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Biochemistry

Expression of a bovine rhodopsin gene in *Xenopus* oocytes: Demonstration of light-dependent ionic currents

(guanine nucleotide binding proteins/glycosylation/11-*cis*-retinal/microinjection/voltage clamp)

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ABSTRACT ***Xenopus* oocytes express a gene encoding bovine rhodopsin as well as its SP6 RNA polymerase-derived transcripts and total retinal mRNA. The opsin produced is in unglycosylated (30 kDa) and two glycosylated (35 kDa and 41 kDa) forms. Incubation of the cells expressing the above proteins with 11-*cis*-retinal generates rhodopsin, which was purified by immunoaffinity chromatography. The purified protein shows the expected UV/visible absorption spectrum and characteristic light-dependent activation of the rod outer segment GTP binding protein. Oocytes expressing rhodopsin exhibit light-dependent ionic currents that are detected by voltage-clamp techniques.**

The process of visual transduction in rod photoreceptor cells begins with photon absorption by rhodopsin, 11-*cis* to all-*trans* isomerization of its retinal chromophore, and relaxation of the protein through a series of metastable photointermediates (1). One of the intermediates, metarhodopsin II, is believed to activate a guanine nucleotide binding protein (G protein)—transducin (G_T)—and thus initiate an enzymatic cascade that results in hyperpolarization of the rod cell (2). Similar interactions between membrane-bound receptors and G proteins occur in many signal transduction systems (3). Sequence homology between the receptors (4) on the one hand and the G proteins (5) on the other hand suggests that the mechanism of interaction between these two classes of proteins has been conserved. However, the structural basis underlying receptor-mediated G-protein activation remains unclear. The visual system provides a highly attractive model for the study of this interaction.

As an approach to rhodopsin structure–function studies, we have expressed a synthetic rhodopsin gene (6) in mammalian cells and have shown that the rhodopsin isolated after addition of 11-*cis*-retinal to the cells is active *in vitro* (7, 8). *Xenopus* oocytes offer the possibility of an alternative expression system in which recombinant proteins can be assayed *in situ*, often by electrophysiological recordings (9). Furthermore, oocytes contain G protein-regulated ionic conductances (9, 10), and coupling to these could form the basis of an assay for rhodopsin. Indeed, a number of cloned receptor genes expressed in oocytes have been assayed by their ability to activate endogenous channels (11–13). In this report we demonstrate that a bovine opsin gene is expressed by *Xenopus* oocytes, and the opsin produced is active *in vitro*. Further, we show that rhodopsin in oocytes produces light-dependent ionic currents that are detected by voltage-clamp techniques.

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MATERIALS AND METHODS

Materials. Poly(A) polymerase and G(5′)ppp(5′)G were from Pharmacia; tunicamycin was from Calbiochem–Behring; *N*-Glycanase, endo-β-*N*-acetylglucosaminidase H (Endo H), and *N*-methyldeoxynojirimycin were from Genzyme (Boston); and SP6 RNA polymerase, DNase 1, and RNasin were from Promega Biotec (Madison, WI). Protein-A-agarose was a gift from Repligen (Cambridge, MA). [^{35}S]Methionine (>1000 Ci/mmol; 1 Ci = 37 GBq) and [γ-^{32}P]GTP were from either Amersham or ICN. Phosphatidylcholine (type V-E) was from Sigma, oligo(dT)-cellulose (type III) was from Collaborative Research (Waltham, MA), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and deoxymannojirimycin were from Boehringer Mannheim. ^{14}C-labeled protein molecular weight standards were from Bethesda Research Laboratories. 11-*cis*-Retinal was a gift from Peter Sorter (Hoffmann–La Roche, Nutley, NJ). Frozen bovine retinas were from J. A. Lawson Co. (Lincoln, NE), and fresh bovine retinas were obtained locally.

Plasmids and RNA. Poly(A)$^+$ mRNA was isolated from fresh bovine retinas as described (14). An *in vitro* transcription plasmid, pOP2, was constructed by inserting the opsin gene with the Kozak sequence at the 5′ end (7) into pSP65 (15). A *Bam*HI–*Not* I linker contained in a 25-base pair (bp) synthetic duplex was inserted into pOP2 at the 3′ end of the opsin gene to give pOP3. The *Eco*RI–*Not* I fragment containing the opsin gene was then cloned downstream of the adenovirus major late promoter in a derivative of pMT2 (8) to generate pMT2-OP3. The nucleotide sequence of all cloned synthetic duplexes was confirmed by the chain-terminator method (16). The pOP2 DNA was linearized with either *Bam*HI, which cuts immediately after the opsin coding sequence, or *Xmn* I, which cuts ≈1.9 kilobases downstream. Capped RNA (SP6 RNA) was synthesized *in vitro* by transcription of the linearized DNA with SP6 RNA polymerase (15) in the presence of G(5′)ppp(5′)G (17). The RNA (4–20 μg) was polyadenylylated *in vitro* [poly(A)$^+$ SP6 RNA] with poly(A) polymerase (1 unit) in the presence of 1600 units of RNasin (18, 19) per ml and was purified by chromatography on oligo(dT)-cellulose. The mean poly(A) tail length ranged from 30 to 80 nucleotides in different preparations.

Oocyte Injections and Immunoprecipitations of Expressed Proteins. Oocytes from proven breeder *Xenopus laevis* (Nasco, Fort Atkinson, WI) were dissociated by treatment with 2 mg of collagenase (Sigma, type 1A) per ml (10). Stage 5 and 6 oocytes were injected with 50 nl of RNA (0.75–1650

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; Endo H, endo-β-*N*-acetylglucosaminidase H; ROS, rod outer segment; G protein, guanine nucleotide binding protein; G_T, ROS guanyl nucleotide binding protein; MBS, modified Barth's saline.

μg/ml) in the cytoplasm or with 20–50 nl of DNA (0.7–5000 μg/ml) in the nucleus (20). Injected cells were incubated at 16–18°C for up to 4 days in Hepes-buffered modified Barth's saline (MBS; see the legend to Fig. 5) supplemented with 5% dialyzed fetal calf serum (20) and [^{35}S]methionine (0.1–0.5 mCi/ml). Oocytes were homogenized in 20–100 μl of PBS (10 mM sodium phosphate, pH 7.4/150 mM NaCl) per cell containing 100 μM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 2% dodecyltrimethylammonium bromide. Insoluble debris was removed by centrifugation at 10,000 × *g* for 20 min; the supernate was incubated overnight at 4°C with anti-opsin IgG and then for 2 hr with protein A-agarose that had been incubated with uninjected oocyte extract. The immunoabsorbed proteins were eluted from protein A-agarose with 2% $NaDodSO_4$ sample buffer, electrophoresed on 10% polyacrylamide gels (21), and visualized by fluorography.

Inhibition of Glycosylation in Oocytes. Oocytes were treated with tunicamycin (20) or were incubated with 2 mM *N*-methyldeoxynojirimycin or 2 mM deoxymannojirimycin in MBS. In all three cases, the oocytes were injected with poly(A)$^+$ SP6 RNA (60 μg/ml). [^{35}S]Methionine was added to the incubation medium 14 hr later, and the oocytes were cultured for 3 days before immunoprecipitation of the proteins.

Deglycosylation of Oocyte Opsin. Immunoprecipitated opsin was enzymatically deglycosylated after elution from the protein A-agarose with 2% $NaDodSO_4$ in 80 mM Tris HCl, pH 6.8/0.1 M dithiothreitol. The eluate was diluted 1:9 and incubated for 24 hr at 37°C with either *N*-Glycanase (at 10 units/ml in 0.2 M sodium phosphate, pH 8.2/0.2% $NaDodSO_4$/1.5% Nonidet P-40/10 mM *o*-phenanthroline) or Endo H (at 50 milliunits/ml in 50 mM sodium phosphate, pH 6.3/0.2% $NaDodSO_4$/10 mM *o*-phenanthroline/1 mM phenylmethylsulfonyl fluoride).

Purification and *in Vitro* Assay of Rhodopsin. Seventeen hundred oocytes were injected with poly(A)$^+$ SP6 RNA (60 μg/ml), incubated for 4 days, washed with MBS, and placed in 50 ml of MBS in dim red light. Three aliquots of 11-*cis*-retinal (500 nmol) were added at 30- to 45-min intervals. The oocytes were then homogenized in 25 ml of PBS containing 100 μM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 1% CHAPS; solubilized overnight at 4°C; and immunopurified (7) by using a monoclonal anti-opsin antibody-conjugated resin (2.4 mg of antibody per ml of Sepharose).

The assay for light-dependent GTPase activity (7) was modified. Rhodopsin was exchanged from PBS/CHAPS into PBS/1% digitonin while bound to the antibody resin. The protein (0.18 μM) was mixed with an equal volume of PBS/1% CHAPS containing 15 mg of phosphatidylcholine per ml and then diluted 1:9 into assay buffer (7) containing 0.26 μM G_T and 0.48 μM [γ-^{32}P]GTP (6 Ci/mmol) in a final volume of 760 μl. The rates of GTP hydrolysis were not corrected for rhodopsin decay.

Electrophysiology. Oocytes were placed in a recording chamber continuously perfused at room temperature with MBS. The cells were impaled with two microelectrodes filled with 3 M KCl. The membrane potential was held at −80mV with a voltage-clamp circuit, and the membrane current was monitored through a virtual ground circuit. Oocytes under voltage clamp were perfused for 30–40 min in the dark with MBS containing 10–50 μM 11-*cis*-retinal. Currents evoked by flashes of white light were low-pass-filtered at 100 Hz with an 8-pole Bessel filter, and the signal was stored on videotape.

Other Methods. Anti-opsin antibodies were generated in female New Zealand White rabbits by repeated subcutaneous injections of bovine opsin (purified on $NaDodSO_4$ gels), and IgG was prepared from the sera (22). G_T was isolated from hypotonic washes of rod outer segment (ROS) membranes (23). Retinal proteins were labeled *in situ* with [^{35}S]methionine in the presence of tunicamycin under conditions that partially inhibit glycosylation, and ROS were prepared as described (23). Dodecyltrimethylammonium bromide (Sigma) was recrystallized three times from hot acetone/methanol, 1:1 (vol/vol), and digitonin (Kodak) was purified by extraction with hot water (24).

RESULTS

Expression of a Bovine Rhodopsin Gene in *Xenopus* Oocytes. *Retinal mRNA.* Initial experiments were performed with the poly(A)$^+$ fraction of the total bovine retinal RNA. Oocytes injected with the mRNA and incubated for 3 days with [^{35}S]methionine showed a major immunoprecipitable product (band I) (41 kDa) (Fig. 1, lane 2). This protein migrated more slowly than rod cell opsin did (Fig. 1, lane 6). The high molecular weight proteins that were also specifically immunoprecipitated are aggregates of opsin. The specificity of the immunoprecipitation was demonstrated by using uninjected oocytes (Fig. 1, lane 1), nonspecific IgG, or by including bovine rhodopsin in the cell extracts.

RNA synthesized in vitro. Oocytes injected with RNA transcribed *in vitro* from the synthetic rhodopsin gene (SP6 RNA) synthesized band I opsin (Fig. 1, lane 3). The amount of protein produced from either mRNA or SP6 RNA was similar, even though opsin mRNA constitutes only a few percent of the total retinal poly(A)$^+$ fraction (25). Since the noncoding regions of the SP6 transcript of the opsin gene differ from those in the mRNA, the influence of the untranslated sequences on the level of expression was investigated. The SP6 RNA containing ≈1.9 kb of noncoding sequence at the 3′ end yielded about 2-fold higher amounts of immunoprecipitated opsin than did the SP6 RNA ending immediately after the termination codon of the opsin gene. The level of expression increased further by 10-fold when SP6 RNA was

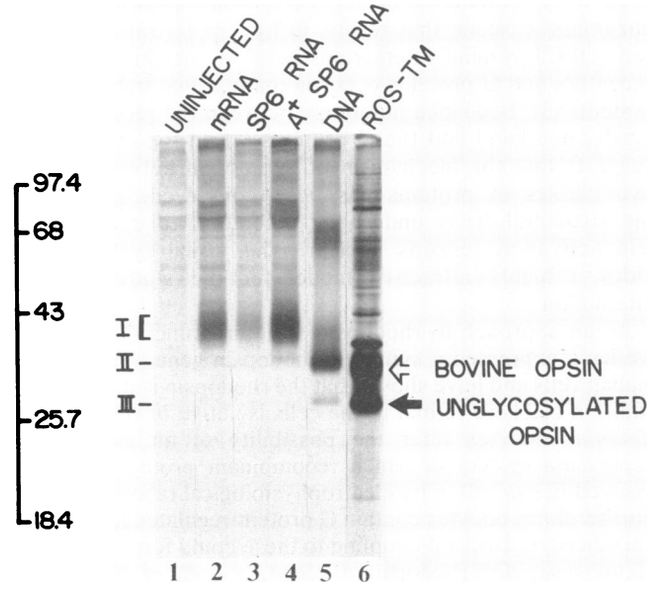

FIG. 1. Immunoprecipitation of bovine opsin synthesized in *Xenopus* oocytes. Detergent extracts of pools of 12 oocytes were immunoprecipitated with anti-opsin IgG, and the proteins were separated by electrophoresis and then fluorographed for 7 hr. The cells were either uninjected (lane 1) or injected with 19 ng of bovine poly(A)$^+$ retinal mRNA (lane 2), 4.5 ng of SP6 RNA transcribed *in vitro* from pOP2 linearized at the *Xmn* I site (lane 3), 4.2 ng of the same SP6 RNA after it was polyadenylylated (lane 4), or 50 ng of pMT2-OP3 (the expression vector) (lane 5). Glycosylated (◊) and unglycosylated (◆) bovine opsin from tunicamycin-treated ROS (ROS-TM) are marked for reference (lane 6).

polyadenylylated prior to injection [poly(A)$^+$ SP6 RNA; Fig. 1, lane 4]. With the increased expression, two additional forms of the opsin were observed: band II (35 kDa) and band III (30 kDa). Band III, which comigrated with unglycosylated bovine opsin, was most easily detected when high concentrations of poly(A)$^+$ SP6 RNA (Fig. 2) or DNA were injected (see below). At optimal poly(A)$^+$ SP6 RNA concentrations (60 μg/ml), the level of opsin continued to increase for 3 days after injection. The amount of opsin synthesized over this period, measured by radioimmunoassay, varied from 2 to 20 ng per cell.

Expression with the vector pMT2-OP3. Oocytes also synthesized opsin in three forms when injected with the expression vector in which the coding sequence is followed by the simian virus 40 early polyadenylylation signal (8) (Fig. 1, lane 5). Nuclear injections of this construct resulted in amounts of protein comparable to the levels produced by cells injected with poly(A)$^+$ SP6 RNA. These proteins were absent in cells that were injected cytoplasmically or when nuclear injections were performed with the parent vector without the opsin gene. Band II, a minor species in cells injected with RNA, was the more prominent species present in DNA-injected cells. Bands II and III comigrated with those formed in RNA-injected cells. The mobility of band I showed variability from frog to frog and most often migrated slightly ahead of band I from RNA-injected cells.

Glycosylation Differences in the Three Forms of Opsin. Treatment of oocytes with tunicamycin, an inhibitor of N-linked glycosylation (26), showed only band III, which comigrated with unglycosylated bovine opsin (Fig. 2*A*, lane 2); bands I and II were not synthesized. Enzymatic deglycosylation using *N*-Glycanase (27) converted both bands I and II to band III (Fig. 2*B*, lane 2). Similar results were obtained with proteins from RNA- or DNA-injected cells and indicate that bands I and II differ from band III by N-linked carbohydrate. Oocytes expressing the three forms of the opsin were treated with Endo H (28). Band II was completely deglycosylated, while the mobility of band I was only slightly increased (Fig. 2*B*, lane 3). When the opsin was expressed in cells treated with deoxymannojirimycin, an inhibitor of Golgi mannosidases (29), band II was the major form observed (Fig. 2*A*, lane 3). Treatment of cells with *N*-methyldeoxynojirimycin, an inhibitor of glucosidases that initiate processing in the endoplasmic reticulum (30), resulted in the appearance of an additional band, core-glycosylated opsin (Fig. 2*A*, lane 4). A temporal relationship in the synthesis of the three forms of opsin was established in a pulse–chase experiment as described in the legend of Fig. 2. The label initially seen in all three forms of opsin (Fig. 2*C*, lane 1) is chased into band I (Fig. 2*C*, lane 2).

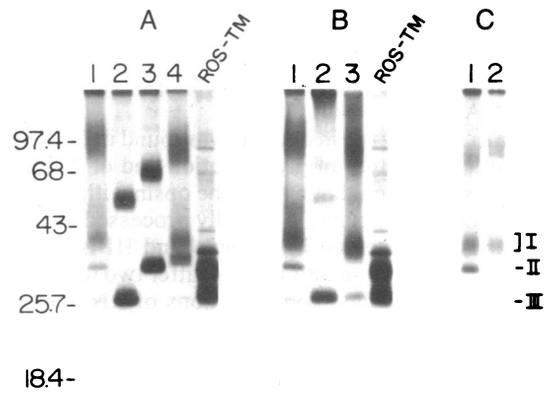

FIG. 2. Glycosylation differences in the three forms of opsin. (*A*) The effects of glycosylation inhibitors. Cells were injected with poly(A)$^+$ SP6 RNA and labeled with [^{35}S]methionine for 3 days in MBS alone (lane 1) or in the presence of tunicamycin (lane 2), deoxymannojirimycin (lane 3), or *N*-methyldeoxynojirimycin (lane 4). Lane ROS-TM contains glycosylated and unglycosylated bovine opsin as in Fig. 1. (*B*) Enzymatic deglycosylation of opsin. Opsin was immunoprecipitated from oocytes injected with poly(A)$^+$ SP6 RNA and incubated overnight without enzyme (lane 1), with *N*-Glycanase (lane 2), or with Endo-H (lane 3). Each lane in *A* and *B* contains the proteins immunoprecipitated from 0.5 oocyte equivalents, except when tunicamycin treatment was involved, in which case 0.7 equivalents was used to correct for the observed 40% inhibition of protein synthesis. The fluorograph was exposed for 36 hr. (*C*) Pulse–chase. Oocytes injected with poly(A)$^+$ SP6 RNA were incubated with [^{35}S]methionine for 12 hr (lane 1) and then washed and incubated for an additional 24 hr in 10 mM nonradioactive methionine (lane 2). Proteins immunoprecipitated from three oocytes were separated by electrophoresis and fluorographed for 48 hr.

Regeneration of the Rhodopsin Chromophore and Assay of the Purified Rhodopsin. Regeneration of rhodopsin from oocyte opsin was carried out by the addition of 11-*cis*-retinal to oocytes expressing the opsin, and the rhodopsin was purified by immunoabsorption. Fig. 3 shows the absorption spectrum obtained for the purified protein. The spectrum was characteristic of rhodopsin, with a maximum at 497 nm. The ratio of absorbance at 280 to 500 nm was 2.3, being somewhat higher than that of ROS rhodopsin (1.6). This indicated contamination by one or more other proteins and/or opsin that did not bind retinal. The yield of purified rhodopsin [assuming ε_{500} = 40,600 $M^{-1}\cdot cm^{-1}$ (31)] was 10 ng per oocyte, in good agreement with the radioimmunoassay. When quantitated by densitometric scanning of a silver-stained $NaDodSO_4$ gel, 85% of the purified protein was found to migrate as band I.

G_T Activation by Oocyte Rhodopsin. The assay of G-protein activation was carried out as described. Oocyte rhodopsin stimulated the GTP hydrolytic activity of bovine G_T in a light-dependent fashion (Fig. 4). The extent of stimulation, 7-fold over that obtained in the dark and the initial rate were similar to those observed with bovine rhodopsin (7).

Light-Induced Ionic Currents in Oocytes Expressing Rhodopsin. A two-electrode voltage clamp was used to monitor the currents flowing through the oocyte's plasma membrane.

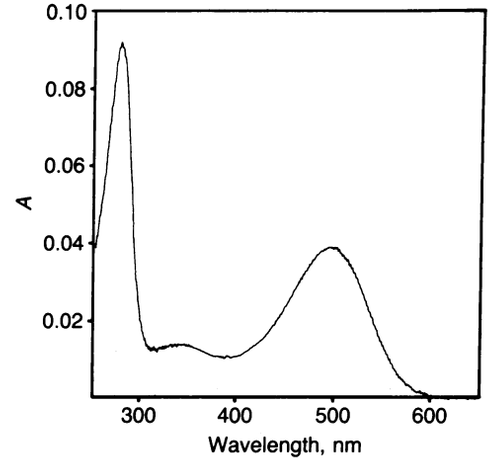

FIG. 3. Absorption spectrum of rhodopsin generated from *Xenopus* oocytes. Oocytes were injected with poly(A)$^+$ SP6 RNA and incubated for 4 days to synthesize opsin. Rhodopsin was generated by the addition of 11-*cis*-retinal to the cells, solubilized in 1% CHAPS, and purified by immunoaffinity chromatography. The UV/visible absorption spectrum of the purified protein is shown. The yield of purified rhodopsin was 17 μg (10 ng per cell).

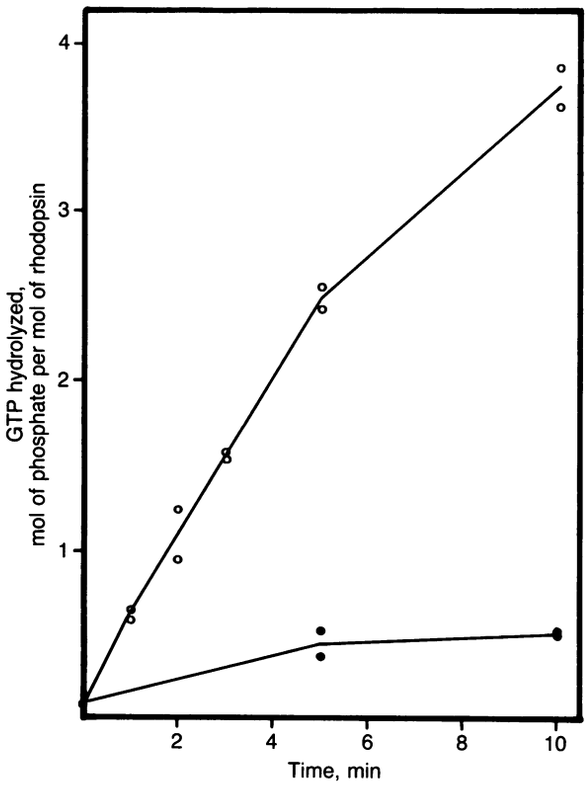

FIG. 4. Light-dependent stimulation of the GTPase activity of G_T by rhodopsin purified from *Xenopus* oocytes. Rhodopsin was incubated with G_T and [γ-^{32}P]GTP in the light (○) or the dark (●), and GTPase activity was assayed by quantitating $^{32}P_i$ released. The initial rates of the GTPase hydrolysis were 0.55 mol of phosphate per mol of rhodopsin per min in the light and 0.08 mol of phosphate per mol of rhodopsin per min in the dark. Bovine rhodopsin controls gave initial rates between 0.17 and 0.85 mol of phosphate per mol of rhodopsin per min in the light.

Cells were impaled with the electrodes, and the membrane potential was clamped at −80mV. The currents were analyzed after each cell was perfused with 11-*cis*-retinal to convert the expressed opsin to rhodopsin. No light-induced currents were detected in uninjected cells (e.g., Fig. 5*A*). In cells expressing rhodopsin, inward currents were observed in response to light. The response of one oocyte is shown in Fig. 5*B* and *C*. A 1-sec flash of light elicited transient inward current fluctuations (Fig. 5*B*). Continuous illumination resulted in a sustained inward current upon which the fluctuations were superimposed (Fig. 5*C*). Although the waveform and amplitude of the current varied somewhat from cell to cell, its onset consistently exhibited a delay following the stimulus, and the direction was inward in cells clamped at −80mV. A detailed analysis of these currents will be published elsewhere.

DISCUSSION

With the aim of developing the oocyte system for the study of visual transduction, we have now reported on the expression of bovine rhodopsin in *Xenopus* oocytes. The opsin synthesized binds exogeneously added 11-*cis*-retinal to generate rhodopsin, which has been purified and characterized. The level of expression of the opsin in oocytes depended upon the amount of RNA injected and was increased by polyadenylylation, as previously observed for the expression of other RNAs in oocytes (32, 33). Nuclear injection of the opsin gene in a mammalian expression vector also resulted in synthesis of the opsin. Under optimal conditions, the amount

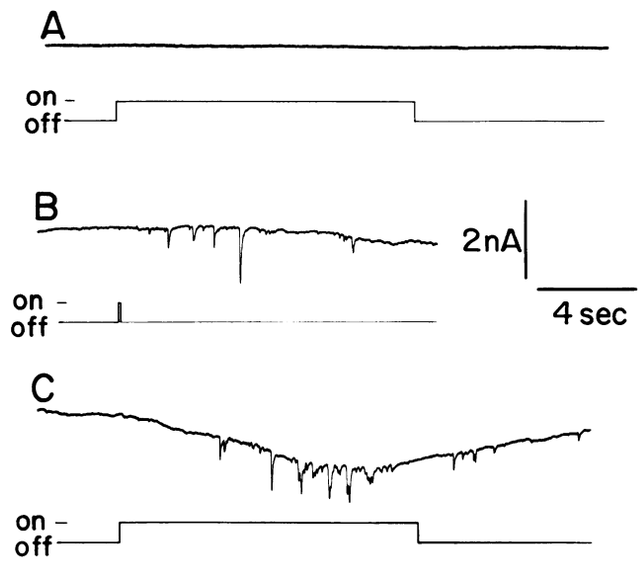

FIG. 5. Light-induced ionic currents in oocytes expressing rhodopsin. Cells were perfused with MBS containing 11-*cis*-retinal, and the currents were recorded as described. The membrane potential of the cells was clamped at −80 mV, and the current signal was low-pass-filtered at 10 Hz upon playback. In each panel, the upper trace is the current record and the lower trace is the light-stimulus monitor. Inward current is represented as a downward deflection of the record. Upward deflections of the stimulus monitor ("on") denote the time the cell was exposed to light. (*A*) The current response of an uninjected cell to 2 min of illumination. (*B*) Currents induced in an oocyte in response to a 1-sec flash of white light. (*C*) The current response of the same cell to continuous (2 min) illumination. [MBS contains 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes·NaOH (pH 7.6), 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 0.82 mM $MgSO_4$.]

of protein synthesized in DNA- or RNA-injected cells was approximately equal, ranging from 2 to 20 ng per oocyte. Direct injection of the opsin DNA as now described offers the advantage that it circumvents the need for *in vitro* transcription of RNA.

The opsin synthesized in oocytes was found in three forms. The action of inhibitors of glycosylation and of deglycosylation enzymes showed that band I, the opsin with the highest apparent molecular weight, is the fully processed form; band II is a partially processed form, and band III is the unglycosylated opsin. Accumulation of the latter two forms in cells injected with the highest concentrations of RNA used suggests that the capacity of the oocyte for posttranslational modification has been exceeded, or alternatively, that opsin aggregation interferes with further processing. However, bands II and III accumulate in oocytes injected with pMT-OP3 at all concentrations tested, suggesting that the form of opsin produced is not determined by expression level alone. Although we have not proven that all three forms of the opsin are equally active, it is clear that band I is functional, since band I comprised 85% of a sample of purified oocyte rhodopsin that had a 280 nm/500 nm absorbance ratio of 2.3. Band I opsin from oocytes is glycosylated differently from ROS rhodopsin, indicating that the exact nature of the carbohydrate is not crucial for activity. The activity of band I suggests that band II and unglycosylated rhodopsin are active as well.

We have observed light-dependent ionic currents in oocytes expressing functional rhodopsin. The mechanism for generating these currents remains to be determined. They may result from a direct contribution of rhodopsin to the plasma membrane conductance or as a consequence of the interaction of rhodopsin with oocyte proteins. The currents

appear to be similar to those underlying the cell's fertilization potential (34) and those induced by muscarinic acetylcholine (11), cloned serotonin (12) and substance K receptors (13) expressed in oocytes. These currents are regulated by an oocyte G protein that stimulates inositol trisphosphate production and intracellular calcium, thus activating chloride channels in the plasma membrane (10, 19, 35, 36). Our results suggest that rhodopsin may also activate this pathway and interact with oocyte G proteins *in vivo*, further highlighting similarities between signal transduction systems suggested by previous *in vitro* experiments (37).

Our ultimate goal is to use *Xenopus* oocytes to express in a single cell the necessary proteins of the visual cascade. This should allow further studies of the mechanisms and controls involved at the different steps in the system.

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