrane enriched fractions were prepared as previously described (Goldin et al., 1980) with the following modifications. Bovine brain gray matter (~150 g) was homogenized in an Osterizer Blender at "mince" setting for 30 s. The homogenate was centrifuged at 3500*g* for 10 min in an SS-34 rotor. The upper two-thirds of the supernatant was then centrifuged at 27000*g* for 20 min in an SS-34 rotor. The pellets were lysed in 10 mM Tris–1 mM EDTA, pH 7.4, and centrifuged as above. Pellets from six tubes were resuspended in 1 L of 15% (w/v) sucrose–5 mM Tris, pH 8.1, and placed in sealable Beckman 50.2 Ti tubes (22 mL/tube), to which a 3-mL 32.5% (w/v) sucrose–10 mM Tris, pH 7.4, cushion was added. After centrifugation at 150000*g* for 50 min the 37.5%/15% sucrose interface was collected and diluted 1:1 (v/v) with 20 mM imidazole, pH 7.0. This material was centrifuged at 110000*g* for 30 min, and the pellets were resuspended in 20 mM imidazole, pH 7.0. The pelleting/resuspension cycle was repeated twice. The resulting pellets were resuspended in 50 mL of 10% (v/v) glycerol and 20 mM imidazole, pH 7.0, and frozen in liquid nitrogen.

Isolation of Mitochondria-Enriched Fraction from Synaptosomal Preparation. A mitochondria-enriched fraction from rat brain synaptosomal preparation was prepared as previously described (Hajos, 1975) with the following modifications. Two rat brains were homogenized (10 strokes, 450 rpm) in 0.32 M sucrose–1 mM EDTA, pH 7.8 (10% w/v). The brain homogenate was centrifuged at 2500*g* for 10 min, and the supernatant (S_1) was saved. The pellet (P_1) was homogenized as described above and centrifuged at 2500*g* for 10 min. The pellet (P_2) was discarded, and the resulting supernatant (S_2) was pooled with the first supernatant (S_1) and centrifuged at 9000*g* for 20 min. This pellet (P_3) was then resuspended in 0.32 M sucrose (5 mL per brain) and homogenized (four strokes, 450 rpm). Five milliliters of this material was layered onto 20 mL of 0.8 M sucrose–1 mM EDTA and centrifuged at 9500*g* for 25 min. The 0.8 M sucrose layer (fraction II of Hajos' protocol) enriched in synaptosomes was collected.

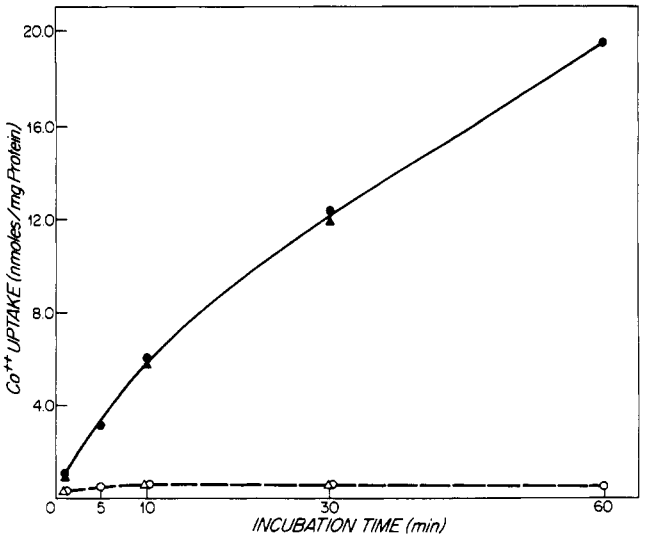

FIGURE 1: Time course of ATP-stimulated Ca^{2+} uptake into "crude" native disks. Crude disks were isolated by 5% Ficoll flotation, and Ca^{2+} uptake was determined as described under Experimental Procedures, in the presence (●, ▲) or absence (○, △) of 2 mM Mg^{2+}ATP. Data from two independently isolated disk preparations are shown (●, ○; ▲, △).

The synaptosomes were further purified as previously described (Cotman, 1974) with the following modifications. The 0.8 M sucrose layer was diluted with 2.5 volumes of 0.32 M sucrose–1 mM EDTA and centrifuged at 25000*g* for 10 min. The pellet (P_5) was resuspended in 2–4 mL of 5 mM Tris–1 mM EDTA, pH 7.8, and homogenized. After homogenization, the membranes were diluted to 80 mL in the same Tris buffer and stirred at 4 °C for 45 min. The osmotically lysed synaptosomes were centrifuged at 25000*g* for 10 min. The pellet (P_6) was resuspended in 10 mL of 0.32 M sucrose–1 mM EDTA and layered onto a discontinuous sucrose/1 mM EDTA gradient composed of 1.3, 1.1, 0.9, and 0.7 M sucrose. The gradients were centrifuged at 107000*g* for 45 min. The resulting pellet (P_7), primarily free mitochondria, was assayed for cytochrome *c* oxidase activity and ATP-dependent Ca uptake activity (see Table I).

Cytochrome Oxidase Assay. Cytochrome *c* oxidase activity was measured as previously described (Yonetani, 1967); a unit is defined as the change in A_{550} per minute. Control experiments demonstrated that most (~62%) of the cytochrome oxidase activity of each fraction was retained over the 3-day period required for the isolation of R-band and W-band membranes.

RESULTS

Purification and Characterization of Disk Membrane Fraction. Crude osmotically sensitive native disk membrane preparations, obtained by the previously reported procedure of flotation on a hypoosmotic 5% w/v Ficoll 400 solution, exhibited an ATP-stimulated Ca^{2+} uptake activity at 24 °C of ~0.52 ± 0.02 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1} (four independent preparations). As shown in Figure 1, the uptake was linear for ~5–10 min and reached ~20 nmol of Ca^{2+}/mg of protein after 1 h of incubation; this was ~30-fold higher than that observed in the absence of ATP.

This crude disk preparation was subsequently further purified by velocity sedimentation on a 5–20% Ficoll 400 linear density gradient. This gradient was formed in a buffer of approximately physiological osmotic strength. Two distinct

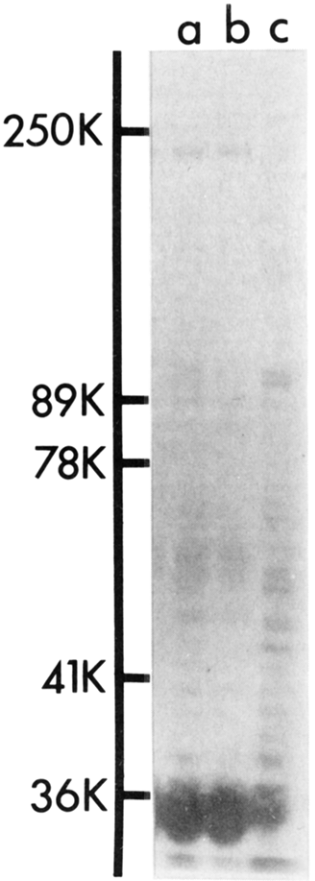

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the crude disk, R-band, and W-band membrane fractions. The 6.5% polyacrylamide gel was stained with Coomassie blue. Lane a, crude disks; lane b, R-band; lane c, W-band. See Experimental Procedures for preparation of each membrane fraction. Human red blood cell ghosts were used as molecular weight standards (Fairbanks, 1971). Each lane contains approximately 25 μg of protein.

bands were always resolved in this gradient system. One band at a density of ~1.05 g/mL, termed the "R-band", contained >90% of the total rhodopsin and ~85% (w/w) of the total protein on the gradient. The other band at $\rho \sim 1.03$ g/mL, the "W-band", contained ~1% of the total rhodopsin and ~15% (w/w) of the total protein in the gradient. A significant and reproducible amount of rhodopsin was present in both the R-band and the W-band membranes. Rhodopsin content quantification showed the R-band had 60 ● 19% w/w rhodopsin/protein ($n = 5$) and the W-band had 14 ± 2% w/w rhodopsin/protein ($n = 4$), as determined by bleaching and regeneration. Because a portion of the rhodopsin may be irreversibly photolyzed, this estimate of the rhodopsin content of these membranes is a lower limit.

SDS/Laemmli gel electrophoresis (Figure 2) demonstrates that a protein component of mobility corresponding to bleached rhodopsin is clearly the preponderant component of the R-band; this is not the case for the W-band. The estimate of rhodopsin content based on SDS/Laemmli gels correlates well with the results of the spectroscopic assay of rhodopsin.

The gradient patterns were highly reproducible; the average net difference in density separating the R- and W-bands on the gradient was 0.02 ± 0.001 g/mL ($n = 7$). The density gradients were not run to equilibrium density; the centrifugation conditions were instead determined empirically to generate the most well-defined separation of the two membrane subfractions. Under these conditions the osmotically active membranes, which when swollen in hypoosmotic media float on 5% Ficoll, reshrink and enter the gradients.

There were distinct differences between the morphology of the R-band and W-band membranes when visualized under thin-section electron microscopy. As shown in Figure 3, the R-band membranes have flattened vesicular structures of size and morphology that are characteristic of native retinal disks (Daemen, 1973; Steinberg et al., 1980); the W-band membranes did not.

Frozen retinas were usually employed for the preparation of the R- and W-bands. To determine whether the resolution of ROS membranes into two components was an artifact resulting from the nature of the initial dissection and/or the disruption of membrane integrity due to freezing, fresh retinal tissue was dissected, and the disks were isolated in parallel with the frozen retinas as starting material. The "fresh" crude disk preparations were also resolved into two distinct bands when centrifuged on a 5–20% Ficoll gradient. The fresh R-band and W-band membranes showed protein content, sedimentation behavior, and Ca^{2+} transport activity similar to those found for the preparations derived from frozen retinas (data not shown).

To determine the distribution of accumulated Ca^{2+} on the gradient, the crude disks were incubated with $^{45}CaCl_2$ in the absence or presence of Mg^{2+}ATP for 1 h prior to centrifugation. Following centrifugation, two peaks of ATP-dependent $^{45}Ca^{2+}$ accumulation were seen. As shown in Figure 4, the peaks of $^{45}Ca^{2+}$ accumulation corresponded to the positions of the R-band and W-band on the gradient. The $^{45}Ca^{2+}$ associated with the R-band was stimulated ~22.5-fold by ATP, (peak uptake ~3.15 nmol of Ca^{2+}/mg of protein). The $^{45}Ca^{2+}$ associated with the W-band was stimulated 18.3-fold by ATP (peak uptake ~11.7 nmol of Ca^{2+}/mg of protein).

In separate experiments, the crude disks were preincubated (1 h) with unlabeled Ca^{2+}- plus Mg^{2+}ATP and thus "loaded" with unlabeled Ca^{2+} prior to centrifugation. After centrifugation, aliquots of each fraction were assayed for $^{45}Ca^{2+}$ uptake activity. The Ca^{2+} uptake activities from these gradients were compared to the uptake activities determined when the crude disks were not preincubated with Ca^{2+}- and Mg^{2+}ATP prior to centrifugation. The ratios of ATP-dependent Ca^{2+} uptake activity of the Ca^{2+} preloaded membranes to that of the nonpreloaded membrane fractions were 1.56 ± 0.33 and 0.74 ± 0.21 for the R-band and W-band fractions, respectively (three separate sets of experiments each). The departure of this ratio from unity was of borderline statistical significance and indicates that the preloading of both membrane subfractions with unlabeled Ca^{2+} before their density gradient fractionation did not dramatically alter their ATP-dependent Ca^{2+} uptake ability; this suggests that substantive inhibition or activation of the transport activity as a result of Ca^{2+} preloading had not occurred.

The R-band and the W-band fractions from the 5–20% Ficoll gradient were pooled separately and further characterized. Both the R-band and W-band exhibited ATP-dependent Ca^{2+} uptake activity; the W-band had ~10-fold higher specific Ca^{2+} uptake activity than the R-band [W-band activity = 2.0 ± 0.4 nmol (mg of protein)$^{-1}$ min^{-1} ($n = 8$); R-band = 0.21 ● 0.04 nmol (mg of protein)$^{-1}$ min^{-1} ($n = 8$)].

The time course of ATP-stimulated Ca^{2+} uptake in pooled R-band membranes (Figure 5) was similar to that of the crude disks (Figure 1). The sum of the ATP-dependent Ca^{2+} uptake activity of the R- and W-bands always accounted for all the uptake activity initially observed in the crude preparation.

Cosedimentation of ATP-Dependent Ca^{2+} Uptake with Disks. To determine whether the apparent association of the ATP-dependent Ca^{2+} uptake with the R-band disks on the first

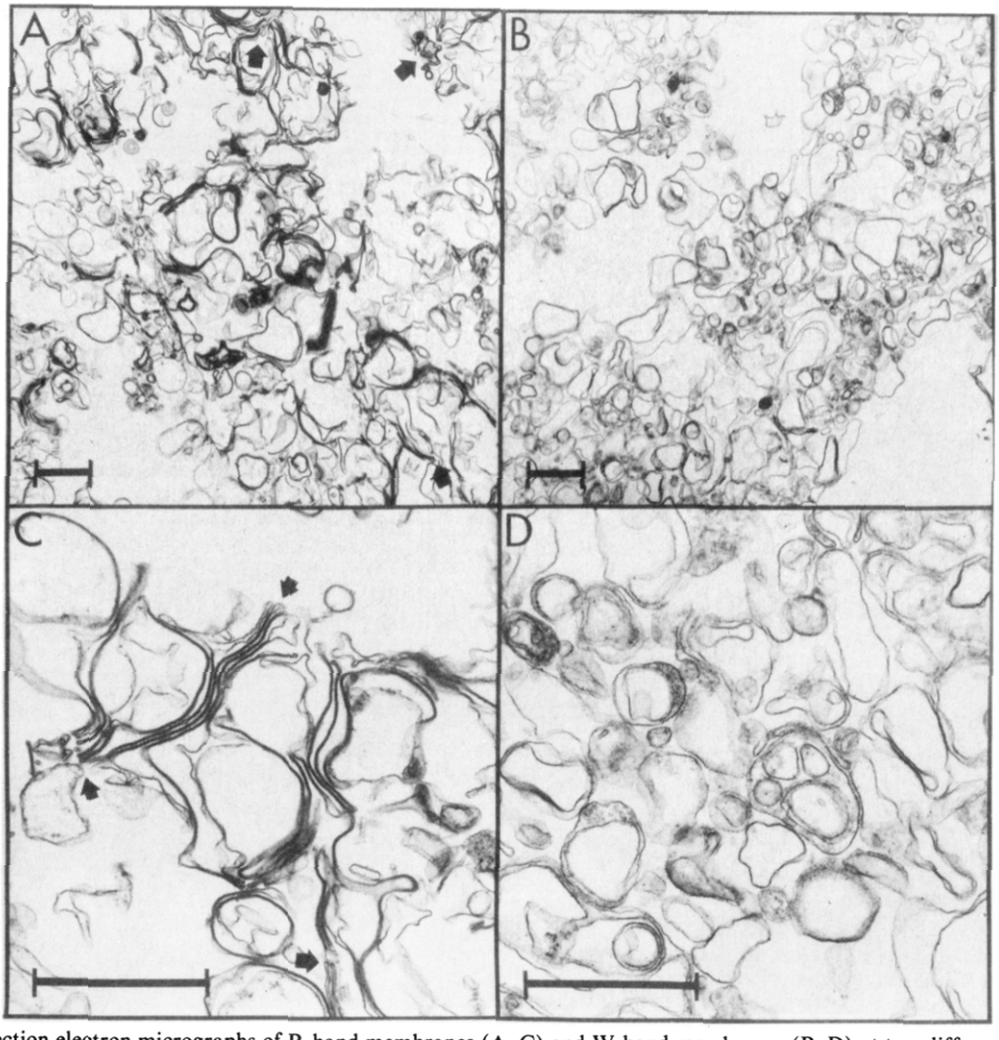

FIGURE 3: Thin-section electron micrographs of R-band membranes (A, C) and W-band membranes (B, D) at two different magnifications. Scale bar = 1 μm. Panels A and B magnified 14599.2× and panels C and D 43797.6×. Arrows show characteristic hairpin loops in the R-band preparation (A, C); these structures are not evident in W-band membranes (B, D). Crude disks (same preparation as employed in the experiment of Figure 1) were centrifuged on a 0–25% Ficoll gradient, and the two components—the R-band and the W-band—were separated. These components were collected separately, washed, and prepared for electron microscopy as described under Experimental Procedures.

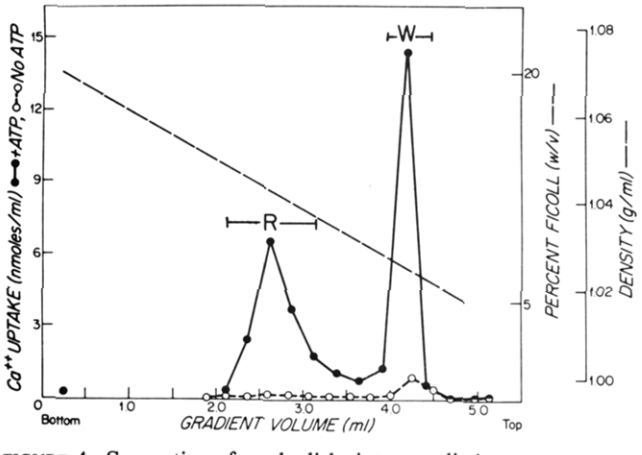

FIGURE 4: Separation of crude disks into two distinct components, both containing Ca^{2+} uptake activity. Crude disks were preincubated with $^{45}CaCl_2$ with (●) or without (○) 1.76 mM Mg^{2+}ATP, prior to centrifugation on a 5–20% w/v Ficoll 400 linear density gradient. Aliquots (25 μL) from each fraction of the gradient were passed through separate Dowex columns to separate free $^{45}Ca^{2+}$ from membrane-associated $^{45}Ca^{2+}$. See Experimental Procedures for details. The diagonal line (- - -) denotes the calculated density profile of the gradient solution. Horizontal bars represent the average positions of the R-band and W-bands, respectively (data from six experiments).

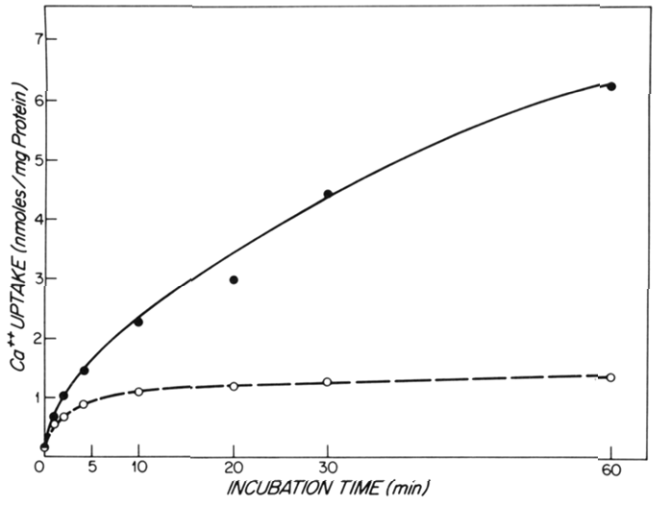

FIGURE 5: Time course of ATP-stimulated Ca^{2+} uptake into R-band disks. The preparation was assayed as described under Experimental Procedures in the presence (●) or absence (○) of 2 mM Mg^{2+}ATP.

density gradient was merely coincidental, we exploited the osmotic properties of these sealed disk membranes. The sedimentation properties of the R-band upon centrifugation on a second 2–15% (w/v) Ficoll gradient were found to vary with the osmotic composition of the gradient solutions. After separation of the W- and R-bands, the R-band membranes were pooled and washed to remove any residual Ficoll from

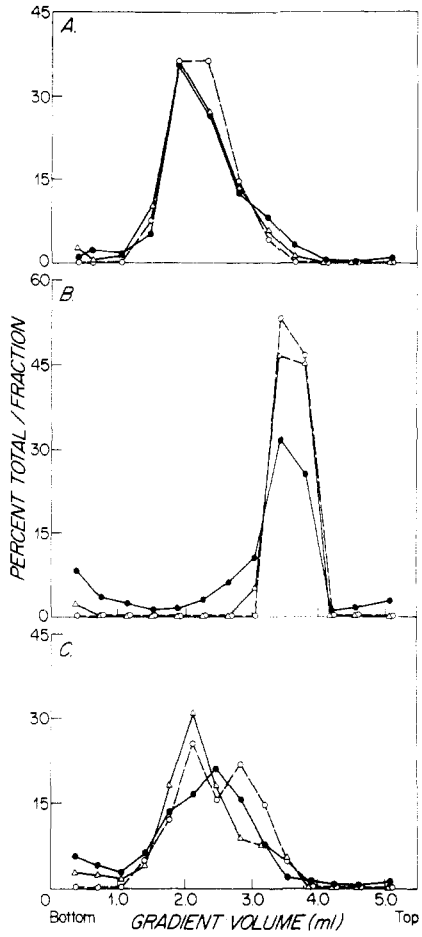

FIGURE 6: Demonstration that the calcium uptake activity is associated with R-band disks. R-band membranes were isolated as described under Experimental Procedures. Disks were washed in three separate buffer solutions and centrifuged on 2–15% Ficoll gradients made up in the following solutions: (A) high phosphate (100 mM KH_2PO_4, 60 mM NaOH, 5 mM βME, pH 7.0); (B) low phosphate (5 mM KH_2PO_4, 3 mM NaOH, 5 mM βME, pH 7.0); (C) mannitol/phosphate (220 mM mannitol, 5 mM KH_2PO_4, 3 mM NaOH, 5 mM βME, pH 7.0). Aliquots from each fraction of each gradient were assayed for ATP-dependent Ca^{2+} uptake activity (●), rhodopsin (○), and protein content (△). Membranes from the low phosphate and mannitol/phosphate gradients were diluted 1:1, resuspended in buffer A, and assayed for Ca^{2+} uptake activity. This was done to enable more direct comparison between the three gradients. All gradients were run in duplicate.

the first linear gradient. Three 2–15% Ficoll linear density gradients ($\rho \sim 1.007$–1.052 g/mL) were formed in parallel, each using a different solution: (1) high phosphate buffer (100 mM KH_2PO_4, 60 mM NaOH, 5 mM βME, pH 7.0); (2) low phosphate buffer (5 mM KH_2PO_4, 3 mM NaOH, 5 mM βME, pH 7.0); (3) mannitol/phosphate buffer (220 mM mannitol, 5 mM KH_2PO_4, 3 mM NaOH, 5 mM βME, pH 7.0). The high phosphate and mannitol/phosphate solutions were calculated to be of the same osmotic strength ($\sim$240 mOsm); the low phosphate solution had a calculated osmotic strength of $\sim$13 mOsm. The R-band membranes from the original 5–20% Ficoll gradient were divided into three equal portions; each portion was pelleted, washed, and resuspended in one of the buffer solutions described above. All gradients were centrifuged in parallel under identical conditions.

After centrifugation, the distribution of membranes on each gradient was determined, as shown in Figure 6. On the high phosphate and the mannitol/phosphate gradients, the R-band sedimented as a single peak to similar positions on each of the gradients. On the low phosphate gradient, the R-band also

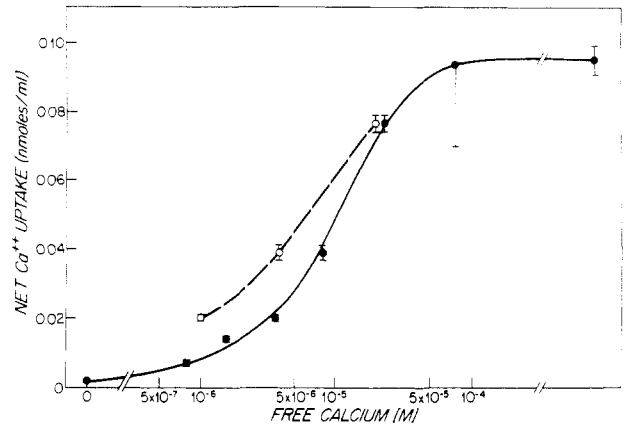

FIGURE 7: ATP-dependent Ca^{2+} uptake as a function of free [Ca^{2+}]. $^{45}Ca^{2+}$ uptake assays on R-band membranes were performed by using suction filtration as described under Experimental Procedures. An EDTA/Ca^{2+} buffering system was employed to control free Ca^{2+} (3 mM EDTA, 0–2 mM added $CaCl_2$). Free Ca^{2+} was determined by a Ca^{2+} selective electrode (○) or calculated from known affinity constants of calcium chelating components in the incubation mixture (●). Each data point was run in triplicate (Error bars denote ±SEM).

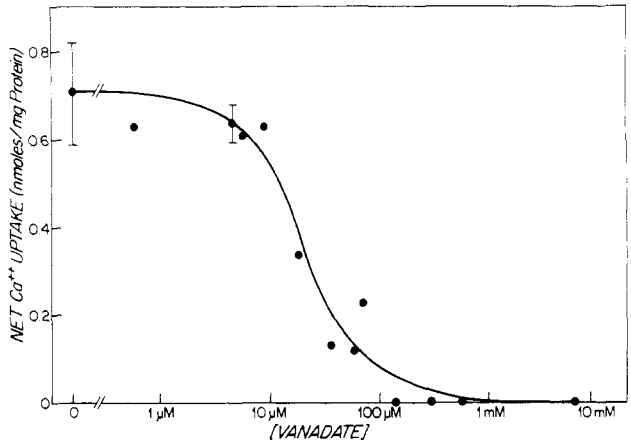

FIGURE 8: ATP-dependent Ca^{2+} uptake activity (5-min incubation at 24 °C) was determined as described under Experimental Procedures except 6 μL of concentrated stock solution of vanadate (0–3 mM VO_4^{2-} in buffer A) was added to membranes prior to the ^{45}Ca solution. The error bars denote ±SEM for those determinations done in duplicate.

sedimented as a single peak, albeit to a lower density. The depression in rhodopsin content at 2.5 mL (Figure 6C) does not define two distinct peaks of rhodopsin; this fluctuation was neither statistically significant nor reproducible.

As is clear from Figure 6, the Ca^{2+} uptake activity, rhodopsin content, and total R-band protein comigrated on each of the three gradients. This shows that, under the three different osmotic/ionic conditions of these gradients, the Ca^{2+} transport activity was always physically associated with the rhodopsin-containing membranes or with a particle of identical osmotic properties.

Further Characterization of the R-Band Ca^{2+} Uptake. The R-band's ATP-dependent Ca^{2+} uptake as a function of external free [Ca^{2+}] is displayed in Figure 7. The Ca^{2+} concentration dependence of Ca^{2+} uptake can be adequately fitted by a Michaelis–Menten kinetic model with an apparent K_m for Ca^{2+} of $\sim$6 μM. Neither GTP (2 mM) nor the nonhydrolyzable ATP analogue AMP-PNP (2 mM) could stimulate uptake activity in the R-band (data not shown).

Vanadate, a general inhibitor of ATPases with phosphorylated intermediates (Simons, 1979), inhibited R-band ATP-dependent Ca^{2+} uptake (Figure 8). This inhibition was consistent with the interaction of vanadate with a single class of sites with an apparent K_i of $\sim$20 μM.

Table I: Cytochrome *c* Oxidase Activity, [^{3}H]Saxitoxin Binding, and ATP-Dependent Ca^{2+} Uptake Activity in Retinal Membrane Fractions[a]

membrane fractions	protein content mg	protein content % of crude ROS	mitochondrial cytochrome *c* oxidase activity units/mg	mitochondrial cytochrome *c* oxidase activity % of crude ROS	[^{3}H]saxitoxin binding activity pmol/mg	[^{3}H]saxitoxin binding activity % of crude ROS	ATP-dependent Ca^{2+} uptake activity A nmol mg^{-1} min^{-1}	ATP-dependent Ca^{2+} uptake activity A % of crude ROS	ATP-dependent Ca^{2+} uptake activity B nmol mg^{-1} min^{-1}	ATP-dependent Ca^{2+} uptake activity B % of crude ROS
crude ROS	114	100	5.80 ± 0.51	100 ± 8.8	0.92 ± 0.42	100 ± 44.9	1.43	100	1.25	100
remainder of disrupted whole retina	1000	881	20.7 ± 0.52	3150 ± 79	0.27 ± 0.07	261 ± 70.3	3.62	2230	2.92	2060
crude disks	57	50.2	0.44 ± 0.04	3.8 ± 0.3	0.21 ± 0.07	11.2 ± 3.8	0.72	25	0.46	18.4
R-band	44.8	39.5	0.04 ± 0.02	0.28 ± 0.1	0.07 ± 0.21	3.0 ± 8.9	0.26	7	0.26	8.2
W-band	2.2	1.9	0.21 ± 0.003	0.07 ± 0.001	0.87 ± 0.34	1.8 ± 0.7	4.81	6.3	3.62	5.5
mitochondria-enriched fraction from brain synaptosomes			1960 ± 90				3.83		0.21	
brain synaptic plasma membranes					16.92 ± 0.64					

[a] Data are expressed as activity per milligram of total protein and as a percent of the crude ROS fraction activity ± standard deviation. See Experimental Procedures for preparation of membranes and specific assay conditions. The ATP-dependent Ca^{2+} uptake activity of each membrane fraction was assayed within 24 h (column A) and at 96 h (column B) after isolation of the R- and W-band membrane fractions. The R-band isolation is a 3-day procedure.

The Ca^{2+} ionophore A23187 (5 μM) was able to completely prevent R-band Ca^{2+} accumulation when the membranes were preincubated with the ionophore prior to the transport assays (6 ± 11% of control uptake, $n = 3$) and released most (59 ± 11%, $n = 3$) of the ATP-dependent accumulation of $^{45}Ca^{2+}$.

Ruthenium red (10 μM), a relatively specific inhibitor of mitochondrial Ca^{2+} uptake (Vasington et al., 1972), did not inhibit the ATP-dependent Ca^{2+} transport activity; uptake was 98 ± 14% of control levels ($n = 4$). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler (Heytler, 1979), and oligomycin (5 μg/mL) did not inhibit ATP-dependent Ca^{2+} transport. In the presence of 1 or 10 μM CCCP, uptake was 100 ± 11% of control levels ($n = 3$) and 103 ± 10% of control levels ($n = 2$) in the presence of oligomycin.

To address the issue of possible mitochondrial contamination more directly, cytochrome *c* oxidase activity was assayed in each membrane fraction resulting from the R-band isolation procedure (Table I). Approximately 3% of the oxidase activity of disrupted whole retina was present in the crude ROS. Upon subsequent centrifugations almost all of this activity pelleted; only ~0.1% of the starting activity was found in the crude disk fraction. The R-band and W-band membranes did not contain significant oxidase activity (<0.01% and 0.002% of total starting activity, respectively).

To investigate the possible presence of contaminating nerve plasma membranes, [^{3}H]saxitoxin binding activity was used as a marker for voltage-sensitive Na^+ channels (Table I). The R-band was devoid of significant STX binding activity, indicating that plasma membranes containing Na^+ channels were not the source of R-band Ca^{2+} uptake. In contrast, the W-band fraction contained a density of STX binding sites ~5% of that of brain synaptic plasma membranes. When detergent was added to expose any hidden STX binding sites, no significant additional [^{3}H]STX binding was found in either the R-band or W-band membranes.

Preliminary data (not shown) indicate that the W-band ATP-dependent Ca^{2+} uptake is not significantly different from the R-band uptake activity in any of the following: the K_m for Ca^{2+}, the K_i for vanadate, the lack of stimulation of Ca^{2+} uptake with either AMP-PNP or GTP, the insensitivity to ruthenium red, and the release of accumulated Ca^{2+} with A23187.

Discussion

Crude native disk membranes displayed ATP-dependent Ca^{2+} uptake. This crude preparation was further purified into two distinct components—the R-band and W-band—when centrifuged on a linear Ficoll density gradient. All of the Ca^{2+} uptake activity seen in the crude disk preparation was reproducibly resolved on density gradients into two components, which exactly corresponded to the positions of the R-band and W-band membranes. There were no other regions of the gradients that contained significant levels of Ca^{2+} transport activity, rhodopsin, or protein.

Subcellular Origin of the R-Band. The R-band membrane fraction was derived by subfractionation of a preparation that, as previously characterized (Clark & Molday, 1979; Amis et al., 1981), primarily contains membrane components of the ROS. The following observations indicate that ROS disks comprise the preponderant component of the R-band: (1) The R-band membranes were the major component of the ROS-derived membrane fraction and disks are, by morphological criteria, clearly the major membrane component of ROS (Kamps et al., 1982). (2) The R-band displayed morphology, in thin-section electron micrographs, that closely resembled that of native disks (Daemen, 1973). (3) Rhodopsin comprised most of the protein of the R-band, and the R-band contained >90% of the total rhodopsin in the crude disk preparation. Previous studies [see Cohen (1972)] have shown that the majority of the rhodopsin of rod cells is localized to ROS disks.

The results reported here do not, however, rule out the possibility that other membranes besides those of disks could be present as minor contaminants of the R-band. For example, rhodopsin is probably also found in the plasma membrane of ROS and in the biosynthetic apparatus localized to the RIS (Basinger & Hoffman, 1982). The absence of significant levels of STX binding indicates minimal contamination of the R-band with nerve plasma membranes.

The origin of the W-band, in contrast, remains unclear. The morphology of the W-band does not resemble that of flattened disks. The significant amount of rhodopsin (~15% w/w) associated with the W-band and the presence of substantial ATP-dependent Ca^{2+} uptake activity with properties similar to those of the R-band suggest that all or part of the W-band may be derived, at least in part, from the photoreceptor cell. The significant density of STX binding sites present in the W-band fraction suggests that this fraction may include electricaly excitable plasma membranes. Further investigation of the origin of the W-band is in progress.

Relationship of the Observed ATP-Dependent Ca^{2+} Uptake to Mitochondrial Ca^{2+} Sequestration. A previous report of

ATP-dependent Ca^{2+} uptake (Bownds et al., 1971) was subsequently attributed by the author to mitochondrial contamination [refer to Szuts (1980)]. George & Hagins (1983) found that the mitochondrial Ca^{2+} uptake inhibitor, ruthenium red, had to be present in their studies of permeabilized ROS in order to localize $^{45}Ca^{2+}$ to disks rather than mitochondria. Pasantes-Morales & Ordonez (1982) also found that ATP-activated Ca^{2+} accumulation in taurine-treated frog ROS was completely inhibited by mitochondrial ATPase inhibitors, including ruthenium red.

The following observations suggest that R-band Ca^{2+} uptake is nonmitochondrial. In our studies ruthenium red, oligomycin, and CCCP caused no inhibition of R-band Ca^{2+} uptake. Ca^{2+} uptake in mitochondria, an energy-linked process stimulated by respiration or ATP hydrolysis (Carafoli, 1980), has an estimated stability in vitro of a few hours (B. Reynafarje, Johns Hopkins University School of Medicine, personal communication). In our hands, only 5.5% of the original ATP-dependent Ca^{2+} uptake activity of mitochondria-enriched brain fractions remained after 3 days. In contrast the R-band uptake activity was completely stable over this time period (Table I); most ($\sim$70%) of the ATP-stimulated Ca^{2+} uptake activity is present 2 weeks following isolation of the crude disks. Finally, the R-band is devoid of significant mitochondrial cytochrome *c* oxidase activity.

A fundamental difference between this disk preparation and those in the studies on ATP-dependent Ca^{2+} uptake previously noted is that, following isolation of the ROS, the plasma membranes are hypoosmotically lysed and only "intact" sealed membranes are collected. It has been determined that a small but significant fraction of vertebrate ROS may break off and reseal just under the cilium (Uhl & Abrahamson, 1981). Mitochondria and/or other membrane contaminants containing Ca^{2+} transport activity may be concentrated in this small portion of the RIS that remains attached to the ROS. In the procedure employed here, the centrifugation step following the hypoosmotic shock that releases the disks should pellet any contaminating mitochondria or membrane fragments.

Evidence for Physical Association of an ATP-Dependent Ca^{2+} Uptake Activity with Disks. Initial observations revealed that a major portion of the ATP-dependent Ca^{2+} uptake activity in the ROS membrane preparation reproducibly and precisely comigrated with the R-band on Ficoll density gradients. However, even though it is apparent that disks are the preponderant component of the R-band, one cannot a priori rule out the possibility that this Ca^{2+} uptake activity is associated with a minor membrane fraction that, by coincidence, happens to cosediment with disks in the gradient system employed. For that reason, we subjected the R-band to additional gradient centrifugation steps. When the solution composition of a second Ficoll gradient was varied, the R-band membranes and rhodopsin were still resolved in each case as a homogeneous peak whose sedimentation characteristics depended primarily on the osmotic strength of the gradient solution. In all cases, the profile of the ATP-dependent Ca^{2+} uptake activity closely paralleled the profile of rhodopsin content and total protein on each gradient.

The reduced sedimentation of the R-band disks in gradients of lowered external osmotic strength can be explained by the resultant swelling of the disks, which would reduce their net buoyant density and velocity of sedimentation. If the Ca^{2+} uptake activity of the R-band is associated with an independent vesicular membrane fraction other than disks, this membrane fraction would have to be precisely identical with disks in its sedimentation characteristics as a function of osmotic strength. We feel that this would be an unlikely coincidence for the following reasons. The effect of an osmotic gradient on vesicular structures is dependent on their size and native morphology. Small spherical vesicles such as 500-Å diameter liposomes are highly resistant to swelling and lysis in the presence of large osmotic gradients (Goldin & Rhoden, 1978; S. M. Goldin, unpublished observations). Larger structures such as synaptosomes (Jones & Matus, 1974) and erythrocytes (Dodge et al., 1963) readily lyse in osmotic gradients. Structures of intermediate size and flattened native morphology, such as 1-μm diameter ROS disks, are relatively resistant to osmotic lysis but are observed to readily swell in an osmotic gradient (Smith et al., 1975). Thus, the precise comigration of this ATP-dependent uptake activity with R-band disks in a variety of gradient systems of differing osmotic strengths is an argument for physical association of this uptake activity with disks that is analogous to the argument that cosedimentation of an enzymatic activity with a purified protein on density gradients indicates that that protein contains this enzymatic activity.

It is possible that, although the ATP-dependent Ca^{2+} sequestration may occur within a site that is physically associated with disks, that site is not the disks themselves but a vesicular structure that is physically attached to disks and hence cosediments with them. The size and degree of association of such vesicular structures with disks would have to be highly reproducible, on the basis of the fact that the SEM for R-band ATP-dependent $^{45}Ca^{++}$ uptake activity was $\pm$ 18.4% (n = 8, six independent preparations of disks from both frozen and fresh retinas). On the basis of thin-section electron microscopy on intact ROS (Steinberg et al., 1980) there is no evidence for the association of vesicles with disks in rod cells. Thus, these vesicles would have to arise from vesicularization of the plasma membrane or attachment of vesicles from elsewhere in the retina upon disruption of the ROS in vitro.

Although this alternative hypothesis should not be discounted, the following argument would appear to favor disks as the site for R-band Ca^{2+} sequestration. R-band ATP-dependent Ca^{2+} uptake reaches a level after 1 h of incubation of at least 6 nmol/mg of protein (Figure 5). Additional experiments involving 2-h incubations (not shown) indicate that the Ca^{2+} uptake plateaus at roughly this level. This corresponds to $\sim$0.3 Ca^{2+} ions per rhodopsin or 30 000 Ca^{2+} per disk and agrees, on an order of magnitude basis, with estimates of total Ca^{2+} that is available for exchange in frog and bovine ROS (Szuts & Cone, 1977; Hagins & Yoshikami, 1975; Nöll et al., 1979). Thus, the hypothesis that R-band disks are the sites for the observed in vitro Ca^{2+} uptake is quantitatively consistent with the in vivo estimates of Ca^{2+} sequestration capacity within the vertebrate ROS.

Possible Physiological Role of R-Band Ca^{2+} Uptake. It has been proposed (Mueller & Pugh, 1983; Hagins, 1972; Kaupp & Schnetkamp, 1982) that Ca^{2+} release from retinal disks is an event critical to the initiation of the hyperpolarizing response of ROS to light. If retinal disks play a role in controlling the response of photoreceptors that is analogous to the role of sarcoplasmic reticulum in regulating muscle contraction, then Ca^{2+} reuptake into disks must be sufficient in rate and extent to remove released Ca^{2+} from the ROS cytoplasm. Yoshikami et al. (1980) have determined that $\sim$400 Ca^{2+} ions are released into the extracellular medium per absorbed photon per rod in a mammalian retina preparation. If the release site is retinal disks, this would constitute a minimum estimate of release because a portion of the released Ca^{2+} may be rese-

questered within disks without ever leaving the ROS. Studies in single isolated ROS of other vertebrates (Baylor et al., 1979; Fain & Dowling, 1973; Mueller & Pugh, 1983) reveal that half-maximal suppression of the dark current occurs when ~30–100 photons are absorbed per ROS. Under the assumption that Ca^{2+} release remains linear with respect to rhodopsin photolysis in this range, this corresponds to ≤40 000 Ca^{2+} ions released at the half-maximal physiological response. In the studies cited above, the dark current is reestablished within several seconds. For resequestration of released Ca^{2+} within the disks to be a possible primary event in terminating the dark current suppression, at least 40 000 Ca^{2+} ions must be pumped into the disks.

We find a rate of ATP-dependent Ca^{2+} uptake by the R-band of, on average, 0.21 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}; this corresponds to ~0.013 Ca^{2+} (rhodopsin molecule)$^{-1}$ min^{-1}. Under the assumption that disks contain ~60% w/w rhodopsin to total protein, 10^5 rhodopsin molecules per disk (Amis et al., 1981), a rhodopsin molecular weight of 36 000, and 1000 disks per ROS (Krebs & Kuhn, 1977), the rate of Ca^{2+} uptake is ~21 000 Ca^{2+} ions ROS^{-1} s^{-1}. This rate is sufficient in principle to resequester Ca^{2+} with a time course that parallels the termination of the photoresponse. It should also be noted that the rate of R-band Ca^{2+} uptake was determined at 24 °C, whereas the studies of Yoshikami et al. (1980) were performed at 37 °C; at 37 °C the Ca^{2+} uptake rate should be higher. However, it is important to note that the pump may be operating below its maximal rate for much of the time due to free Ca^{2+} levels below its K_m for Ca^{2+}, as described below.

Yoshikami et al. (1980) calculate that the amount of Ca^{2+} released by the absorption of ~110 photons by a single rod would elevate the cytoplasmic [Ca^{2+}] by about 3 μM. On the basis of estimates of cytoplasmic [Ca^{2+}] of ~1 μM for rat rods in the dark (Hagins & Yoshikami, 1974, 1977), the total [Ca^{2+}] would be ~4 μM, which approximates our determined value for the K_m for Ca^{2+} of the R-band Ca^{2+} pump. This correspondence between the Ca^{2+} affinity of the pump and the estimated transient ROS cytoplasmic [Ca^{2+}] at light intensities that substantially suppress the dark current constitutes additional circumstantial evidence for the physiological significance of the R-band Ca^{2+} pump.

Our results are consistent with, but by no means prove, the hypothesis that Ca^{2+} release from and reuptake into disks are critical for the short-term regulation of photocurrent. The potential physiological importance of this Ca^{2+} pump in the regulation of cytosolic Ca^{2+} levels in rod cells is not dependent on the validity of this hypothesis. The pump may, alternatively or in addition, play a role in longer term mechanisms such as visual adaptation.

Preliminary attempts to identify the ATPase activity responsible for this Ca^{2+} uptake system indicate that the activity is a minor component of the total R-band-associated ATPase activity. The purification of the R-band Ca^{2+} pump should be an important step toward identifying the Ca^{2+} transport ATPase activity and for defining the pump's mechanism and regulation. Immunocytochemical localization of the Ca^{2+} pump would be a valuable test of our hypothesis that this pump is associated with disks of the bovine rod outer segment.

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Registry No. ATP, 56-65-5; Ca, 7440-70-2.

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Light-Driven Protonation Changes of Internal Aspartic Acids of Bacteriorhodopsin: An Investigation by Static and Time-Resolved Infrared Difference Spectroscopy Using [4-^{13}C]Aspartic Acid Labeled Purple Membrane†

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ABSTRACT: The molecular events during the photocycle of bacteriorhodopsin have been studied by the method of time-resolved and static infrared difference spectroscopy. Characteristic spectral changes involving the C=O stretching vibration of protonated carboxylic groups were detected. To identify the corresponding groups with either glutamic or aspartic acid, BR was selectively labeled with [4-^{13}C]aspartic acid. An incorporation of ca. 70% was obtained. The comparison of the difference spectra in the region of the CO_2^- stretching vibrations of labeled and unlabeled BR indicates that ionized aspartic acids are influenced during the photocycle, the earliest effect being observed already at the K_{610} intermediate. Taken together, the results provide evidence that four internal aspartic acids undergo protonation changes and that one glutamic acid, remaining protonated, is disturbed. The results are discussed in relation to the various aspects of the proton pumping mechanism, such as retinal isomerization, charge separation, pK changes, and proton pathway.

The elucidation of the mechanism of bacteriorhodopsin (BR),[1] the light-driven proton pump of the halophilic bacterium *Halobacterium halobium* [for a review, see Stoeckenius & Bogomolni (1982)], still represents a challenge. Its chromophore, which is *all-trans*-retinal in its light-adapted func-

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[1] Abbreviations: BR, bacteriorhodopsin; ^{13}C-Asp-BR, [4-^{13}C]aspartic acid labeled bacteriorhodopsin; FTIR spectroscopy, Fourier transform infrared spectroscopy.

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