Localization of the Cyclic ADP-Ribose–Dependent Calcium Signaling Pathway in Bovine Rod Outer Segments

Isabella Panfoli,[1] *Silvia Ravera,*[1] *Andrea Fabiano,*[1] *Raffaella Magrassi,*[2,3] *Alberto Diaspro,*[2,3] *Alessandro Morelli,*[1] *and Isidoro M. Pepe*[1]

Purpose. Calcium ions play a pivotal role in phototransduction. In this study, the presence and functional role of the adenosine diphosphoribosyl (ADPR)-cyclase– cyclic ADP-ribose (cADPR) system in bovine retinal rod outer segments (ROS) was investigated.

Methods. A Ca^{2+} release from osmotically intact ROS discs elicited by cADPR was studied in the presence of the Ca^{2+} tracer fluo-3. Endogenous cyclic guanosine diphosphate ribose (cGDPR) formation in discs was investigated by spectrophotometric detection of its synthesis from nicotinamide guanine dinucleotide (NGD^+). ADPR-cyclase was also investigated at a structural level on mildly denaturing SDS-PAGE by production of cyclic inosine diphosphate ribose from nicotinamide hypoxantine dinucleotide (NHD^+). Western immunoblot analysis with a specific antibody was conducted to verify the presence of ryanodine-sensitive Ca^{2+} channels (RyRs) in ROS discs.

Results. cADPR-dependent Ca^{2+} release was a linear function of extravesicular free Ca^{2+} concentration, between 200 and 900 nM Ca^{2+}. When free Ca^{2+} was 203 ± 10 nM the mean Ca^{2+} release was 23 ± 3 pmol/mL per milligram protein. The average rate of cGDPR production was 13 ± 2 nmol cGDPR/min per milligram protein, by a putative enzyme with an apparent molecular mass of 53 ± 1 kDa. ROS ADPR-cyclase was localized in the membranous fraction. No nicotinamide adenine dinucleotide glycohydrolase (NADase) activity was detected. The presence of RyR channels in pure disc preparations was confirmed by confocal laser scanning microscopy.

Conclusions. A cADPR metabolism may be present in retinal ROS discs, which may be Ca^{2+} stores operated by cADPR. A model is proposed for the physiological role of cADPR-mediated Ca^{2+} release in bovine ROS. (*Invest Ophthalmol Vis Sci.* 2007;48:978–984) DOI:10.1167/iovs.06-0543

Ca^{2+} signaling is the most ubiquitous signal transduction mechanism, mediating the intracellular effects of extracellular signals into a diverse array of specific cellular responses.[1] Several metabolites of nicotinamide adenine dinucleotide (NAD^+) are second messengers in cell calcium signaling. Cyclic adenosine diphosphoribose (cyclic ADP-ribose, cADPR) modulates calcium movements from ryanodine-sensitive endoplasmic reticulum stores.[2] A novel Ca^{2+}-release mechanism activated by nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) has been shown to act on different stores of calcium (for review, see Ref. 3). ADPR has been shown to modulate the activity of a member of the transient receptor potential channels family (TRPM2).[4] cADPR, abundant in the brain,[5] has been shown to modulate calcium movements from ryanodine-sensitive stores, in both neurons[6,7] and astrocytes.[8]

Ryanodine-sensitive Ca^{2+} receptors (RyR) are homotetrameric peptides[9] modulating calcium release from intracellular stores. Together with inositol 1,4,5-trisphosphate (IP_3) receptors,[10] they belong to a family displaying calcium-induced calcium release (CICR),[11] the process accounting for cytosolic free Ca^{2+} spatiotemporal dynamics. Three RyR isoforms are known,[10–12] all found in the nervous system.[13] cADPR is involved in calcium-dependent neural processes, including synaptic transmission, plasticity, and neuronal excitability.[6,8,14–16] cADPR-dependent signaling has been identified within the nucleus in several cell types.[17] The cADPR-synthesizing enzymes known to date form a growing family of conserved proteins.[18] The first identified enzyme of this family was isolated from the synaptic terminal cytosol of the mollusc *Aplysia californica*.[19] In mammalian systems, the ectoenzymes CD38 and CD157 (or BST-1), membrane-associated[20] and -anchored,[21] proteins, respectively appear to be the major enzymes with ADP-ribosyl cyclase (ADPR-cyclase) activity.[18] The two proteins share approximately 30% sequence identity with the *Aplysia* cyclase. CD38 is expressed in the nervous system,[20] principally in the soma of neural cells, and so it is unlikely to play a significant role in relation to ryanodine-sensitive internal stores. Recently, the existence of a novel endocellular mammalian ADPR-cyclase, involved in brain development and functioning,[22]was demonstrated in neural cells from *Cd38*$^{-/-}$ mice.[22,23] Basile et al.[24] demonstrated that, in addition to producing cADPR, ADPR, and NAADP, ADPR-cyclases can generate other adenine dinucleotides that are known to exist naturally and to have biological activity. Therefore, there is incipient evidence of the existence of other types of ADPR-cyclases in mammalian cells.

Vertebrate retina, with its laminar composition, has been considered a model of the central nervous system, from which it derives. In vertebrate visual phototransduction, free Ca^{2+} concentration has long been known to play a pivotal role, mediating guanosine 3′,5′-cyclic monophosphate (cGMP) metabolism,[25,26] and light adaptation in rods and cones.[27] We have characterized an active ($^{45}Ca^{2+}$) uptake in ROS discs, a sarcoendoplasmic reticulum type of Ca^{2+}-ATPase (SERCA) in bovine ROS discs[28,29] and a Ca^{2+}ion-sensitive adenylate kinase activity associated with disc membranes.[30] The cited results are consistent with the notion of discs as dynamic organelles able to store and release calcium ions, consistent with the idea that the nearly all calcium measured in the ROS is sequestered within the discs.[28,31]

In the present study, we investigated the presence and functional role of the ADPR-cyclase– cADPR system in ROS.

From the [1]Department of Biology, [2]LAMBS (MicroScoBiO Research Center and Department of Physics), University of Genoa, Genova, Italy; and the [3]INFM-CNR (Istituto Nazionale per la Fisica della Materia and Centro Nazionale delle Ricerche), Genova, Italy.

Supported by the Italian Ministry for University and Scientific and Technologic Research (MIUR).

Submitted for publication May 17, 2006; revised October 13, 2006; accepted January 10, 2007.

Disclosure: **I. Panfoli**, None; **S. Ravera**, None; **A. Fabiano**, None; **R. Magrassi**, None; **A. Diaspro**, None; **A. Morelli**, None; **I.M. Pepe**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Isabella Panfoli, Università di Genova, Dipartimento di Biologia, Viale Benedetto XV, 3, 16132 Genova, Italy; isabella.panfoli@unige.it.

Toward this end, subfractions of ROS, i.e., purified discs, crude discs, and cytosolic fractions were screened. Results suggest that discs (1) possess a consistent ADPR-cyclase activity; (2) are able to release Ca^{2+} in response to cADPR; and, (3) contain RyR Ca^{2+} channels. Based on these data, a role for cADPR-mediated Ca^{2+} signaling in mammalian phototransduction is proposed.

METHODS

Chemicals

cADPR, nicotinamide guanine dinucleotide (NGD^+), nicotinamide hypoxantine dinucleotide (NHD^+), calmodulin (CaM), and A23187 were purchased from Sigma-Aldrich (St. Louis, MO); fluo-3 from Molecular Probes (Eugene, OR); goat polyclonal antibody IgG, (200 μg/mL) against ryanodine-sensitive Ca^{2+} receptors from Santa Cruz Biotechnology (sc-8169; Santa Cruz, CA); and mouse monoclonal antibody raised against rabbit Na/K ATPase α1 (C464.6) from Santa Cruz Biotechnology (sc-21712). All other reagents were of analytical grade.

ROS and Disc Preparations

ROS were isolated from 20 bovine retinas (from a local abattoir), by sucrose gradient centrifugation[32] in the presence of protease inhibitor cocktail (Sigma-Aldrich). Osmotically intact discs were obtained, after bursting ROS for 3 hours in a 5% single-density gradient (Ficoll; Sigma-Aldrich) solution in distilled water with 5 mM dithiothreitol (DTT) and 70 μg/mL leupeptin, by collecting them at the solution surface after centrifuging for 2 hours at 25,000 rpm in a rotor (FW-27; Beckman, Fullerton, CA).[33] To obtain crude disc membranes and cytosolic fractions, ROS were homogenized in a glass homogenizer (1 mL) and centrifuged for 4 minutes at 14,500 rpm in a centrifuge (Eppendorf, Fremont, CA), and the supernatant (cytosol) and membranes were separated.

Rhodopsin Determination

Rhodopsin concentration was determined spectrophotometrically (molar extinction coefficient of 41,000 $M^{-1} \cdot cm^{-1}$)[34] by measuring the difference in 500 nm absorption between spectra recorded before and after exhaustive bleaching (green light) for 5 minutes. The A_{280}/A_{500} ratio was 2.9 $\pm$ 0.6 for ROS and 1.8 $\pm$ 0.2 (average $\pm$ SD) for discs, respectively. Absorbance spectra were recorded with a dual-beam spectrometer (UV2; Unicam, Cambridge, UK).

Calcium Measurements by Flame Atomic Absorption Spectrometry

Total calcium content of osmotically intact discs was assayed by flame atomic absorption spectrometry (AAS; Perkin Elmer, Wellesley, MA) on 0.3 mL of packed discs (0.8 mM rhodopsin) with a hollow cathode lamp for Ca (422.7 nm), as previously described.[35]

Transmission Electron Microscopy of Osmotically Intact Disc Preparations

For ultrastructural analysis, osmotically intact discs (1.2 mg total protein) were centrifuged at 6000 rpm for 2 minutes in the centrifuge (Eppendorf). The pellet was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 minutes. Then the sample was centrifuged and washed with 0.1 M cacodylate buffer (pH 7.4) and 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) was added, for 10 minutes. After the osmium solution was discarded, the sample was treated with a 1% aqueous solution of uranyl acetate, dehydrated through a graded ethanol series, then embedded in Epon resin and polymerized at 42°C, overnight followed by 48 hours at 70°C. Ultrathin sections were obtained by using a microtome (Ultracut E; Leica, Heidelberg, Germany) and analyzed with an electron microscope[36] (FEI CM10 or Tecnai 12 G2 (FEI, Eindhoven, the Netherlands).

Sample Preparation and Ca^{2+} Background Tuning for Fluorometric Free Ca^{2+} Measurements

Freshly prepared disc suspensions (11 mg protein, 0.8 mM rhodopsin concentration) were left overnight at 4°C (freezing caused disc damage) in 2 mM $CaCl_2$ (200 μL discs plus 4 μL of 0.1 M $CaCl_2$), as discs tend to loose intravesicular Ca^{2+} in the hours after their isolation. Preloading was not necessary when disc suspensions were used immediately after preparation. It was impossible to load disc vesicles during the experiment, as the Ca^{2+}-ATPase of ROS is inactive at nanomolar calcium concentrations ($K_{0.5}$ for Ca^{2+} = 10 μM).[28,29,35] Before experiments, 0.2 mL discs were rapidly centrifuged, the supernatant discarded, and the remnant (~40 μL) washed in resuspended in double-distilled water (10 times the volume). The procedure was repeated twice (605-fold dilution), and the discs were resuspended in 0.4 mM rhodopsin at a 6 mg/mL protein concentration.

Fluorometric Free Ca^{2+} Measurements

Protein disc suspension (0.15 mL of 6 mg/mL) was added to the incubation medium (0.45 mg/mL final). The corresponding dilution of initial Ca^{2+} in the suspension was 8067-fold, and so in the experiment, free Ca^{2+} was ~0.2 μM, which is the background level over which it is possible to observe cADPR-evoked Ca^{2+} release.[37] Incubation was conducted at room temperature in the presence of 30 mM HEPES (pH 7.1), 120 mM KCl, 10 μg/mL CaM, and 2.5 μM fluo-3. Recordings (excitation, 505 nm; emission, 534 nm) were performed with a spectrophotometer (LS 50B; Perkin Elmer) with continuous stirring. Seeking to study CICR, we determined the dependence of the entity of cADPR-evoked Ca^{2+} release from discs on the extravesicular free Ca^{2+} concentration ($[Ca^{2+}]$) in the experimental mixture, producing an elevation of background $[Ca^{2+}]$ in the medium by addition of a few picomoles of $CaCl_2$. These additions randomly modified $[Ca^{2+}]$ (ranging from ~200–900 nM). $[Ca^{2+}]$ quantitation was achieved by probe calibration at the end of each experiment.[37] Water, first deionized by passage through two consecutive ion-exchange columns and second passed through a water purification system (Milli-Q Ultrapure Water Purification System; Millipore Corp., Billerica, MA), was used throughout.

Western Blot Analysis

ROS homogenates were subjected to SDS-PAGE.[38] Proteins were transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Uppsala, Sweden) in a mini transblot device (Bio-Rad Laboratories, Hercules, CA). Sheets were blocked for 1 hour at room temperature in TBS-BSA 1% (25 mM Tris-HCl [pH 7.4], 150 mM NaCl plus 1% BSA).[39]

Overlay Assays

Samples on nitrocellulose were probed with specific antibody diluted 1:1000 in TBS-BSA 3.5%. After extensive washing, the binding of the antibody was revealed by an enhanced chemiluminescence (ECL) detection system (Roche Diagnostics, Mannheim, Germany) using HRP-conjugated monoclonal secondary anti-goat antibody (GE Healthcare) diluted 1:1000 in TBS-BSA 1%. Blots were then autoradiographed (Hyperfilm ECL; GE Healthcare) and images acquired.

Assays for ADPR-Cyclase Activity

Putative ADPR-cyclase was assayed by using NGD^+, a β-NAD^+ analogue, as a substrate, according to Graeff et al.[40] Aliquots of bovine ROS homogenates, or purified disc suspensions (300 μg protein) were incubated with the same reaction mixture as was used for Ca^{2+} measurements (pH 7.2). The reaction was started by addition of 1 mM NGD^+, conducted at 37°C, and stopped by addition of 50 μL of 25% perchloric acid (PCA). Absorbance of neutralized (50 μL 2 M K_2CO_3) and clarified supernatants was read at 300 nm and optical densities converted to rates of cGDPR production (molar extinction coefficient of 3758 $M^{-1} \cdot cm^{-1}$).

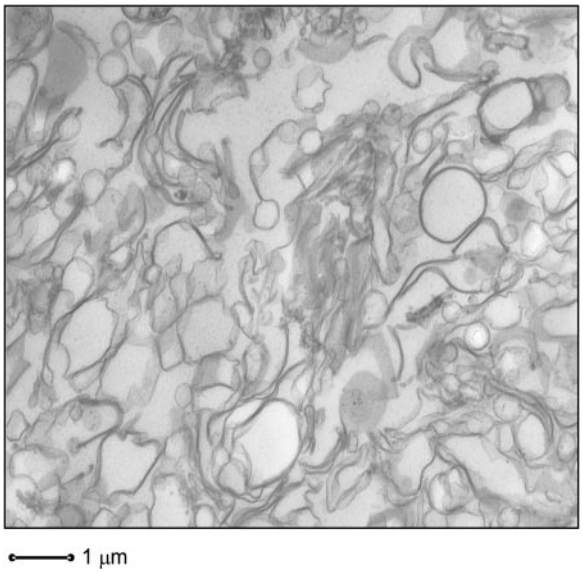

FIGURE 1. Morphologic analysis of osmotically intact ROS disc membranes. Osmotically intact disc suspensions were observed by TEM. The fraction mainly contained closed membrane vesicles, in which mitochondria, endoplasmic reticulum, melanosomes, and other cellular subfractions are absent. Because of elevated protein concentration, some membranes did not appear as vesicles but as more or less parallel membrane sheets. The length of the membrane profiles was ~1 μm.

Detection of ADPR-Cyclase Activity by SDS-PAGE

Samples were incubated at 25°C for 5 minutes, with a modified Laemmli sample buffer containing 1.5% SDS (instead of the usual 8%) and no β-mercaptoethanol, and run on SDS-PAGE (12% polyacrylamide).[41] To remove excess SDS, gels were washed in 50 mM Tris-HCl (pH 7.0), with repeated buffer changes. NHD^+ (0.2 mM) in 50 mM Tris-HCl (pH 7.0) was then added, and after the gels were incubated for 15 minutes in the dark at 20°C, the fluorescent bands, corresponding to locally produced cyclic inosine diphosphate ribose, were visualized under UV light and images acquired (Electrophoresis Documentation and Analysis System EDAS 290; Eastman Kodak, Rochester, NY).

Assay for NAD^+ Glycohydrolase Activity

NADase activity was assayed by spectrophotometric measures of decrement of β-NAD^+, according to Wu et al.,[42] with minor modifications. Briefly, the reaction mixture contained 0.05 M potassium phosphate buffer (pH 7.3), 5 mM β-NAD, 1 mM $MgCl_2$ and 200 μg of either crude or purified disc preparations in a total volume of 1 mL. The reaction was terminated by addition of 0.2 mL 25% PCA. Clarified and neutralized supernatants were added of 0.25 M Tris-HCl, 35 mM semicarbazide, 1% (vol/vol) ethanol. Initial absorbance at 340 nm was recorded, and then 60 μg alcohol dehydrogenase (ADH, EC 1.1.1.1; Roche) were added and the increase in A_{340} monitored.

Skeletal Muscle Preparations

Three grams of mouse muscle were homogenized in 10 mL of 0.25 M sucrose, 5 mM HEPES buffer, 1 mM EDTA (pH 7.2), and protease inhibitor cocktail (Sigma-Aldrich). Homogenate was centrifuged for 10 minutes at 500*g*, the precipitate discarded, and the supernatant centrifuged for 20 minutes at 20,000*g* (Heraeus centrifuge, Hanau, Germany). A microsomal pellet was collected after the 20,000*g* supernatant was centrifuged for 60 minutes at 100,000*g* (SW40 Rotor; Beckman).

Confocal Laser Scanning Microscopy

Image acquisition was performed with a confocal microscope (SP2 AOBS TCS; Leica) equipped with a 405-nm 10-mW laser diode. Images were collected with a 100× oil immersion numeric aperture 1/4 1.4 objective lens (HCX PL APO; Leica Microsystems SpA, Milan, Italy). Images of the sample were obtained with the 514-nm line of a 20-mW argon ion laser. To detect the presence or absence of the red dye (CY3), we analyzed the images to determine the related mean intensity values. A spectral evaluation was made possible by the spectral characteristics of the microscope scanning head (SP2 AOBS; Leica). The spectral window used for collecting fluorescence was 500 to 700 nm, according to reported emission spectra.[43] The resultant images were acquired, stored, elaborated, and visualized with the confocal software (Leica).

Protein Assay

Protein content was determined by bicinchoninic acid (BCA) protein assay kit from Pierce Biotechnology, Inc. (Rockford, IL), with BSA as the standard.

RESULTS

Osmotically intact disc suspensions purified from bovine ROS by single-density gradient flotation (Ficoll; Sigma-Aldrich), were characterized for purity and rhodopsin content. For osmotically intact discs, the A_{280}/A_{500} ratio was found to be 1.8 ± 0.1 (average ± SD). Rhodopsin concentration in the original preparation of the preloaded discs was 0.8 mM, whereas it was 0.4 mM after dilution. Mitochondrial contamination of the purified disc fractions was negligible, as shown by transmission electron microscopy (TEM) imaging (Fig. 1). An immunoblot analysis of ROS and discs with a mouse monoclonal antibody raised against rabbit sodium/potassium ATPase α1, a ubiquitous protein of the inner segment, was conducted. Figure 2 shows that, with respect to the muscle homogenates used as the positive control, cross-reactivity with Na/K ATPase antibody was present in the ROS but not in the purified discs.

Disc suspensions (0.45 mg/mL) displayed a Ca^{2+}-releasing activity of cADPR (10 μM final concentration), monitored in the presence of the calcium-reporting dye fluo-3 (Fig. 3). Initial free Ca^{2+} concentrations in the incubation medium were ~200 nM. To vary the Ca^{2+} background levels, we added a few

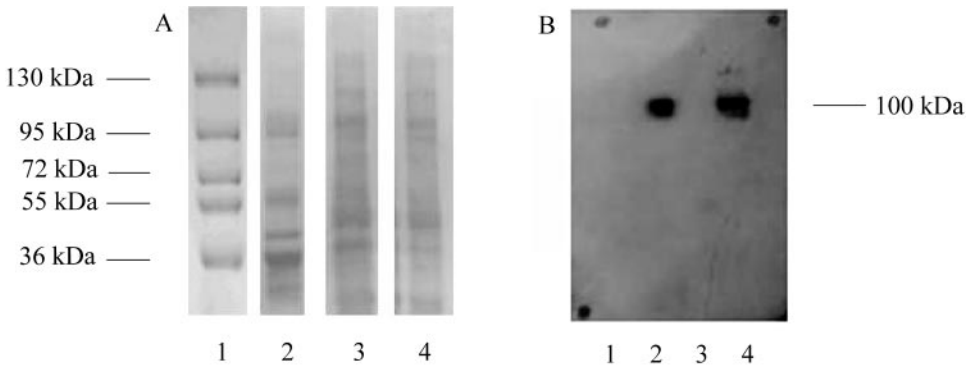

FIGURE 2. Western blot with anti Na/K-ATPase. The presence of Na/K-ATPase was determined by Western blot analysis with a mouse monoclonal antibody raised against rabbit Na/K-ATPase-α1. (**B**). Thirty micrograms protein was loaded in each lane for SDS-PAGE. (**A**) Ponceau-S staining of proteins after blot and dots of secondary antibody made for and overlay.

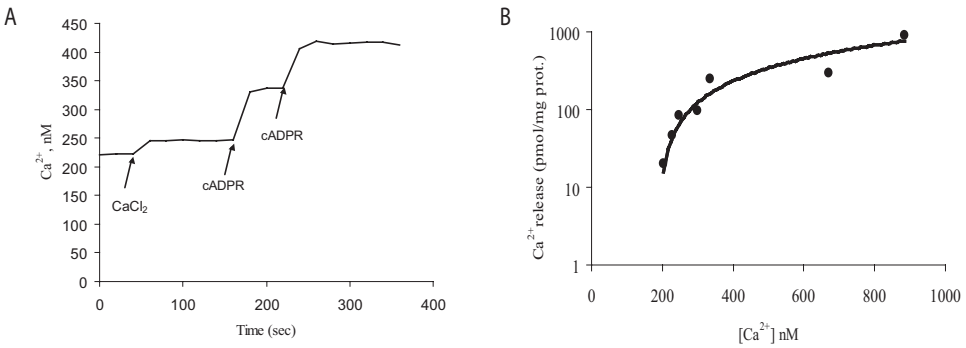

FIGURE 3. (**A**) cADPR evoked Ca^{2+} release from retinal rod disc vesicles. cADPR-induced Ca^{2+} release was performed on a suspension (2 mL) of osmotically intact bovine ROS discs (0.45 mg/mL). Ca^{2+} release was conducted at pH 7.2 in the presence of the Ca^{2+} tracer Fluo-3. The fluorometric trace from a typical experiment depicts an increase in free Ca^{2+} after addition of 0.2 picomoles $CaCl_2$ and after two consecutive additions of cADPR (20 nmol each) to the disc suspension (*arrows*). (**B**) $[Ca^{2+}]$-dependent Ca^{2+} release from retinal rod disc vesicles. A plot of cADPR-dependent peak Ca^{2+} release as a function of free extravesicular Ca^{2+} levels. Osmotically intact disc vesicles were incubated in the presence of the Ca^{2+} tracer fluo-3 in experiments similar to those in (**A**). Peak Ca^{2+} release data show a linear trend ($R^2 = 0.85$).

picomoles of $CaCl_2$ (see the Methods section). In the case of Figure 3A, 0.1 nmol $CaCl_2$ was added. Then at the 247-nM free Ca^{+2} level, the first cADPR addition was performed and elicited a release of 46 pmol/mL per milligram protein. When free $[Ca^{2+}]$ was 203 ± 10 (mean ± SD) nM, Ca^{2+} release was 23.0 ± 3.0 pmol/mL per milligram protein (mean ± SD, n = 5). Addition of the calcium ionophore A23187 at the end of the experiment for calibration (not shown, for clarity) showed that the Ca^{2+} plateau was far from probe saturation. The stored Ca^{2+} released by A23187 was 75.00 nM (i.e., 150 nanomoles). Total Ca^{2+} stored in the 0.150 mL discs in the experiment was 160 nanomoles, being total Ca^{2+} concentration in the original preloaded preparation 2 mM, as measured by flame atomic absorption spectrometry. Therefore, the fraction of the stored Ca^{2+} released by the A23187 was >90%. The ratio of Ca^{2+} to rhodopsin was 2.4.

cADPR-evoked Ca^{2+} release was strengthened by increasing extravesicular free $[Ca^{2+}]$. Experiments from the same preparation as shown in Figure 3A showed that Ca^{2+} release induced by cADPR was a linear function of extravesicular free $[Ca^{2+}]$ (Fig. 3B) in approximately the physiological range. A comparison of Figure 3A with 3B shows that, at 247 nM, Ca released was 84 nM, and at 336 nM was 70 nM, whereas it would be expected to be 250 nM at 336 nM. So, desensitization to sequential additions of cADPR was little.

To investigate endogenous cADPR formation, a spectrophotometric assay of GDP-ribosyl cyclase (GCPR-cyclase) activity in ROS discs was conducted (Fig. 4). This method is based on NGD^+ conversion in abundance by most known ADPR cyclases into a cyclic derivative, cGDPR, resistant to hydrolysis.[40,41] Both crude and purified ROS discs contained readily detected ADPR-cyclase activity, with an average initial rate of 13 ± 2 nanomoles cGDPR/min per milligram protein (mean ± SD, n = 7). In samples in which proteins were denatured by adding 25% PCA just before addition of NGD^+, there was no cGDPR production.

A putative NAD^+ glycohydrolase activity, spectrophotometrically assayed in both crude and purified ROS discs measuring the decrement of β-NAD^+, was not found (0.0 nmol β-NAD consumed/min per milligrams). The enzyme was tested with the purpose of better understanding whether the putative ROS enzyme was similar to the novel enzyme from brain which, in contrast with CD38, does not display NADase activity.[22,23]

To obtain conclusive evidence of the presence of ADPR-cyclase in bovine discs, we investigated the enzyme at a structural level. For this purpose, ADPR-cyclase of discs was detected on mildly denaturing SDS-PAGE by way of its production of fluorescent cyclic inosine diphosphate ribose from NHD^+.[41] One gel representative of at least 10 separated experiments is shown in Figure 5, where black and white are inverted for the sake of clarity. The resultant apparent molecular mass (MW) of the enzyme, seen as a fluorescent band in the gel was 53 ± 1 (mean ± SD). To seek the subcellular localization of the ADPR-cyclase, we tested three fractions (Fig. 5): osmotically intact discs (lane 3), crude discs (lane 2), and cytosolic ROS fractions (lane 1). ADPR-cyclase activity appeared to be located only within membranes, being detectable in both crude and purified discs and not detectable in the soluble ROS fraction.

The dependence of cADPR-elicited Ca^{2+} release on free extradiscal $[Ca^{2+}]$ (Fig. 3B) prompted us to investigate the presence in the discs of the RyR channels to which cADPR is known to bind. Samples were screened with an antibody (Ab) against an epitope mapping at the C-terminal region of human RyR, where the calcium release channel activity resides. Pictures obtained in confocal microscopy (Fig. 6A) show the presence in purified discs of RyR channels, stained by indirect immunocytochemistry (in red, a secondary Ab coupled to Cy3). The spectrum of Cy3-labeled secondary Ab is shown in Figure 6B. Incubation of discs with secondary Ab yielded only negligible immunoreactivity, confirming the specificity of disc surface interaction of the Ab with the RyRs (data not shown). In Figure 6C, Western blot analysis with the same Ab shows that a protein with the apparent mass of 520.0 ± 60 kDa

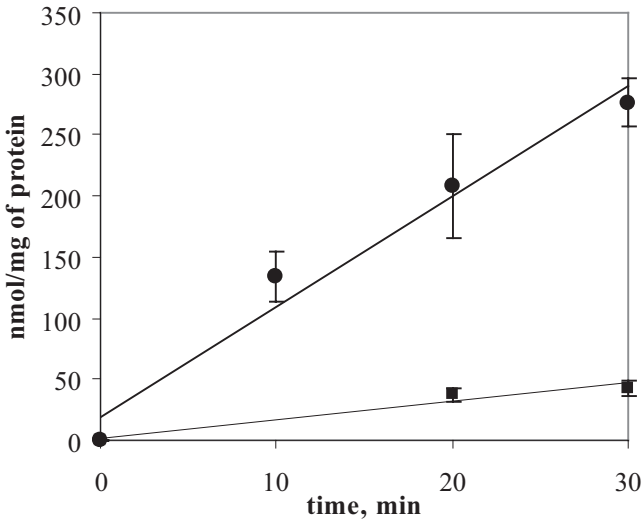

FIGURE 4. Assay of ADPR-cyclase activity in osmotically intact bovine ROS discs. The time course of generation of cGDPR by crude discs suspended with 1 mM NGD^+ in the same reaction mixture as for the Ca^{2+} measurements (●). cGDPR production was assayed by spectrophotometric analysis of absorbance of neutralized supernatants at 300 nm wavelength. Optical densities were converted to rates of cGDPR production ($\varepsilon = 3758\ M^{-1} \cdot cm^{-1}$). A deproteinized sample showed no variation after NGD^+ addition (■). Each point represents the mean ± SD of results in five different experiments.

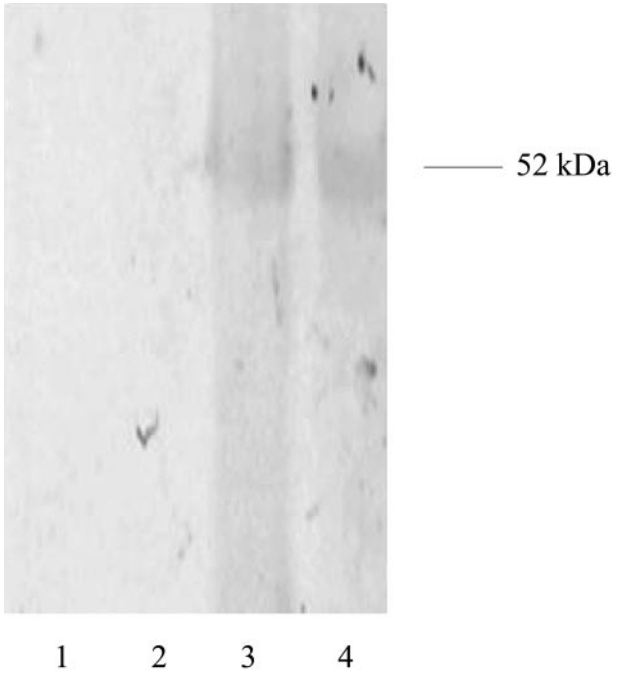

FIGURE 5. Detection of ADPR-cyclase activity on mildly denaturing SDS-PAGE. Shown are the fluorescent bands (inverted for clarity) corresponding to cyclic inosine diphosphate ribose locally produced by ADPR-cyclase activity, as visualized under UV light. Samples (60 μg/lane) were loaded onto a 12% polyacrylamide gel and run on a modified Laemmli SDS-PAGE. The gel was then incubated in the dark for 15 minutes with 0.2 mM NHD^+. *Lane 1*: cytosolic ROS fraction; *lane 2*: crude discs; *lane 3*: osmotically intact discs. Apparent molecular mass of the enzyme was 53 ± 1 (average ± SD, n = 10).

(mean ± SD) was present in osmotically intact discs (lane 1) but not in ROS cytosol. Mouse skeletal muscle microsomes, used as a positive control (lane 1) showed a band of 560 ± 75 kDa (mean ± SD).

DISCUSSION

The results presented in this work point to the existence of a cADPR metabolism in retinal ROS. cADPR elicited a Ca^{2+} release from osmotically intact discs that was modulated by extravesicular [Ca^{2+}] (Fig. 3). Osmotically intact bovine ROS disc preparations were shown to be devoid of mitochondria and endoplasmic reticulum (Fig. 1) and negligibly contaminated by inner segments, as suggested by the lack of cross-reactivity with a mouse monoclonal antibody raised against rabbit Na/K ATPase in purified discs (Fig. 2). The trend of the dependence was linear (Fig. 3B) around the physiological levels in dark-adapted ROS.[44,45] Saturation is likely reached for higher Ca^{2+} concentrations that cannot be explored by means of fluo-3. In our experimental conditions, Ca^{2+} reuptake into discs was not observed (Fig. 3), because the ATP-driven Ca^{2+} pump of ROS discs does not operate at nanomolar Ca^{2+} concentrations.[28,29] Data indicate that in the discs, self-inactivation due to consecutive additions of cADPR was minimal (Fig. 3).

CaM is one of the accessory proteins that modulating calcium release from RyR channels along with cADPR, calcium and magnesium ions.[12] CaM and CaM-like proteins were found in ROS.[25]

A GDPR-cyclase activity is present in both crude and purified ROS discs, with initial rate of ~13 ± 1 namomoles cGDPR formed/min per milligram of protein (Fig. 4). Such a result is comparable to the activity found in pancreatic homogenate (35.5 ± 4 nanomoles cGDPR formed/min per milligram of protein)[46] and in mouse brain synaptosomes, where the initial

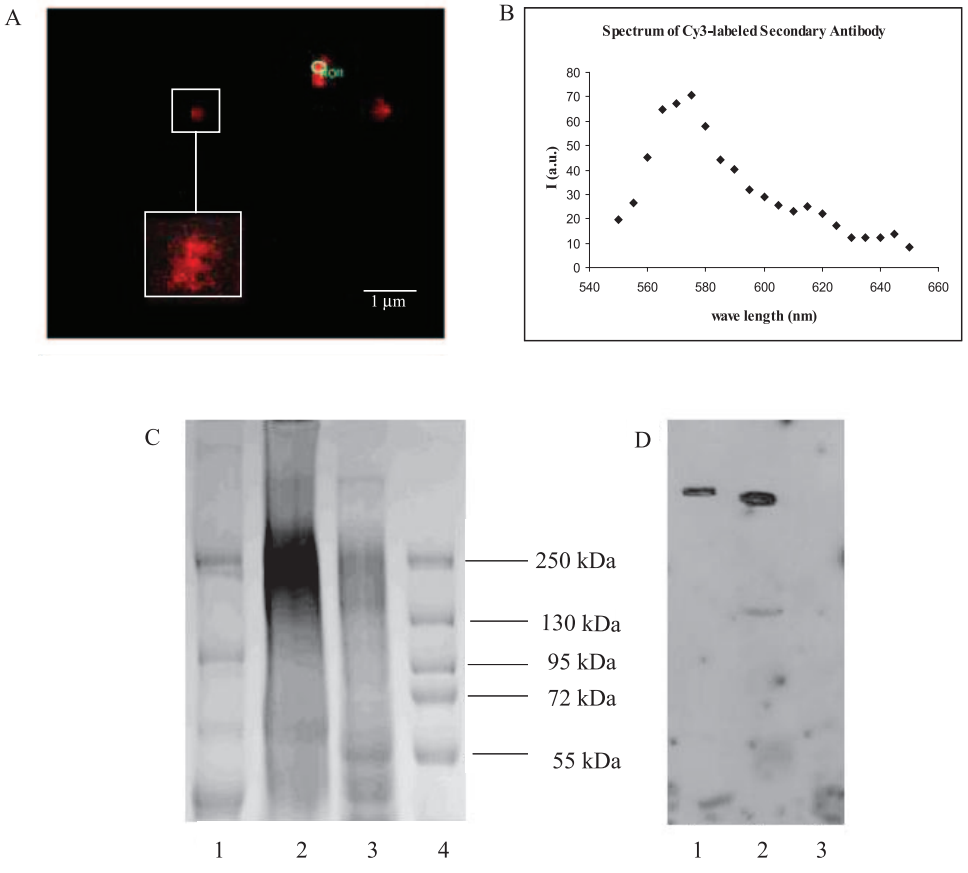

FIGURE 6. Localization of RyR channels with isoform-specific antibody on ROS discs. (**A**) Confocal fluorescence imaging of intact discs stained with a secondary anti-mouse antibody CY3 bound to a goat polyclonal antibody against RyR Ca^{2+} receptors. The resultant image was taken in the 512 × 512 format for a scanned area of 150 μm^2. *White box*: a magnified view of a single disc. As shown by the spectral analysis (*inset*), elaborated from several spectral acquisitions within the 560- to 660-nm range with a wavelength resolution of 5 nm, the fluorescence intensity of the discs is due only to the *red* CY3 (plotted in **B**). (**C**, **D**) Western blot analysis. Samples (80 μg/lane) were separated by 7% SDS-PAGE, transferred to nitrocellulose membranes, and then probed with the same antibody as used in (**A**). *Lane 1*: mouse skeletal microsomes, used as positive control; *lane 2*: crude discs; *lane 3*: osmotically intact discs; *lane 4*: cytosolic ROS fraction. Antibody recognized a protein with the apparent mass of 500 ± 70 kDa (average ± SD; n = 3). (**D**) Colloidal Coomassie blue staining of proteins after the run in (**C**).

reaction rate was 25 nanomoles cADPR formed/min per milligram of protein.[22] In isolated cardiac myocytes[47] and scallop muscle supernatants,[37] activity was lower (~2.5 nanomoles/mg per minute). The mouse brain enzyme[22,23] was unable to catalyze the cyclization reaction with NGD^+, similar to an ADPR-cyclase identified in T-cell lymphocytes.[48] Conversely, the ROS putative ADPR-cyclase can use the NAD-analogue NGD^+ as a substrate as can most ADPR-cyclases (CD38, CD157, and the *Aplysia* cyclase[39,40,49]). When the ADPR-cyclase activity of discs was investigated on mildly denaturing SDS-PAGE with NHD^+, a fluorescent band was observed on gels at the apparent molecular mass of 53 ± 1 kDa (Fig. 4). M_r of both CD38 and BST1, as observed in Western Blot analyses, is 45 kDa, whereas M_r of *Aplysia* ovotestis cyclase is approximately 35 kDa.[19]

Confocal laser imaging of intact disc preparations showed that RyR channels are expressed in purified discs of the bovine retina (Fig. 6A). To explore the molecular mass of RyRs we performed Western immunoblot analysis by means of the same Ab on both intact and crude discs. Results (Fig. 6B) suggest that discs possess RyRs with a molecular mass similar to that of most RyRs.

ADPR-cyclase is a ubiquitous enzyme.[49] In lower metazoan tissues, such as *Aplysia* ovotestis, ADPR-cyclase activity is typically cytosolic[2,19,50] whereas, in sea urchin eggs, it is localized partially in the cytosol and partially in the membranes.[51] In vertebrate cells and tissues, the ADPR-cyclase activity was found to be associated with plasma membranes.[2] This association also occurs for both the ectocellular multifunctional CD38-ADPR-cyclase glycoprotein[2,14,15] and the BST-1/BF-3 antigen.[8]

Recently, scientists have sought to identify ADPR-cyclase activities located in intracellular membranous structures, not in contact with the vertebrate cell surface. Ceni et al.[22,23] have described the cited novel endocellular ADPR-cyclase inside synaptosomes from $Cd38^{-/-}$ mouse brain. Purified ROS discs are exquisitely intracellular organelles, whose protein composition has been shown to differ from that of the plasma membrane.[52] The ROS disc ADPR-cyclase activity was detectable only in crude or purified discs but not in the cytosolic ROS fraction, and so the enzyme appears to be intracellular, but not cytosolic (Fig. 5). It has been shown that the $Cd38^{-/-}$ mouse ADPR-cyclase displays high intracellular ADPR-cyclase activity and a very low NADase activity in vitro.[22] Of note, the ROS enzyme does not display NAD-glycohydrolase activity, unlike CD38 and CD157. More experiments that go beyond the scope of the present work are needed to compare the biochemical characteristics of the enzyme found in the ROS with the other ADPR cyclases described up to now.

We propose that a novel mammalian ROS ADPR-cyclase regulates the production of cADPR and therefore calcium levels within ROS. In the dark, the cGMP-gated channel mediates an inflow of Ca^{2+}, keeping ROS cytosolic [Ca^{2+}] relatively high.[44] During the excitation phase of the photoresponse, light stimulates an enzymatic cascade culminating in cGMP hydrolysis leading to the closure of light-sensitive plasma membrane channels, generating the nervous signal.[25,26] In resting bovine ROS cADPR would elicit a [Ca^{2+}]-dependent Ca^{2+} release from discs, thanks to the relatively high (~500 nM)[44] cytosolic dark-adapted ROS free Ca^{2+} concentration, which would keep RyRs in an activatable state. This hypothesis is in accordance with our results showing that the disc RyR channels are in an activated state over which desensitization to cADPR is partially ineffective (Fig. 3A). The physiological role of the cADPR pool generated within the ROS in the dark would therefore be to implement Ca^{2+} release from discs in the dark, contributing to the maintenance of elevated free [Ca^{2+}] in the dark.

After light absorption, the decrease in internal [Ca^{2+}] plays a key role in the recovery of photoreceptors through the regulation of guanylyl cyclases (GC1 and -2) catalyzing the conversion of GTP to cGMP.[25,26] [Ca^{2+}] inside the photoreceptor was calculated to drop to the theoretical level of 10^{-10} M[53]; however, experiments have shown that, after illumination, Ca^{2+} drops to approximately 140 nM.[45] Light was also thought to increase [Ca^{2+}] initially.[54] Moreover, Schnetkamp[55] reported that the internal Ca^{2+} concentration is maintained at relatively high levels (83 nM) in isolated bovine ROS kept in Ca^{2+}-free medium, by a combined inactivation of the Na^+/Ca^{2+}-K^+ exchanger coupled with Ca^{2+}release from discs.[55] It is tempting to presume that the cADPR-dependent calcium extrusion keeps the extradiscal Ca^{2+} decline less steep immediately after illumination, considering that, at 200 nM, RyR channels are still sensitive to cADPR (Fig. 3). Ultimately, somewhere below this value it is reasonable to think that the fall in cytosolic ROS free [Ca^{2+}] drives the RyRs to a nonresponsive state; otherwise Ca^{2+} would never reach the measured level of 83 to 140 nM.

cGMP was found to stimulate cADPR synthesis by activating its synthetic enzyme through the cGMP-dependent protein kinase type I (cGKI).[56] Mammals have two cGKs: cGKI, expressed in some brain regions and in retina,[57,58] and cGKII, widely distributed in the mammalian brain. Fluctuations in cGMP concentration, higher in ROS than in any other vertebrate cell,[25] may further control ADPR-cyclase activity. cADPR has been reported to operate either as a second messenger in Ca^{2+} signaling through the activation of its synthetic enzyme, or as a RyR channel modulator, as in muscle cells.[2] In fact, in skeletal muscle sarcoplasmic reticulum cADPR is bound to RyR1, because basal ADPR-cyclase activity and Ca^{2+} ion dynamics cause the rapid switching on and off of the channels. Instead, according to our model, cADPR acts in ROS as a messenger. In conclusion, ROS discs would be Ca^{2+} stores operated by cADPR.

Acknowledgments

The authors thank Carlo Tacchetti and Consuelo Venturi (University of Genova, Italy) for performing the TEM imaging.

References

1. Berridge MJ, Bootman MD, Lipp P. Calcium-a life and death signal. *Nature*. 1998;395:645–648.
2. Higashida H, Hashii M, Yokoyama S, et al. Cyclic ADP-ribose as a second messenger revisited from a new aspect of signal transduction from receptors to ADPR-cyclase. *Pharmacol Ther*. 2001;90:283–286.
3. Rutter GA. Calcium signalling: NAADP comes out of the shadows. *Biochem J*. 2003;373:e3–e4.
4. Perraud AL, Fleig A, Dunn CA, et al. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature*. 2001;411:595–599.
5. Walseth TF, Wong L, Graeff RM Jr, Lee HC. Bioassay for determining endogenous levels of cyclic ADP-ribose. *Methods Enzymol*. 1997;280:1287–1294.
6. Hua SY, Tokimasa T, Takasawa S, et al. Cyclic ADP-ribose modulates Ca^{2+} release channels for activation by physiological Ca^{2+} entry in bullfrog sympathetic neurons. *Neuron*. 1994;12:1073–1079.
7. Hashii M, Minabe Y, Higashida H. cADP-ribose potentiates cytosolic Ca^{2+} elevation and Ca^{2+}entry via L-type voltage-activated Ca^{2+} channels in NG108-15 neuronal cells. *Biochem J*. 2000;345:207–215.
8. Verderio C, Bruzzone S, Zocchi E, et al. Evidence of a role for cyclic ADP-ribose in calcium signalling and neurotransmitter release in cultured astrocytes. *J Neurochem*. 2001;78:646–657.
9. Takeshima H. Physiological roles of ryanodine receptor subtypes. *Neurosci Res*. 1998;31:20–30.
10. Marks AR. Intracellular calcium-release channel. *Am J Physiol*. 1997;272:C597–C605.

11. Roderick HL, Berridge MJ, Bootman MD. Calcium-induced calcium release. *Curr Biol*. 2003;13:R425.
12. Sorrentino V. *Ryanodine Receptors*. Boca Raton, FL: CRC Press; 1995.
13. Furuichi T, Furutama D, Hakamata Y, Nakai J, Takeshima H, Mikoshiba K. Multiple types of ryanodine receptor/Ca^{2+} release channels are differentially expressed in rabbit brain. *J Neurosci*. 1994; 14:4794–4805.
14. Morikawa K, Khodakhah J, Williams T. Two intracellular pathways mediate metabotropic glutamate receptor-induced Ca^{2+} mobilization in dopamine neurons. *J Neurosci*. 2003;23:149–157.
15. Hotta T, Asai K, Fujita K, Kato T, Higashida H. Membrane-bound form of ADPR-cyclase in rat cortical astrocytes in culture. *J Neurochem*. 2000;74:669–675.
16. Futatsugi A, Kato K, Ogura H, et al. Facilitation of NMDAR-independent LTP and spatial learning in mutant mice lacking ryanodine receptor type 3. *Neuron*. 1999;24:701–713.
17. Khoo KM, Han MK, Park JB, et al. Localization of the cyclic ADP-ribose dependent calcium signaling pathway in hepatocyte nucleus. *J Biol Chem*. 2000;275:24807–24817.
18. Schuber F, Lund FE. Structure and enzymology of ADPR-cyclases: conserved enzymes that produce multiple calcium mobilizing metabolites. *Curr Mol Med*. 2004;4:249–261.
19. States DJ, Walseth TF, Lee HC. Similarities in amino acid sequences of Aplysia ADPR-cyclase and human lymphocyte antigen CD38. *Trends Biochem Sci*. 1992;17:495.
20. Yamada M, Mizuguchi M, Otsuka N, Ikeda K, Takahashi H. Ultrastructural localization of CD38 immunoreactivity in rat brain. *Brain Res*. 1997;756:52–60.
21. Itoh M, Ishihara K, Tomizawa H, et al. Molecular cloning of murine BST-1 having homology with CD38 and Aplysia ADPR-cyclase. *Biochem Biophys Res Commun*. 1994;203:1309–1317.
22. Ceni C, Pochon N, Villaz M, et al. The CD38-independent ADP-ribosyl cyclase from mouse brain synaptosomes: a comparative study of neonate and adult brain. *Biochem J*. 2006;395:417–426.
23. Ceni C, Muller-Steffner H, Lund F, et al. Evidence for an intracellular ADP-ribosyl cyclase/NAD+-glycohydrolase in brain from CD38-deficient mice. *J Biol Chem.* 2003;278:40670–40678.
24. Basile G, Taglialatela-Scafati O, Damonte G, et al. ADP-ribosyl cyclases generate two unusual adenine homodinucleotides with cytotoxic activity on mammalian cells. *Proc Natl Acad Sci USA*. 2005;102:14509–14514.
25. Stryer L. Molecular mechanism of visual excitation. *Harvey Lect*. 1991;87:129–143.
26. Palczewski K, Kumasaka T, Hori T, et al. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science*. 2000;289:739–745.
27. Pepe IM. Recent advances in our understanding of rhodopsin and phototransduction. *Prog Retin Eye Res*. 2001;20:733–759.
28. Panfoli I, Morelli A, Pepe IM. Characterization of Ca^{2+}-ATPase in Rod outer segment disk membranes. *Biochem Biophys Res Commun*. 1994;204:813–819.
29. Pepe IM, Panfoli I, Notari L, Morelli A. ATP synthesis in rod outer segments of bovine retina by the reversal of the disk calcium pump. *Biochem Biophys Res Comm*. 2000;268:625–627.
30. Notari L, Morelli A, Pepe IM. Studies on adenylate kinase isoform bound to disk membranes of the rod outer segment of bovine retina. *Photochem Photobiol Sci*. 2003;12:1299–1302.
31. Fain GL, Schröder WH. Calcium content and calcium exchange in dark-adapted toad rods. *J Physiol* (*Lond*). 1985;368:641–645.
32. Schnetkamp PP, Daemen FJ. Isolation and characterization of osmotically sealed bovine rod outer segments. *Methods Enzymol*. 1982;81:110–116.
33. Smith G, Stubbs GW, Litman BJ. The isolation and purification of osmotically intact discs from retinal rod outer segments. *Exp Eye Res.* 1975;20:211–217.
34. Wald G, Brown PK. The molar extinction of rhodopsin. *J Gen Physiol*. 1954;37:189–200.
35. Panfoli I, Morelli A, Pepe IM. Calcium pump in the disk membranes isolated from bovine retinal rod outer segments. *J Photochem Photobiol B Biol*. 1994;24:187–194.
36. Stigliani S, Zappettini S, Raiten L, et al. Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. *J Neurochem*. 2006;96:656–668.
37. Panfoli I, Burlando B, Viarengo A. Cyclic ADP-ribose-dependent Ca^{2+} release is modulated by free [Ca^{2+}] in the scallop sarcoplasmic reticulum. *Biochem Biophys Res Commun*. 1999;257:57–62.
38. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
39. Guse AH, Tsygankov AY, Weber K, Mayr GW. Transient tyrosine phosphorylation of human ryanodine receptor upon T cell stimulation. *J Biol Chem*. 2001;276:34722–34727.
40. Graeff RM, Walseth TF, Fryxell K, Branton WD, Lee HC. Enzymatic synthesis and characterizations of cyclic GDP-ribose: a procedure for distinguishing enzymes with ADP-ribosyl cyclase activity. *J Biol Chem*. 1994;269:30260–30267.
41. Zocchi E, Carpaneto A, Cerrano C, et al. The temperature-signaling cascade in sponges involves a heat-gated cation channel, abscisic acid, and cyclic ADP-ribose. *Proc Natl Acad Sci USA*. 2001;98: 14859–14864.
42. Wu SD, Liu Y, Xu X, Zhu Z. Purification of NAD glycohydrolase from *Agkistrodon acutus* venom. *Prot Express Purif*. 2002;25: 319–322.
43. Pawley JB. *Handbook of Biological Confocal Microscopy.* 3rd ed. New York: Plenum Press-Springer; 2006.
44. Gray-Keller MP, Detwiler PB. The calcium feedback signal in the phototransduction cascade of vertebrate rods. *Neuron*. 1994;13: 849–861.
45. Ratto GM, Payne R, Owen WG, Tsien RY. The concentration of cytosolic free calcium in vertebrate rod outer segments measured with fura-2. *J Neurosci*. 1988;8:3240–3246.
46. Takasawa S, Nata K, Yonekura H, Okamoto H. Cyclic ADP-ribose in insulin secretion from pancreatic beta cells. *Science*. 1993;259: 370–373.
47. Meszaros V, Socci R, Meszaros LG. Heterogeneity of the cardiac calcium release channel as assessed by its response to ADP-ribose. *Biochem Biophys Res Commun*. 1995;210:452–456.
48. Guse AH, da Silva CP, Berg I, et al. Regulation of calcium signalling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature*. 1999;398:70–73.
49. Fernandez JE, Deaglio S, Donati D, et al. Analysis of the distribution of human CD38 and of its ligand CD31 in normal tissues. *J Biol Regul Homeostasis Agents*. 1998;12:81–91.
50. Mothet JP, Fossier P, Meunier FM, Stinnakre J, Tauc L, Baux G. Cyclic ADP-ribose and calcium-induced calcium release regulate neurotransmitter release at a cholinergic synapse of Aplysia. *J Physiol*. 1998;507:405–414.
51. Glick DL, Hellmich MR, Beushausen S, Tempst P, Bayley H, Strumwasser F. Primary structure of a molluscan egg-specific NADase, a second-messenger enzyme. *Cell Regul*. 1991;2:211–218.
52. Bauer PJ. Evidence for two functionally different membrane fractions in bovine retinal rod outer segments. *J Physiol*. 1988;401: 309–327.
53. Cervetto L, Lagnado L, Perry RJ, Robinson DW, McNaughton PA. Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature*. 1989;337:740–743.
54. Matthews HR, Fain GL. A light-dependent increase in free Ca^{2+} concentration in the salamander rod outer segment. *J Gen Physiol*. 2001;532:305–321.
55. Schnetkamp PP. How does the retinal rod Na-Ca^{+}K exchanger regulate cytosolic free Ca^{2+}? *J Biol Chem*. 1995;270:13231–13239.
56. Feil R, Kellermann J, Hofmann F. Functional cGMP-dependent protein kinase is phosphorylated in its catalytic domain at threonine-516. *Biochemistry*. 1995;34:13152–13158.
57. Gamm DM, Barthel LK, Raymond PA, Uhler MD. Localization of cGMP-dependent protein kinase isoforms in mouse eye. *Invest Ophthalmol Vis Sci*. 2000;41:2766–2773.
58. Snellman J, Nawy S. cGMP-dependent kinase regulates response sensitivity of the mouse on bipolar cell. *J Neurosci.* 2004;24:6621–6628.