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Specific labeling and permanent activation of the retinal rod cGMP-activated channel by the photoaffinity analog 8-*p*-azidophenacylthio-cGMP

(rod outer segments/cyclic nucleotide-binding proteins/patch-clamping/ligand-gated ion channels)

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ABSTRACT **Cyclic nucleotide-gated ion channels play a key role in visual excitation and a variety of other signaling pathways. The photoaffinity probe 8-*p*-azidophenacylthio-cGMP (APT-cGMP) has been developed for structural and functional studies of the cGMP-activated channel of retinal rod outer segments. Using this analog, we have demonstrated both specific labeling of the channel in a partially purified biochemical preparation from bovine rod outer segments and permanent activation of the channel current in excised membrane patches from salamander outer segments. After UV illumination, a ^{32}P-labeled version of APT-cGMP was shown by SDS/PAGE and autoradiography to be covalently attached to the 63-kDa channel subunit. This incorporation was significantly reduced by 8-Br-cGMP but was not reduced by 5′-GMP. In patch-clamp experiments APT-cGMP was a potent activator of the channel; APT-cGMP typically opened half of the channels in a patch at a 10-fold lower concentration than cGMP. Exposure of membrane patches to UV light in the presence of APT-cGMP resulted in a persistent current observed in the absence of bath-applied nucleotide. This current increased with repeated exposure of the patch to both UV light and fresh APT-cGMP, approaching the maximum current originally evoked by saturating (500 μM) cGMP. At this point, addition of 500 μM cGMP caused a negligible increase in current. The persistent current had several other properties expected of current through cGMP-activated channels: it was outwardly rectifying; outward current was blocked >90% by 2 mM internal Mg^{2+}, whereas inward current was blocked much less efficiently; a low concentration of cGMP caused a larger increase in current atop a half-maximal persistent current than it did originally. We conclude that the persistent current was caused by the covalent tethering of cGMP moieties to channel binding sites, resulting in irreversible channel activation. APT-cGMP should prove useful for further studies of these and similar cGMP-binding sites and in the identification of unknown cGMP-binding proteins.**

The electrical response of retinal rods to light is generated by cGMP-activated cation channels in the surface membrane (1–3). In the dark, the cytoplasmic concentration of cGMP is relatively high, and the rod is held in a partially depolarized state by a steady influx of Na^+ and Ca^{2+}. A light-activated enzymatic cascade lowers the cGMP concentration, causing channels to close and the cell to hyperpolarize (for review, see refs. 4–6).

The cGMP-activated channel of bovine rod outer segments (ROS) has recently been purified (7), and its cDNA has been cloned and expressed in *Xenopus* oocytes (8). The purified channel subunit runs as a 63-kDa protein on SDS/polyacrylamide gels. The deduced primary structure of the channel contains an 80-amino acid region that exhibits sequence similarity to both of the tandem cGMP-binding domains of cGMP-dependent protein kinase. Although a site-specific mutagenesis study has shown that Thr-560 in this region of the channel influences binding-site specificity for cGMP over cAMP (9), little else is known about either the three-dimensional structure of the cGMP-binding site or the specific amino acids that interact with cGMP. Furthermore, although it is known that activation of the rod channel requires the binding of at least three cGMP molecules (10–12), the detailed mechanism of channel activation remains to be determined.

As a first step toward both identifying amino acid residues involved in cGMP binding and studying the multiple-site regulation of channel opening, we have developed a photoaffinity analog of cGMP, 8-*p*-azidophenacylthio-cGMP (APT-cGMP). In the presence of UV light, this analog specifically labeled the 63-kDa channel subunit in biochemical preparations and permanently activated channels in excised membrane patches.

MATERIALS AND METHODS

Materials. [^{32}P]cGMP (25 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. cGMP, 8-Br-cGMP, and 5′-GMP (all sodium salts) were purchased from Sigma. *p*-Azidophenacyl bromide was from ICN. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from either Sigma or Boehringer Mannheim.

Thin-Layer Chromatography Methods. Two TLC methods were used to monitor reaction progress and to characterize products. The first system was silica gel (Kieselgel 60 F254; 0.25 mm; EM Science) developed with 1-butanol/acetic acid/water, 5:3:2. Preparative TLC was performed on silica plates containing a 2-mm-thick layer, developed with the same solvent system. The second system was polyethyleneimine-cellulose (0.1 mm; Brinkmann) developed with 200 mM LiCl/50 mM triethanolamine, pH 7.9. Both matrices contained a fluorescent dye excited at 254 nm, and products were usually visualized by UV shadowing using short-wavelength UV excitation from a mineral light.

Synthesis and Purification of APT-[^{32}P]cGMP. Bromination of [^{32}P]cGMP (1.6 mCi) was done as described by Geahlen *et al.* (13). The 8-Br-[^{32}P]cGMP was purified by anion-exchange HPLC developed with a 5–500 mM ammonium acetate gradient (pH 5 with acetic acid), and dried repeatedly in a Speed-Vac.

The purified 8-Br-[^{32}P]cGMP was dissolved in 200 μl of redistilled dimethyl sulfoxide and treated with 24 mg of thiourea at 115°C for 20 hr in a screw-cap Eppendorf tube on a Temp-Block. Polyethyleneimine-cellulose TLC and auto-

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Abbreviations: APT-cGMP, 8-*p*-azidophenacylthio-cGMP; ROS, rod outer segments.

radiography showed that the reaction was virtually complete, forming an isothiuronium salt adduct of [^{32}P]cGMP at the C8 position. This intermediate product was decomposed to 8-thio-[^{32}P]cGMP by the addition of 800 μl of methanol and 20 mg of sodium methoxide. All subsequent steps were done under reduced room light. Alkylation of this product with 124 mg of *p*-azidophenacyl bromide at room temperature for several hours produced APT-[^{32}P]cGMP. Complete conversion of 8-thio-[^{32}P]cGMP to APT-[^{32}P]cGMP was verified by silica TLC and autoradiography. The product was then purified by preparative silica TLC. The appropriate region of silica was identified by autoradiography, scraped from the plate, and extracted twice with 60 ml of methanol. The APT-[^{32}P]cGMP was repeatedly dried in a Speed-Vac and dissolved in 500 mM Tris·HCl, pH 7.6. Specific activity of the product was adjusted by the addition of unlabeled APT-cGMP.

Synthesis of Unlabeled APT-cGMP. Fifty milligrams of 8-Br-cGMP and 90 mg of thiourea were dissolved in 800 μl of dimethyl sulfoxide and heated as above. The reaction mixture was then diluted with 9 ml of methanol, and 65 mg of sodium methoxide was added. Twenty-five milligrams of crude 8-thio-cGMP was alkylated with 140 mg of *p*-azidophenacyl bromide as above. The mixture was dried in a Speed-Vac, redissolved in 2 ml of methanol, and purified by preparative TLC (band visualized by UV shadowing at plate edges). For chemical analysis and patch-clamp experiments, APT-cGMP was further purified by reversed-phase HPLC (C_{18} column), developed with a 0–40% gradient of methanol in water (5 mM ammonium acetate, pH 5.0 with acetic acid, throughout).

Purification of the cGMP-Activated Channel of Bovine Retinal Rods. Frozen bovine retinas were purchased from J. A. and W. L. Lawson (Lincoln, NE). ROS were purified by the procedure of Fung and Stryer (14), followed by centrifugation in a 28–38% sucrose gradient (15). The cGMP-activated channel was partially purified for photoaffinity labeling by the procedure of Cook *et al.* (7) through the DEAE-Fractogel TSK chromatography step. Retention of channel activity was verified by functional reconstitution (7).

Photoaffinity Labeling of the cGMP-Activated Channel with APT-[^{32}P]cGMP. CHAPS-solubilized and partially purified cGMP-activated channels from bovine retinal rods were photoaffinity labeled with APT-[^{32}P]cGMP in the presence or absence of competing 8-Br-cGMP. Labeling was done in DEAE-Fractogel TSK elution buffer (7). Two and seven-tenths milliliters of DEAE-Fractogel TSK-purified channel, derived from ROS containing 42 mg of rhodopsin, was divided into two 1.35-ml aliquots. 8-Br-cGMP was added to one sample to a final concentration of 100 μM. Sixty-five microliters of APT-[^{32}P]cGMP (2.5 Ci/mmol) was then added under dim light to both samples to a final concentration of 2.5 μM. Samples were transferred into 1.6-ml quartz cuvettes and were photolyzed for 30 min by using a 200-W mercury lamp (Leitz) screened with a 360-nm bandpass filter (UG 1; Schott). This filter was chosen to allow photolysis of APT-cGMP while avoiding wavelengths of light absorbed by aromatic amino acid residues. One hundred and twenty-microliter aliquots from each sample were precipitated with 10% trichloroacetic acid and spun in a microcentrifuge. The pellets were redissolved in gel solubilization buffer [10% SDS/7 M urea/195 mM Tris·HCl, pH 8.8/20% (wt/vol) glycerol/20 mM dithiothreitol/5% (vol/vol) 2-mercaptoethanol/0.01% bromophenol blue] and electrophoresed on a SDS/9% polyacrylamide gel (16). Proteins were visualized by staining the gel with Coomassie brilliant blue R-250. Gels were then dried and exposed to Kodak XAR film for 6–12 hr at −80°C with an intensifying screen.

Electroelution of the Channel Protein and Cleavage with Cyanogen Bromide. The ^{32}P-labeled protein band at 63 kDa was further confirmed as the cGMP-activated channel by sequence analysis of cyanogen bromide (CNBr) fragments. After separation by SDS/9% PAGE, the 63-kDa band was identified by copper staining (17) and excised from the gel. The protein was electroeluted at 100 V for 4 hr in 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), pH 11/0.1% SDS, with a Centrilutor apparatus (Amicon). The protein was concentrated to 50 μl and added to 500 μl of 0.25 M CNBr in 70% formic acid; this digestion mixture was incubated in the dark for 24 hr at room temperature. After lyophilization, the mixture of peptides was separated by *N*-tris(hydroxymethyl)methylglycine (Tricine)/SDS/16% PAGE by the procedure of Schägger and von Jagow (18), and electroblotted in 20 mM CAPS, pH 11, and 20% methanol onto poly(vinylidene difluoride) membrane (Bio-Rad). A prominent, ^{32}P-labeled band identified by autoradiography was excised from the blot and subjected to sequence analysis.

Patch-Clamp. Electrophysiology experiments were done at 22°C on excised inside-out membrane patches from ROS of the larval tiger salamander, *Ambystoma tigrinum*, using described methods (19). cGMP- and APT-cGMP-induced currents were measured in a control solution containing 130 mM NaCl/0.02 mM EDTA/2 mM Hepes, pH 7.6, in the patch pipette and experimental chamber. For permanent activation studies, patches bathed in control solution with 10 μM APT-cGMP were illuminated through the open front of the experimental chamber (≈100-μl vol) from a distance of 0.6 m with the light source described above. During photolysis the chamber was perfused with fresh 10 μM APT-cGMP at 2-min intervals to replenish the supply of unphotolyzed APT-cGMP. After each period of illumination, the patch was washed extensively with cGMP-free control solution. Currents that remained were compared with currents induced by saturating cGMP.

RESULTS

Structural, Chromatographic, and Spectral Characteristics of APT-cGMP. The structure of APT-cGMP is shown in Fig. 1. The molecule was identified by fast-atom-bombardment MS as a negative ion with the expected *m/e* ratio of 535. APT-cGMP eluted with a R_f of 0.46 on our silica TLC system, compared with 0.36 for 8-Br-cGMP. Fig. 2 shows that the absorbance profile in 10 mM Hepes, pH 7.4, exhibited a broad peak with a maximum at 284 nm. This absorbance was sensitive to UV irradiation, which caused a 33% reduction in the peak absorbance with a half-time of photolysis of 3 min under the conditions used. The extinction coefficient was estimated to be 3.2×10^4 $M^{-1} \cdot cm^{-1}$ from the sum of the spectra of its two unconjugated component chromophores, 8-methylthio-cGMP (λ_{max} 273, $\varepsilon = 1.77 \times 10^4$ $M^{-1} \cdot cm^{-1}$; ref. 20) and azidoacetophenone (λ_{max} 290, $\varepsilon = 2.08 \times 10^4$ $M^{-1} \cdot cm^{-1}$; ref. 21). This extinction coefficient was used in all subsequent experiments to estimate the concentration of APT-cGMP.

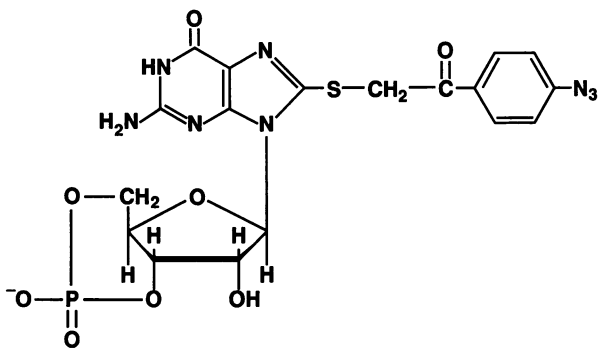

FIG. 1. Structure of APT-cGMP.

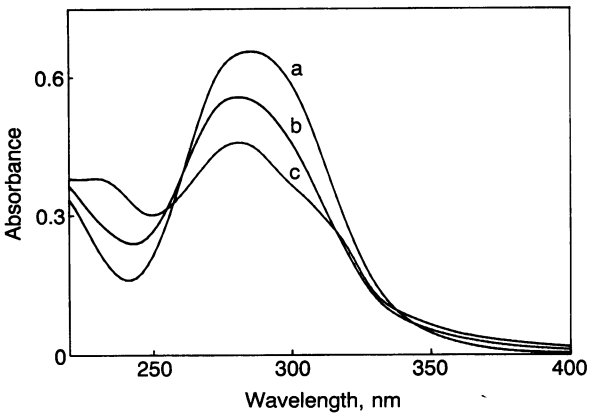

FIG. 2. Absorption spectra of 20 μM APT-cGMP before photolysis (trace a) and after partial (trace b) and complete photolysis (trace c). Peak absorbance at 284 nm progressively decreased with UV illumination centered at 360 nm (trace b; 3 min) until reaching a limiting value at 67% of the original absorbance (trace c; 30 min). The spectra were recorded on a diode-array spectrophotometer; the path length was 1 cm.

Specific Photoaffinity Labeling of the cGMP-Activated Channel. As shown in Fig. 3, a 63-kDa protein was photoaffinity labeled by APT-[^{32}P]cGMP in a way that was blocked significantly by a 40-fold excess of unlabeled 8-Br-cGMP. This protein was identified as the cGMP-activated channel subunit by comparison of partial amino acid sequences of CNBr fragments (see *Materials and Methods*) with the sequences predicted from the cloned cDNA (8). Most ^{32}P label in the CNBr digest coincided with a protein band of 8 kDa (data not shown). This labeling was nearly abolished when excess 8-Br-cGMP was present during photolysis, as was observed for the intact subunit. When this band was analyzed for sequence, two prominent fragments generated by cleavage at Met-213 and Met-514 were identified from 10 sequencing cycles. The precise location of the ^{32}P label within one or both of these fragments remains to be determined.

When gel slices containing the ^{32}P-labeled cGMP-activated channel subunit were excised and counted for radioactivity by liquid scintillation, the sample labeled in the absence of 8-Br-cGMP consistently contained 2.5- to 3-fold more counts than the sample labeled in its presence. In the experiment of Fig. 3, we estimate that 0.3–1% of the channel subunits were labeled, based on the specific activity of APT-[^{32}P]cGMP and the amount of cGMP-activated channel in the gel band (2 ± 1 μg from a comparison with the standards). UV illumination was required for labeling; identical samples incubated in the absence of UV illumination did not covalently incorporate the ^{32}P label. 5′-GMP (100 μM) did not affect labeling of the channel protein, indicating that protection by 8-Br-cGMP was specific to this nucleotide and not an artifact due to increased UV-absorbing material in the sample (data not shown). The results suggest that some channel labeling was nonspecific; in other experiments a 600-fold excess of 8-Br-cGMP was included during photolysis, but this led to little further reduction of ^{32}P incorporation. Dithiothreitol (1 mM), which would be expected to scavenge long-lived (but reactive) photolysis intermediates, was always present in the photolysis mixture; 5 mM glutathione, also a known scavenger, had little or no effect on the nonspecific labeling. Shorter illumination times (10 min) did not seem to reduce nonspecific labeling. The amount of nonspecific labeling, however, should not interfere with the identification of specifically labeled peptides or amino acid residues.

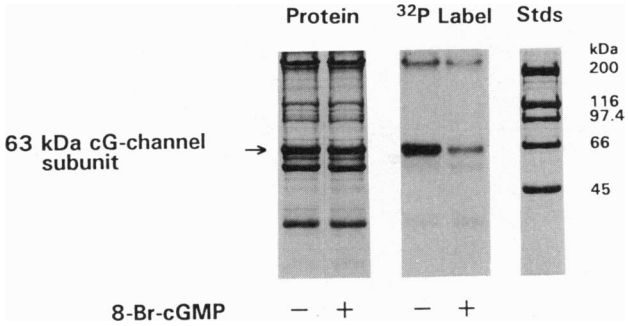

FIG. 3. Specific photoaffinity labeling with APT-[^{32}P]cGMP of the cGMP-activated channel in detergent micelles. The Protein lanes are from a Coomassie-stained SDS/9% polyacrylamide gel of the partially purified cGMP-channel preparation after photoaffinity labeling with APT-[^{32}P]cGMP; the ^{32}P Label lanes are from an autoradiogram taken of the same gel after a 6-hr exposure. 8-Br-cGMP (100 μM) was present or absent from the labeling mixtures as indicated. Molecular-size standards (Stds) myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin (2 μg each) are shown at right for comparison.

A protein band of ≈240 kDa also consistently exhibited labeling that was blocked by 100 μM 8-Br-cGMP but was not blocked by 100 μM 5′-GMP. Other prominent protein bands at 39 kDa and 55 kDa were faintly labeled, but this labeling was unaffected by the addition of either 8-Br-cGMP or 5′-GMP. Similar labeling patterns were obtained with six other channel preparations and two different APT-[^{32}P]-cGMP batches.

We used a partially purified channel preparation rather than ROS membranes to demonstrate the usefulness of APT-[^{32}P]cGMP for two reasons: (*i*) at reasonable membrane concentrations, bleached rhodopsin would absorb most of the light needed to photolyze APT-cGMP; and (*ii*) because of its abundance, rhodopsin would severely limit the amount of other proteins that could be loaded on SDS/polyacrylamide gels. The potential value of APT-cGMP for identifying unknown cGMP-binding proteins in complex mixtures is still illustrated by the observation that there were at least 30 Coomassie-stained proteins in the gel of Fig. 3; yet only two of these were specifically labeled by APT-[^{32}P]cGMP.

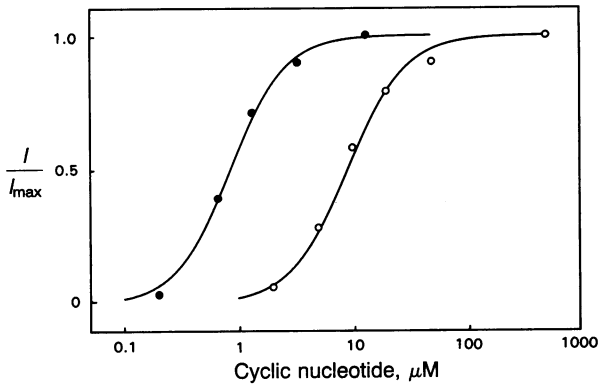

FIG. 4. Dose–response relations for APT-cGMP (●) and cGMP (○). Steady-state cyclic nucleotide-activated currents were measured at +50 mV. Plotted is the fraction of the saturating current (I_{max}) versus cyclic nucleotide concentration on a logarithmic scale. The smooth curves are fits to the Hill equation: $I/I_{max} = [G]^n/(K_{1/2}^n + [G]^n)$, where $[G]$ is the cyclic nucleotide concentration. The APT-cGMP data were fit with $K_{1/2} = 0.86$ μM and $n = 1.85$; cGMP data were fit with $K_{1/2} = 9.1$ μM and $n = 1.7$. The cGMP relation is taken from a patch having a similar sensitivity to cGMP as the patch on which APT-cGMP was tested. Response of the latter patch to 5 μM cGMP was 0.234 I_{max}, and the response of the former was 0.286 I_{max}. I_{max} was 1307 pA for the APT-cGMP patch and 1600 pA for the cGMP patch. When tested on the same patch, cGMP and APT-cGMP gave nearly identical I_{max} values. The values of n used to fit the data are lower than the value of 3 required to fit very small currents (10, 11).

APT-cGMP Is a Potent Activator of cGMP-Activated Channels in Excised Membrane Patches. The dose–response relation for APT-cGMP is compared with that for cGMP in Fig. 4. The smooth curves are fits to the Hill equation (see legend). The concentrations of APT-cGMP that gave a half-maximal current ($K_{1/2}$) was 0.86 μM. The dose–response relation shown for cGMP was taken from a patch with a similar sensitivity to cGMP as the patch on which APT-cGMP was measured ($K_{1/2}$ = 9.1 μM). Although patch sensitivities to cGMP varied (11, 22), the $K_{1/2}$ for APT-cGMP was consistently 10-fold lower than the $K_{1/2}$ for cGMP on three other patches. Several other C_8-substituted cGMP analogs have been shown to be more effective than cGMP itself (23, 24).

Permanent Activation of cGMP-Activated Channels in the Presence of APT-cGMP and UV Light. When an excised membrane patch was exposed to UV light in the presence of 10 μM APT-cGMP (freshly applied every 2 min), there was a time-dependent increase in a current that remained even after extended bath perfusion with a cGMP-free solution. Both UV light and APT-cGMP were required for this effect; intense UV light centered at 351 nm was previously shown to have no effect on patch currents (12). As shown in Fig. 5, the persistent current was 40% of the total patch current after 8 min of exposure, 77% after 16 min, and 98% after 29 min, where the total current is defined as the sum of persistent current (which includes leak current through the seal resistance) and any additional current induced by saturating cGMP at +50 mV. The persistent and total currents were nearly identical after 29 min of exposure, suggesting that most of the persistent current results from permanent channel activation caused by covalent attachment of cGMP moieties to the binding sites of the channel. Four additional lines of evidence support this conclusion. (*i*) Persistent outward currents were largely abolished by including 2 mM $MgCl_2$ in the internal bath solution (91% reduction at +50 mV; Fig. 5, at 29-min exposure). Although millimolar concentrations of divalent cations can improve the seal resistance slightly (unpublished observations), this effect does not account for the dramatic reduction in current. Na^+ current through fully-liganded cGMP-activated channels is known to be blocked by internal Mg^{2+} with an apparent K_d of 120–220 μM at +30 mV (19, 25, 26). If the leak current remained stable near its initial level (20 pA), a 90–94% reduction in the remaining persistent current would leave a current of 65–89 pA, consistent with the measured value of 72 pA. The effect of 2 mM Mg^{2+} on a total patch current that was 40% persistent and 60% cGMP-induced was very similar (Fig. 5, after 8-min exposure). (*ii*) As shown in Fig. 6, 2 mM internal Mg^{2+} blocked inward current much less strongly than outward current (43% reduction at −40 mV). This result is consistent with previous reports of the apparent K_d for block by internal Mg^{2+} at −30 mV (2.1 mM; refs. 25 and 26). (*iii*) As shown in Fig. 6, the persistent current exhibited a marked outward rectification similar to that previously reported for the cGMP-activated channel at saturating cGMP. The magnitude of the current at +100 mV was 1.97 times that at −100 mV, nearly identical to values reported for the channel in the presence of saturating cGMP (25, 27). (*iv*) The patch was shown to have enhanced sensitivity to low concentrations of bath-applied cGMP when the persistent current was 40% of the total. Two micromolar cGMP applied to the patch of Figs. 5 and 6 after 8 min of exposure to APT-cGMP and UV light caused an increase

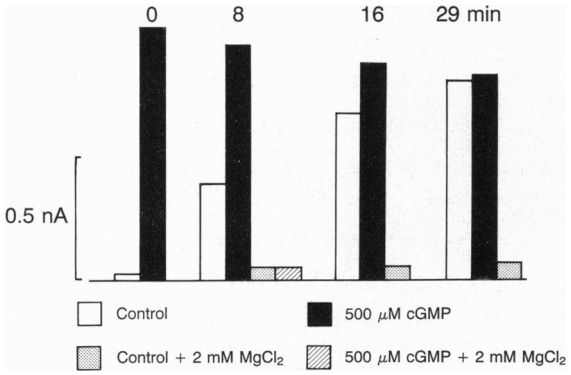

FIG. 5. Evidence that cGMP-activated channels in an excised membrane patch are permanently activated by APT-cGMP in the presence of UV light. The initial leak current in control solution and the current induced by 500 μM cGMP are shown before exposure of the patch to APT-cGMP and UV light (0 min). Steady-state currents were measured at +50 mV. The patch was then exposed to 10 μM APT-cGMP in the presence of UV illumination. After the total exposure times shown at top, currents were measured in the indicated solutions. Data were averaged from eight to ten trials. The original seal resistance was 2.5 GΩ, and currents were not corrected for small series-resistance errors.

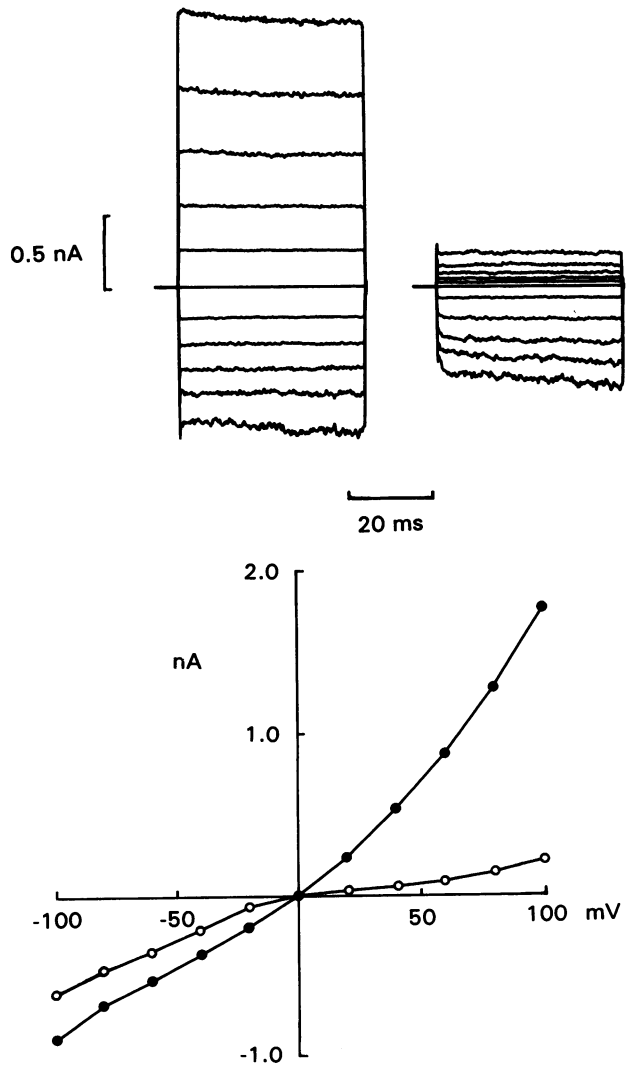

FIG. 6. (*Upper*) Persistent currents at different membrane potentials in the absence (*Left*) and presence (*Right*) of 2 mM Mg^{2+} in control solution. Data are from the patch of Fig. 5 after 29 min of exposure to APT-cGMP and UV light. Currents were evoked by 45-ms pulses from a holding potential of 0 mV to potentials ranging from −100 mV to +100 mV in 20-mV steps. (*Lower*) Plot of the steady-state currents from above as a function of membrane potential in the absence (●) and presence (○) of Mg^{2+}. Currents were averaged from three trials and were not corrected for leak or small series-resistance errors. Traces were filtered at 2 kHz and sampled at 10 kHz.

of 278 pA at +50 mV. Before exposure, 10 μM cGMP induced a 662-pA current, whereas the current at saturating cGMP (500 μM) was 990 pA (both currents corrected for leak). For a typical patch with this sensitivity to cGMP, a 75- to 115-pA response to 2 μM cGMP is predicted by the Hill equation. The observed increase of 278 pA is more consistent with the current increase expected for a 2-μM cGMP increment in a steeper region of the dose–response relation, arguing that the persistent current arose from fully liganded channels and that a large fraction of the remaining closed channels were already partially liganded.

Similar results were obtained on another patch in which the persistent current reached 66% of the total current before the patch broke. On this patch there was no time-dependent decline in the total current.

DISCUSSION

Comparison with Previous Photoaffinity Labeling Results. The results presented here indicate a striking labeling of the cGMP-activated channel by APT-[^{32}P]cGMP. In contrast, Thompson and Khorana (28) reported no photoaffinity labeling of the channel in bovine rods using 8-azido-[^{32}P]cGMP, whereas the cGMP-specific phosphodiesterase and a cGMP-dependent protein kinase were identified. Although 8-azido-cGMP has been shown to activate the bovine channel (24), the photolyzed azide may not be in an appropriate orientation to attach to the protein covalently. In this regard the longer and somewhat flexible linker arm on APT-cGMP may afford it a greater selection of targets after photolysis. Because unbound nucleotide was removed before photolysis in the previous study, the absence of channel labeling might also be explained by the experimental conditions that favored labeling of proteins with very high affinities and/or slow binding off rates. Shinozawa *et al.* (29) have reported the identification of a 66-kDa cGMP-binding membrane protein from frog rods by direct photoaffinity labeling with [^{3}H]cGMP. It is not entirely clear that this protein was the cGMP-activated channel, and the autoradiogram exposure time required to visualize this band indicated that incorporation of the nucleotide was very inefficient. Furthermore, direct photoaffinity labeling, which would be expected to perturb the core structure of the nucleotide, has not been reported to cause permanent activation of the channel.

Specific Labeling of a 240-kDa Protein. The result shown in Fig. 3 suggests that a high-molecular-mass protein (≈240 kDa) was also labeled by APT-cGMP. Although the difference in the amount of label incorporated with and without 8-Br-cGMP was not as pronounced as the difference seen with the channel, some specific labeling was consistently seen in seven preparations. 5′-GMP was ineffective as a competing nucleotide. This protein may be the retinal rod version of the cytoskeletal protein spectrin, which has been shown to copurify with the cGMP-activated channel in this preparation (30). It is not clear whether this protein is specifically labeled because it has a cyclic nucleotide-binding site or because it is in very close proximity to the binding sites of the channel. It should be noted that Shinozawa *et al.* (31) identified a 250-kDa protein by direct photoaffinity labeling with [^{3}H]cGMP.

APT-cGMP, a Structural and Functional Probe for cGMP-Binding Proteins. Because APT-cGMP not only specifically labels the channel from retinal rods but also causes permanent activation, we plan to exploit this analog both to identify amino acids in the cGMP-binding site and to investigate the allosteric mechanism of channel opening. Channels directly activated by cyclic nucleotides have also been identified in retinal cones (32), olfactory cilia (33), cochlear hair cells (34), pinealocytes (35), and renal epithelial cells (36). APT-cGMP promises to be useful as a probe both to study known cGMP-binding proteins and to identify new ones.

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