A Derivative of Amiloride Blocks Both the Light-regulated and Cyclic GMP–regulated Conductances in Rod Photoreceptors

G. D. NICOL, P. P. M. SCHNETKAMP, Y. SAIMI, E. J. CRAGOE, Jr., and M. D. BOWNDS

From the Laboratory of Molecular Biology and the Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706; the Department of Medical Biochemistry, Health Sciences Centre, University of Calgary, Calgary, Alberta T2N 4N1, Canada; and the Department of Medicinal Chemistry, Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

ABSTRACT Vertebrate rod photoreceptors in the dark maintain an inward current across the outer segment membrane. The photoresponse results from a light-induced suppression of this dark current. The light-regulated current is not sensitive to either tetrodotoxin or amiloride, potent blockers of Na^+ channels. Here, we report that a derivative of amiloride, $3',4'$-dichlorobenzamil (DCPA), completely suppresses the dark current and light response recorded from rod photoreceptors. DCPA also blocks a cyclic GMP–activated current in excised patches of rod plasma membrane and a cGMP-induced Ca^{++} flux from rod disk membranes. These results are consistent with the notion that the Ca^{++} flux mechanism in the disk membrane and the light-regulated conductance in the plasma membrane are identical. DCPA also inhibits the Na/Ca exchange mechanism in intact rods, but at a 5–10-fold-higher concentration than is required to block the cGMP-activated flux and current. The blocking action of DCPA in 10 nM Ca^{++} is different from that in 1 mM Ca^{++}, which suggests either that the conductance state of the light-regulated channel may be modified in high and low concentrations of Ca^{++}, or that there may be two ionic channels in the rod outer segment membrane.

INTRODUCTION

The ionic channels that conduct the light-regulated current in rod photoreceptors have several unique features (see the recent review by Attwell, 1986). The single channel conductance is small, between 0.1 and 0.5 pS (Gray and Attwell, 1985; Fesenko et al., 1985; Bodoia and Detwiler, 1985), but the channel seems to be relatively nonselective. The permeability ratio for Na^+ and K^+ is ~1:0.6 (Yau and Nakatani, 1984; Hodgkin et al., 1985). The mean open times are brief, ~2 ms, as measured in the intact rod (Gray and Attwell, 1985). The probability

of the channel being open in the dark is low and has been estimated to be only between 1 and 5%.

The pharmacology of the channel is not well understood. The light-regulated current is not sensitive to either the voltage-dependent Na^+ channel blocker tetrodotoxin or the epithelial Na^+ channel blocker amiloride (Fain and Lisman, 1981). Recently, an inhibitor of the light-regulated conductance has been identified. Kaupp and his colleagues have found that l-cis-diltiazem blocks a cyclic GMP–activated conductance in excised patches of rod membrane (Stern et al., 1986), as well as a cGMP-induced Ca^{++} release from rod disk membranes (Koch and Kaupp, 1985). However, when applied externally to the intact rod, l-cis-diltiazem only suppresses the light-regulated current by threefold (Stern et al., 1986).

Here we describe the effects of an amiloride derivative that effectively inhibits the light-regulated current in frog rod photoreceptors. The properties of this new inhibitor are different from those of diltiazem. The derivative, 3',4'-dichlorobenzamil (DCPA), has been shown to be an effective inhibitor of the Na/Ca exchange mechanism in other cellular systems (Kaczorowski et al., 1985). We initially employed DCPA to inhibit the Na/Ca exchange mechanism in rod photoreceptors, but found that DCPA was a much better inhibitor of the cGMP-induced Ca^{++} release from rod disk membranes, the cGMP-activated conductance in excised membrane patches, and the light-regulated current in the intact rod. These results are consistent with the notion that the Ca^{++}-release mechanism, the cGMP-activated conductance, and the light-regulated conductance observed in the intact rod are similar. A portion of this work has been presented in abstract form (Nicol et al., 1987b).

MATERIALS AND METHODS

Preparation of Frog Rod Photoreceptors

Rod photoreceptors were prepared according to the method of Biernbaum and Bownds (1985) with slight modifications. Isolated dark-adapted retinas from the bullfrog, Rana catesbeiana, were gently shaken in 0.6 ml of Ringer's solution that contained (millimolar): 105 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 $NaHCO_3$, 5 glucose, 10 HEPES, and 5% iso-osmotic Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), pH 7.6, which had been oxygenated before use. Retinas were then shredded into fine particles and gently vortexed. The rod suspensions were layered onto a linear Percoll density gradient (30–70% Percoll Ringer's). After centrifugation at 10,000 rpm for 1 min, there were three bands in the gradient. A top band contained retinal debris with little rhodopsin, a middle band contained rod outer segments (ROS) whose plasma membrane was osmotically leaky and stained with didansyl cysteine (Yoshikami et al., 1974), and a lower band contained osmotically intact ROS and intact ROS that retained the mitochondria-rich ellipsoid portion of their inner segments (OS-IS).

Ca^{++} Flux Measurements in Frog ROS and OS-IS

The release of Ca^{++} from ROS and OS-IS suspensions was determined spectroscopically with the Ca^{++}-indicating dye arsenazo III. For details of the spectroscopic assay for Ca^{++} fluxes in frog and bovine rods, see Koch and Kaupp (1985), Schnetkamp (1986), and Schnetkamp and Bownds (1987). ROS and OS-IS bands were removed from the gradient

(~3 ml) and each was diluted with 2 vol of 125 mM tetramethylammonium-chloride (TMA-Cl) and 20 mM HEPES, adjusted to pH 7.4 with arginine. The suspension was gently pelleted (5,000 rpm for 10 s) and resuspended in ~0.85 ml of 125 mM TMA-Cl, 2.5 mM KCl, and 30 mM HEPES, adjusted to pH 7.4 with arginine.

For experiments examining the Na/Ca exchange mechanism, an aliquot (typically 200 μl) from an intact OS-IS suspension was placed in a cuvette containing a medium of 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, 100 μM arsenazo III, and 1 μM FCCP, adjusted to pH 7.4 with arginine. The Na^+-dependent Ca^{++} release was initiated by the addition of 50 mM NaCl to the cuvette. A spectrophotometer (Aminco DW2C, SLM Instruments, Inc., Urbana, IL) was used to measure arsenazo III absorbance changes and was used in the dual-wavelength mode so that [ΔA(650 nm) $-$ ΔA(750 nm)] was recorded. The arsenazo III absorbance change was calibrated with known additions of Ca^{++} to the cuvette containing rod membranes. The rhodopsin concentration was determined by dark-light difference spectroscopy.

For experiments examining the cGMP-induced Ca^{++} release, an aliquot from a suspension of ROS with plasma membranes leaky to small solutes was placed in a cuvette containing a medium of 127.5 mM KCl, 30 mM HEPES, 100 μM arsenazo III, and 1 μM FCCP, adjusted to pH 7.4 with arginine. The release of Ca^{++} was initiated by the addition of 250 μM cGMP to the cuvette. The arsenazo III absorbance changes were recorded as described above.

Ca^{++} Flux Measurements in Bovine ROS

Intact bovine ROS were isolated according to methods previously described by Schnetkamp (1986). The Ca^{++}-indicating dye arsenazo III was used to measure the Na^+-stimulated Ca^{++} release under conditions that optimize the rate of Na/Ca exchange (see Schnetkamp, 1986, for details). The Ca^{++} release was initiated with the addition of NaCl to the cuvette. NaCl was used from concentrated stocks: the 50 mM NaCl was from a 2.5 M stock and the 12 mM NaCl was from a 0.6 M NaCl plus 1.9 M TMA-Cl stock. It is important to keep constant the surface potential, which depends on ionic strength—hence the mixture of NaCl and TMA-Cl.

To examine the cGMP-induced Ca^{++} release, a suspension of intact bovine ROS was frozen and thawed. The ROS were then hypotonically shocked by dilution in 20 vol of 10 mM HEPES adjusted to pH 7.4 with arginine. After 3 min, TMA-Cl was added to a final concentration of 25 mM. The ROS disks were then sedimented (10,000 rpm for 20 s) and resuspended in 300 mM sucrose, 2.5% Ficoll-400, 0.5 mM dithioerythritol, and 20 mM HEPES, adjusted to pH 7.4 with arginine.

The binding of DCPA to bovine ROS membranes was determined in the following manner. DCPA has an absorbance maximum at ~380 nm, so its concentration can be measured spectroscopically. Bovine ROS were incubated with a known concentration of DCPA. The ROS membranes were sedimented rapidly (12,000 rpm for 30 s), and the supernatant was removed and diluted with a TMA-Cl medium containing 1% Triton X-100 (to eliminate any light-scattering by the ROS). The absorbance of the supernatant was then measured to determine the quantity of DCPA remaining in the supernatant; the difference was the amount of DCPA bound to the ROS membranes.

Electrophysiological Measurements

Experiments involving excised membrane patches. Intact OS-IS were isolated and purified as described above; cells were used directly from the gradient without further resuspension. A high-resistance seal (typically 5–8 GΩ) was obtained from OS-IS suspended in an intracellular medium containing (millimolar): 10 NaCl, 90 KCl, 0.1 $CaCl_2$, 0.11

EGTA, 0.1 μM free Ca^{++}, 10 $MgCl_2$, 30 sucrose, 5 glucose, and 10 HEPES, adjusted to pH 7.6 with KOH. The high-Mg^{++} solution was washed away with intracellular medium lacking Mg^{++}. The membrane patch was then excised and superfused with the intracellular medium containing the test drugs. Membrane current was measured with a patch-clamp amplifier (model 8900, Dagan Corp., Minneapolis, MN) and recorded on a chart recorder (Gould Inc., Cleveland, OH). The membrane patch was held at 0 mV, and the voltage was stepped to −50 mV, where a ramp was begun that stepped the voltage to +50 mV over a time of 2 s (see inset to Fig. 4B for the ramp). The voltage was controlled with a ramp generator (model 26G1, Tektronix, Inc., Beaverton, OR). The patch pipette was filled with a solution that contained (millimolar): 110 NaCl, 2 KCl, 1 $CaCl_2$, 5 glucose, and 10 HEPES, at pH 7.6.

Current recordings from intact OS-IS. The light-regulated current from intact OS-IS was recorded by using the suction electrode technique (Baylor et al., 1979). The recording technique and setup are described in detail elsewhere (Nicol et al., 1987a).

Solutions

DCPA and 5-(N-ethyl-N-isopropyl)amiloride (EIA) were synthesized as described previously (Cragoe et al., 1967). DCPA and EIA were dissolved in 1-methyl-2-pyrrolidinone; A23187 and FCCP were dissolved in ethanol. These compounds were used from concentrated stock solutions. The NaCl used to initiate Na/Ca exchange was spectroscopic grade. All solutions used in the spectroscopic assay were made with distilled water that was further purified with a NANOpure II filtration system (Barnstead Co., Newton, MA).

RESULTS

We initially sought to investigate the contributions of Na/Ca exchange to the normal physiology of rod photoreceptors by using a derivative of amiloride, DCPA, designed as an inhibitor of the Na/Ca exchange mechanism in other cellular systems (Kaczorowski et al., 1985).

DCPA Inhibition of Na/Ca Exchange

The Na/Ca exchange mechanism and its inhibition by DCPA, as shown in Fig. 1, were assayed by measuring Na^+-stimulated Ca^{++} release from a suspension of intact ROS and OS-IS, using the Ca^{++}-indicating dye arsenazo III. The Na^+-stimulated Ca^{++} release was reduced progressively with increasing concentrations of DCPA and had a half-maximal inhibition (apparent K_i) of ~50 μM. In each case, the total Ca^{++} content was determined by the addition of A23187; this calcium ionophore caused the release of >90% of the total intracellular Ca^{++}. In untreated OS-IS, this release (Na^+ and A23187) corresponded to 1.2 mol Ca^{++}/ mol rhodopsin.

DCPA Inhibition of a cGMP-induced Ca^{++} Release

DCPA proved to be a much more effective inhibitor of a cGMP-induced Ca^{++} efflux from frog rod disk membranes than expected. The action of cGMP on Ca^{++} flux has been described previously for bovine and frog rod disk membranes (Caretta and Cavaggioni, 1983; Koch and Kaupp, 1985; Schnetkamp and Bownds, 1987). Fig. 2 illustrates the effect of DCPA on a cGMP-induced Ca^{++} release from disks measured in a suspension of ROS with plasma membranes leaky to small solutes. DCPA blocked the cGMP-activated Ca^{++} release in a dose-

dependent manner and had an apparent K_i of ~10 μM. Subsequent addition of
A23187 caused the release of the remaining intradiskal Ca^{++}. The total Ca^{++}
present in the leaky ROS amounted to ~1.2 mol Ca^{++}/mol rhodopsin.

We investigated the selectivity of DCPA in inhibiting these Ca^{++}-release
processes by comparing its effects with those of EIA, an amiloride derivative
designed to inhibit Na/H exchange (Vigne et al., 1984; Zhuang et al., 1984).
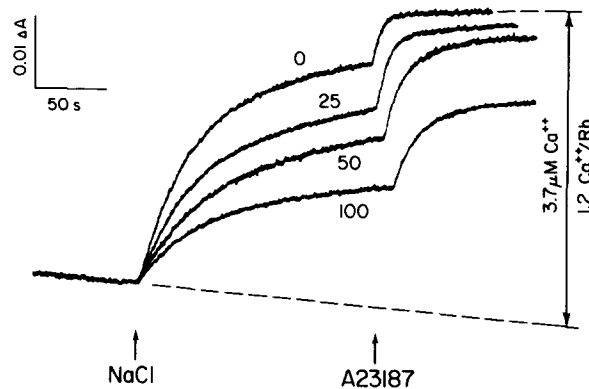This inhibitor is a 5-amino nitrogen-substituted analogue of amiloride and, unlike

FIGURE 1.   The concentration dependence for inhibition of the Na/Ca exchange rate by
DCPA. The Na^+-dependent Ca^{++} release was initiated by the addition of 50 mM NaCl to
the cuvette (at the first arrow), which contained the intact OS-IS suspension (3.2 μM
rhodopsin) in a medium consisting of 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES,
100 μM arsenazo III, and 1 μM FCCP, adjusted to pH 7.4 with arginine. DCPA was
added to the cuvette before Na^+ addition. The numbers next to the traces of Ca^{++} release
indicate the DCPA concentration. For this particular experiment, the Na^+-dependent
Ca^{++} release was 4.3×10^7 Ca^{++}/s·ROS; similar values were obtained in five other
experiments. The drift in absorption before addition of Na^+ varied somewhat between
different preparations; the decrease in absorption may reflect Ca^{++} uptake by reverse Na/
Ca exchange (cf. Schnetkamp, 1986). After most of the Ca^{++} was released by Na^+, the
total Ca^{++} content of the OS-IS suspension was determined by adding 2 μM A23187 to
the cuvette (at the second arrow). For this experiment, the total Ca^{++} content was 1.2 mol
Ca^{++}/mol rhodopsin. At the higher concentrations of DCPA (50 and 100 μM), the total
Ca^{++} content of the OS-IS suspension was lower than for the untreated condition. (OS-IS
isolated and purified in normal Percoll Ringer's plus 100 μM DCPA contained ~30–50%
as much Ca^{++} as untreated OS-IS [data not shown]. The smaller Ca^{++} content does not
result entirely from a DCPA-induced lowering of arsenazo III sensitivity to Ca^{++}, because
the sensitivity to known amounts of Ca^{++} in the absence of ROS membranes plus 100 μM
DCPA was decreased by only 10%.)

DCPA, it is structurally identical to amiloride at the guanidino group (see
Kaczorowski et al., 1985). EIA slightly inhibited the Na^+-stimulated Ca^{++} release
by 15 and 24% at concentrations of 50 and 100 μM, respectively (data not
shown). The cGMP-activated Ca^{++} efflux was reduced by 12 and 19% at 10 and
20 μM EIA, respectively (data not shown). In comparison, 100 μM DCPA
inhibited the Na/Ca exchange by ~66%, and 20 μM DCPA completely blocked
the cGMP-induced Ca^{++} release.

DCPA Has Similar Inhibitory Effects in Bovine ROS

The ability of DCPA to inhibit the Na/Ca exchange mechanism was also examined in intact bovine ROS and is shown in Fig. 3, *A* and *B*. The effects of DCPA on bovine ROS were similar to those on frog intact ROS and OS-IS. The concentration dependence for the inhibition of the Na/Ca exchange rate by
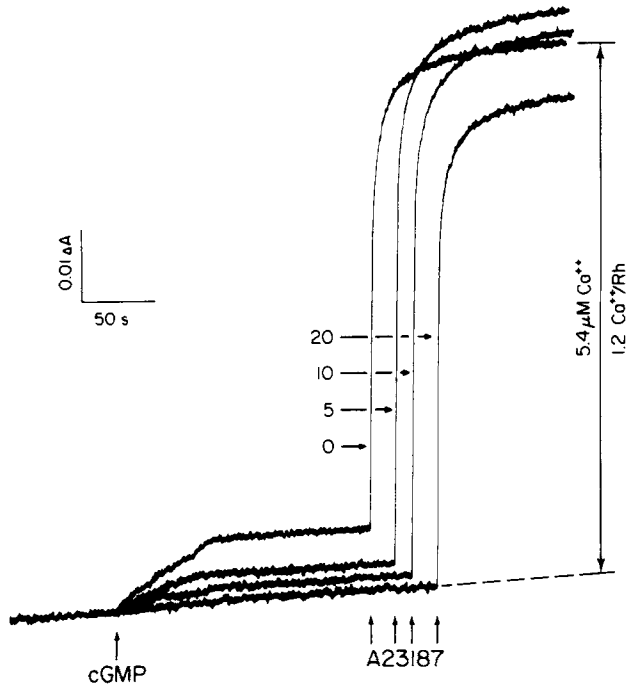

FIGURE 2.   The concentration dependence for inhibition of the cGMP-induced Ca^{++} release by DCPA. The release of Ca^{++} was initiated by the addition of 250 μM cGMP to the cuvette, which contained ROS with plasma membranes leaky to small solutes (4.6 μM rhodopsin) suspended in a medium consisting of 127.5 mM KCl, 30 mM HEPES, 100 μM arsenazo III, and 1 μM FCCP, adjusted to pH 7.4 with arginine. DCPA was added to the cuvette before the addition of cGMP. The numbers next to the traces indicate the DCPA concentration. For this particular experiment, cGMP released 6.7 × 10⁶ Ca^{++}/s·ROS; similar values were obtained in four other experiments. The slight drift in absorption before the addition of cGMP reflects a slow leakage of Ca^{++} across the disk membranes, in contrast to the slow uptake by the intact ROS and OS-IS in Fig. 1. We never observed an apparent uptake of Ca^{++} by ROS disks. The total Ca^{++} content of the ROS was determined by the addition of 2 μM A23187 to the cuvette. For this experiment, the total Ca^{++} content was 1.2 mol Ca^{++}/mol rhodopsin.

DCPA is presented in Table I. The effects of DCPA did not depend on the Na^+ concentration, which suggests that the inhibition was not competitive. These values yield an apparent K_d of ~50 μM, in agreement with the apparent K_i for frog Na/Ca exchange. It was determined that ~66% of the DCPA in the intact bovine ROS was bound to the ROS membranes. When the apparent K_d is corrected for the amount bound, a value of 17 μM is obtained. These effects of
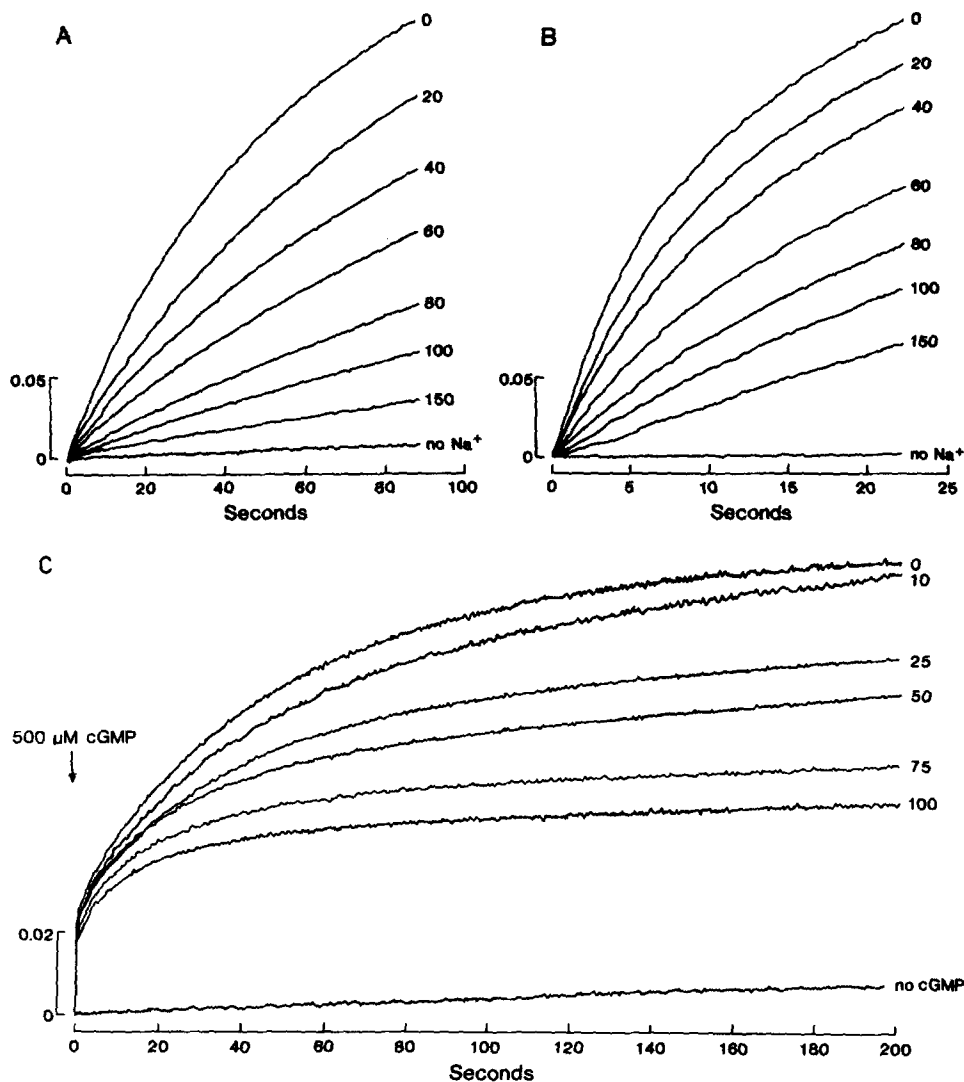
FIGURE 3.   DCPA inhibition of Ca^{++} flux in bovine ROS. The concentration dependence for inhibition of the Na/Ca exchange rate by DCPA in intact bovine ROS is shown for two different Na$^+$ concentrations. Intact bovine ROS were suspended in a medium that consisted of 600 mM sucrose, 2 mM KCl, 200 μM arsenazo III, 1 μM FCCP, 1 μM valinomycin, 4.9 μM rhodopsin, and 20 mM HEPES, adjusted to pH 7.4 with arginine, at 25°C. The Ca^{++} release was initiated (at time zero) with the addition of either 12 (A) or 50 (B) mM NaCl to the cuvette. The concentrations of DCPA are given to the right of each trace in micromolar. The 0.05 absorbance unit is equivalent to 0.43 mol Ca^{++}/mol rhodopsin. In C, the concentration dependence for the inhibition of the cGMP-induced Ca^{++} release in intact bovine ROS by DCPA is shown. In the cuvette, the ROS disks were suspended in a medium that consisted of 150 mM KCl, 200 μM arsenazo III, 20 mM HEPES, adjusted to pH 7.4 with arginine, and 15 μM rhodopsin, at 5°C. The cGMP-induced Ca^{++} release was initiated (time zero) with the addition of 500 μM cGMP. The initial rate of the slow component of the cGMP-induced Ca^{++} release was 1.0 × 10^5 Ca^{++}/ s·ROS. The 0.02 absorbance unit is equivalent to 0.06 mol Ca^{++}/mol rhodopsin. Addition of DCPA to either the intact ROS or ROS disks did not cause any Ca^{++} release by itself or an altered rate of Ca^{++} leakage. Also, as with the frog ROS, addition of DCPA slightly lowered the total Ca^{++} content, as judged from the A23187-induced Ca^{++} release.

DCPA on Na/Ca exchange in rod photoreceptors are in close agreement with those obtained for DCPA inhibition of Na/Ca exchange in heart sarcolemma (Kaczorowski et al., 1985).

The inhibitory effect of DCPA on the cGMP-induced Ca^{++} release in bovine ROS was similar to that described for frog ROS; these results are shown in Fig. 3C. DCPA appears to lower the total amount of Ca^{++} released; however, DCPA does not cause a significant release of Ca^{++} by itself. In this experiment, of the total DCPA added, ~90% was bound to the ROS membranes. The cGMP-induced Ca^{++} release in bovine ROS exhibits an initial rapid phase that is absent in frog ROS (compare Figs. 2 and 3C). The origin of this fast phase of release is unknown at present. A large portion of the rapid Ca^{++} release in bovine ROS was not blocked by DCPA. These results suggest that there may be two distinct components of the cGMP-induced Ca^{++} release in bovine ROS disks.

TABLE I

DCPA Inhibition of the Na/Ca Exchange Rate in Bovine ROS

[DCPA]	12 mM Na⁺	50 mM Na⁺
μM	$Ca^{++}/s \cdot ROS$	$Ca^{++}/s \cdot ROS$
0	100% = 2.3 × 10^6	100% = 7.5 × 10^6
20	70% of control	72% of control
40	53	62
60	37	41
80	23	28
100	15	19
150	7	11

DCPA Blocks the cGMP-activated Conductance in Excised Membrane Patches

Because of the blocking effects of DCPA on the cGMP-induced Ca^{++} release, we examined the ability of DCPA to block a cGMP-activated conductance in excised patches of rod membrane (Fesenko et al., 1985; Haynes et al., 1986; Zimmerman and Baylor, 1986). These results are shown in Fig. 4. Addition of 100 μM cGMP to the perfusate enhanced the current recorded from membrane patches by 4–16-fold; this current was rapidly and reversibly abolished upon removal of cGMP. In the experiment shown in Fig. 4A, the current recorded from an excised patch was increased by about sixfold upon perfusion with 100 μM cGMP. Addition of 1 μM DCPA to the perfusate containing cGMP reduced the current by ~40%. Upon removal of DCPA, the current recovered almost completely. Subsequent addition of 10 μM DCPA in the presence of 100 μM cGMP resulted in nearly total blockage of the cGMP-activated current. Again, after washing out DCPA, the return was slow, with almost full recovery. In experiments with other membrane patches, the average reduction in the cGMP-activated current upon addition of 1 μM DCPA was 50% ($n = 6$), while 10 μM completely suppressed this current and in some cases even lowered it to levels below that measured in the absence of cGMP.

DCPA does not appear to block the cGMP-activated current by a competitive antagonist mechanism, i.e., compete with cGMP for a channel-binding site. After the cGMP-activated current had been completely suppressed by 10 μM DCPA

(as illustrated in Fig. 4A), the addition of 1 mM cGMP to the 10 μM DCPA perfusate did not relieve the DCPA-induced block of the cGMP-activated current (data not shown).

Fig. 4B illustrates the current-voltage relation for another membrane patch in the absence and presence of cGMP, and for the subsequent addition of 1, 2, and 10 μM DCPA. In this patch, the current was enhanced 16-fold with the addition of 100 μM cGMP, and shows rectification properties for the light-regulated and cGMP-regulated currents similar to those described by others (Bader et al., 1979; Nicol et al., 1984; Nakatani and Yau, 1985; Stern et al., 1986). With increasing concentrations of DCPA, the current magnitude was reduced at all voltage levels. The current-voltage relation for the cGMP-activated current in the presence of 10 μM DCPA was nearly identical to that recorded in the absence of cGMP. This is consistent with the idea that the channels activated by cGMP were completely blocked by DCPA.

The selectivity of DCPA in blocking the cGMP-activated conductance was examined by comparing its effects with those of EIA, which is structurally identical to amiloride at the 2 position of the pyrazine ring. The addition of the Na/H inhibitor EIA (10 μM) to the perfusate reduced the cGMP-activated current by only ~18% (data not shown), as compared with the complete suppression of the cGMP-activated current by an equivalent concentration of DCPA. Application of the solvent in which DCPA was dissolved (methyl-pyrrolidinone) reduced the cGMP-activated current by ~10% (data not shown).

A summary of the concentration dependence for inhibition of the cGMP-activated current, the cGMP-activated Ca^{++} release, and the Na^+-stimulated Ca^{++} release is illustrated in Fig. 5. This figure demonstrates the relative effectiveness of DCPA in inhibiting the cGMP-activated current or Ca^{++} flux. DCPA appears to be most effective in suppressing the cGMP-activated current in excised membrane patches, having a half-maximal inhibition constant (apparent K_i) of ~1 μM. For the cGMP-induced Ca^{++} release, the apparent K_i is ~9 μM and that for the Na^+-stimulated Ca^{++} release is ~50 μM. These results illustrate that DCPA can block the cGMP-activated current in excised patches in a relatively specific manner and that DCPA is about fivefold more effective at blocking the cGMP-activated Ca^{++} release than the Na^+-stimulated Ca^{++} release. The differences between the apparent K_i's for the ROS suspension and the excised patch may be accounted for by nonselective binding or partitioning of DCPA into ROS membranes. For ROS suspensions, the ratio of membranes to DCPA is much higher than for the excised patches. The differences in K_i's may also reflect differences in the efficacy of DCPA to bind or block the different target proteins. This possibility seems less likely because the percentage of DCPA bound to bovine ROS is independent of the DCPA concentration up to ~100 μM DCPA. This would imply that DCPA is partitioning nonselectively into ROS membranes rather than selectively binding to these different target proteins (data not shown).

DCPA Suppresses the Light-regulated Current in OS-IS

DCPA is capable of completely blocking the cGMP-activated current in excised patches of rod membrane (Fig. 4A). However, can DCPA suppress the light-regulated current recorded from the physiologically intact rod photoreceptor?
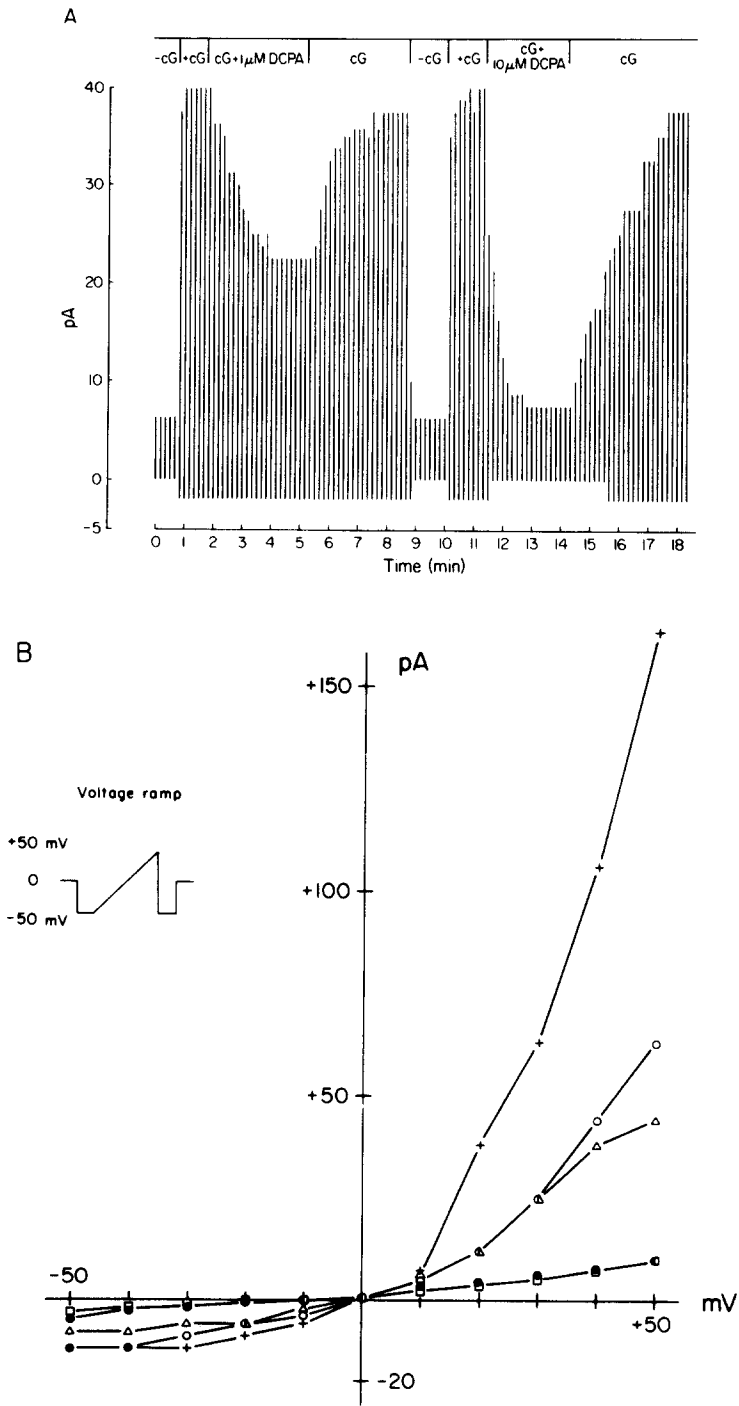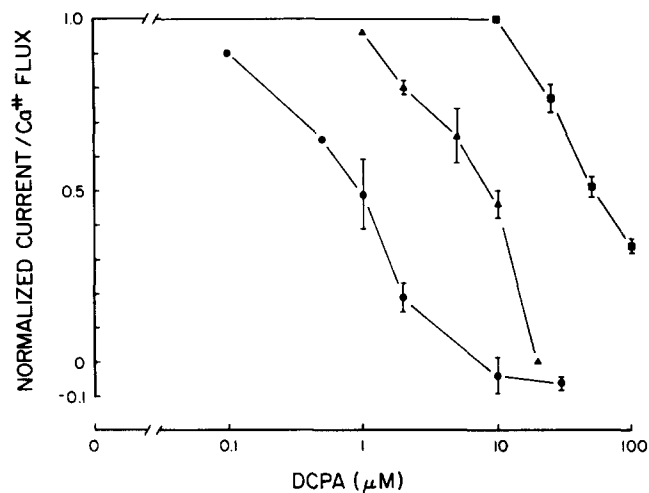
FIGURE 4.

NORMALIZED CURRENT/Ca⁺⁺ FLUX

$$1.0$$

$$0.5$$

$$0$$

$$-0.1$$

$$0 \quad 0.1 \quad 1 \quad 10 \quad 100$$

DCPA (μM)

FIGURE 5. Summary of the inhibitory effects of DCPA on the cGMP-activated current from excised patches (●), the cGMP-induced Ca^{++} release (▲), and the Na^+-stimulated Ca^{++} release (■). The current or Ca^{++} flux values are normalized to the maximal current or Ca^{++} flux observed. The data points represent means ± standard deviation; points without error bars represent the mean of only two values, except for the filled triangle at 20 μM DCPA, where all three values were zero.

FIGURE 4. (*opposite*) DCPA inhibition of the cGMP-activated current recorded from an excised inside-out patch of rod membrane. In *A*, the patch was perfused with an intracellular medium (see Materials and Methods for details). The upper trace reflects changes in the perfusate bathing the patch; the intracellular medium is designated as −cG. At +cG, the perfusate was changed to the intracellular medium containing 100 μM cGMP; this caused a sixfold elevation in the current and was readily reversed with the removal of cGMP. The length of the lines represents the magnitude of current obtained at +50 mV of the voltage ramp (see inset in *B*); outward current is shown as positive. Addition of 1 μM DCPA to the cGMP medium slowly decreased the current from 40 to 22.5 pA, a reduction of 44%. Removal of DCPA resulted in a slow and almost complete recovery of the membrane current. Addition of 10 μM DCPA to the cGMP medium rapidly reduced the current from 40 to 7.5 pA, close to the value recorded for the current in the absence of cGMP (6.3 pA). With the removal of DCPA, the current slowly returned to the pre-DCPA value, again with nearly complete recovery. In *B*, the current-voltage relation for the cGMP-activated current and its inhibition by DCPA in a different excised patch is shown. The current-voltage relation was obtained from the voltage ramp. The different symbols represent: (□) 0 cGMP, (+) 100 μM cGMP, (○) 100 μM cGMP plus 1 μM DCPA, (△) 100 μM cGMP plus 2 μM DCPA, and (●) 100 μM cGMP plus 10 μM DCPA, all in the intracellular medium. In this patch, addition of 1 μM DCPA decreased the current from 163 to 63 pA at +50 mV, a reduction of 61%. Addition of 10 μM DCPA reduced the cGMP-activated current to the value recorded in the absence of cGMP for all voltage values.

A case in point is the action of diltiazem; this drug effectively blocks the cGMP-gated current in excised patches of rod membrane, but when applied to the intact photoreceptor, the light-regulated current is blocked only by three- to fourfold (Stern et al., 1986), even for high concentrations of diltiazem (see below).

The effects of DCPA on the light-regulated current recorded from intact OS-IS were investigated with the use of the suction electrode technique (Baylor et al., 1979). As shown in Fig. 6A, an OS-IS suspension was bathed in normal Na^+ Ringer's containing 1 mM Ca^{++}. With the addition of 10 μM DCPA to the perfusate, the photoresponse amplitude slowly declined. After 15 min in 10 μM DCPA, the response was reduced by ~50% (from 13 to 7 pA) and, after 35 min, a 65% reduction (to 4 pA) was observed. The peak amplitude of the photoresponse did not change with the application of DCPA; this is consistent with the notion that the dark current was being suppressed by DCPA. DCPA at 1 μM caused partial suppression of the photoresponse. After 15 min in 1 μM DCPA, the photoresponse was reduced by ~25% (from 15 to 11 pA; data not shown).

DCPA at a concentration of 25 μM completely suppressed the light-regulated
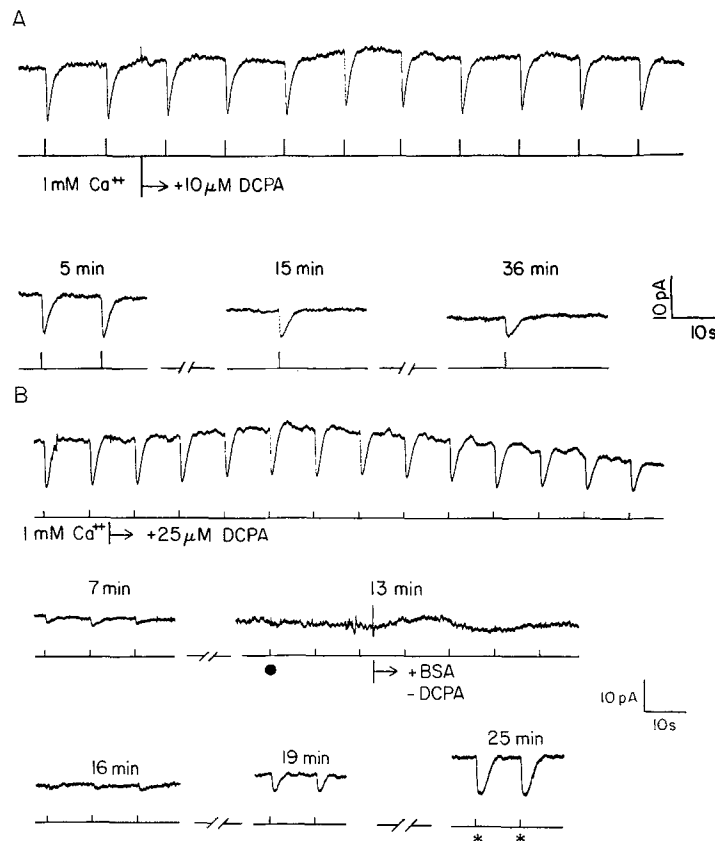

FIGURE 6.

current recorded from OS-IS; these results are shown in Fig. 6B. The addition of 25 μM DCPA to the perfusate caused a slow decline in the photoresponse amplitude. After 7 min in DCPA, the photoresponse had been suppressed by ~83% (from 14 to 2 pA). At 13 min, the photoresponse was completely blocked and a response could not be elicited, even by a flash bleaching 1,700 Rh*. The inhibitory effects of DCPA were reversible; however, it was necessary to add a carrier to aid in the removal of the lipophilic DCPA from the OS-IS membrane. After suppression of the light-regulated current, the perfusate was changed to normal Ringer's containing bovine serum albumin (2 mg/ml) without DCPA. With the washout of DCPA, the photoresponse slowly began to recover. After 25 min, the dark current had recovered nearly to the control value (11 vs. 14 pA; see figure legend for details).

The inhibitory action of DCPA in the excised membrane patch and the slow effect in the intact OS-IS are consistent with the notion that DCPA blocks the light-regulated channel and that the blocking site for DCPA is located at the cytoplasmic side of the rod plasma membrane. In support of this contention, we observed that the blocking action of DCPA on the light-regulated current was greatly delayed when the outer segment of the OS-IS was drawn into the suction

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FIGURE 6. (*opposite*) Inhibition of the light-regulated current by DCPA as recorded from intact OS-IS using the suction electrode technique. The inner segment of an OS-IS was drawn into the electrode, permitting the outer segment to be superfused with different solutions (the turnover time was 10–15 s). In A, the OS-IS was bathed in normal Na$^+$ Ringer's containing 1 mM Ca^{++}. At the bar (note the artifact in the current trace), the perfusate was changed to 1 mM Ca^{++} Ringer's containing 10 μM DCPA. With the addition of DCPA, the photoresponse to a flash bleaching 160 rhodopsins (Rh*) slowly declined. After 15 min in 10 μM DCPA, the response was reduced by ~50% (from 13 to 7 pA) and after 36 min a 65% reduction (to 4 pA) was observed. The current trace is a continuous record that has been interrupted for the indicated times. In B, the effect of 25 μM DCPA on the light-regulated current recorded from another OS-IS is shown. The initial conditions were the same as those described in A. The addition of 25 μM DCPA to the perfusate (defined as time zero) caused a slow decline in the photoresponse amplitude. After 7 min in DCPA, the photoresponse had been suppressed by ~83% (from 14 to 2 pA). At 13 min, the photoresponse was completely blocked and a response could not be elicited, even by a flash bleaching 1,700 Rh* (the filled circle). At the second bar, the perfusate was changed to normal Ringer's containing bovine serum albumin (BSA) (2 mg/ml) without DCPA. With the washout of DCPA, the photoresponse slowly began to recover. Similar results were obtained in five other cells. Prolonged perfusion with BSA Ringer's and the aspiration used to remove it from the chamber resulted in the formation of small bubbles at the top of the chamber, causing attenuation of the light flash. Normally a light flash bleaching 160 Rh* is just sufficient to yield a maximal photoresponse. The photoresponses obtained at 25 min were to flashes bleaching 7,700 Rh* (designated by asterisks) and are of maximal amplitude (note the plateau in the response). This indicates that the magnitude of the dark current had recovered nearly to the control value (11 vs. 14 pA), so the inhibitory effects of DCPA were reversible. The traces below the current recordings mark the flashes of light (12 ms duration). Current records were low-pass filtered (eight pole) at 20 Hz.

electrode (data not shown). In this case, DCPA must first cross the inner segment plasma membrane and then diffuse into the outer segment, where the light-regulated channels are located.

The selectivity of DCPA in blocking the light-regulated current in intact OS-IS was examined by comparison with the action of the Na/H inhibitor EIA. Addition of 25 μM EIA to the perfusate caused partial inhibition of the photoresponse (data not shown). After 7 min in EIA, the photoresponse was reduced by only 25% (from 16 to 12 pA). At 14 min, the response was decreased by 56% (to 7 pA). At an equivalent concentration of DCPA, the response was reduced by 83% at 7 min and was completely blocked at 13 min. The inhibitory action of EIA could result from a combination of effects: (a) 20 μM EIA reduces the cGMP-induced Ca^{++} release by ~20%, (b) 10 μM EIA reduces the cGMP-activated current by ~20%, and (c) inhibition of Na/H exchange might result in acidification of the intracellular medium, thus decreasing the magnitude of the light-regulated current. In support of this notion, protons have been shown to suppress the light-regulated current (Liebman et al., 1984).

Mechanism of DCPA Inhibition

These results demonstrate that DCPA leads to suppression of the light-regulated current in the physiologically intact cell. At least two mechanisms for its action can be considered. One is that DCPA lowers the ability of the OS-IS to remove intracellular Ca^{++} by inhibiting the Na/Ca exchange mechanism (Figs. 1 and 3A). In this instance, the light-regulated current would be suppressed because the cell fills with Ca^{++}. This mechanism seems unlikely since OS-IS isolated and purified in the presence of 100 μM DCPA contained two- to threefold less total Ca^{++} than OS-IS under normal conditions (data not shown). (This reduced value for the total Ca^{++} would be consistent with the notion that Ca^{++} enters the outer segment through the light-regulated channels, while the major pathway of Ca^{++} efflux is via Na/Ca exchange [MacLeish et al., 1984; Yau and Nakatani, 1984; Hodgkin et al., 1985]. Since DCPA is a more effective blocker of the cGMP-activated and light-regulated currents than of the Na/Ca exchanger, the entry of Ca^{++} should be greatly reduced, whereas the Ca^{++} efflux was only slightly reduced, thus lowering the total Ca^{++} content.) Also, the concentrations of DCPA needed to block the cGMP-activated conductance in the excised patch and the light-regulated current in the intact OS-IS are much lower than that required to significantly inhibit the Na/Ca exchange rate. A second possible mechanism would be the direct block by DCPA of the light-regulated channel, i.e., the cGMP-activated channel (Figs. 2, 3B, and 4).

To differentiate between these two alternatives, the effect of DCPA on the light-regulated current of OS-IS in low-Ca^{++} Ringer's was examined. Prolonged exposure to low Ca^{++} causes bovine ROS and frog OS-IS to lose nearly all their intracellular Ca^{++} via Na/Ca exchange (Schnetkamp, 1986; Nicol et al., 1987a) and it is unlikely that Na/Ca exchange could have a great effect on rod physiology under such conditions. As illustrated in Fig. 7A, addition of 50 μM DCPA to the 10 nM Ca^{++} Ringer's perfusate initially caused suppression of the photoresponse.

Thus, the light-regulated current can be blocked by DCPA in the absence of significant Na/Ca exchange.

In low-Ca^{++} Ringer's, the addition of DCPA resulted in a further unexplained observation. A photoresponse of the opposite polarity developed, which subse-
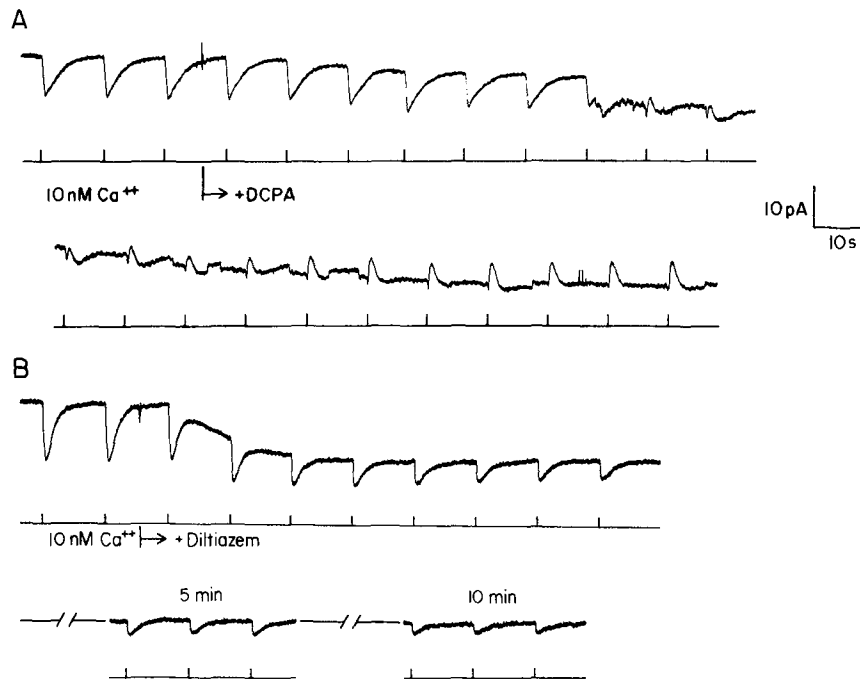

FIGURE 7. Effects of DCPA on the light-regulated current recorded from OS-IS in low Ca^{++}. An OS-IS suspension was bathed in 10 nM Ca^{++} Ringer's (0.1 mM Ca^{++} plus 0.39 mM EGTA) for 10 min before any recording. This caused the depletion of >90% of the intracellular Ca^{++} content (Nicol et al., 1987a). The outer segment of an OS-IS was perfused with 10 nM Ca^{++} Ringer's, and at the bar the perfusate was changed to one containing 50 μM DCPA. The photoresponse to flashes bleaching 7,700 Rh* was slightly reduced and then blocked suddenly. From this point, the photoresponse was biphasic, having a rapid initial negative component and a slower positive component. With time, the negative component was abolished, leaving only the positive component, which then achieved a stable amplitude. The Mg^{++} concentration (2 mM) was unchanged during this experiment. The first trace is continuous with the second. In other OS-IS, suppression of the light-regulated current by DCPA was more gradual than illustrated above. In these OS-IS, the current was completely blocked, with a slow transition to a positive component that grew larger with time and reached a stable amplitude. In *B*, the addition of 50 μM diltiazem (at the bar) to the 10 nM Ca^{++} Ringer's perfusate reduced the amplitude of the photoresponse by fourfold (from 13 to 4 pA). The current traces are a continuous recording and have been broken for ease of presentation. In *B*, a different OS-IS suspension was used under the same starting conditions described in *A*. Current and time scales are the same as in *A*; each flash bleached 1,700 Rh*. Current records were low-pass filtered (eight pole) at 20 Hz.

quently reached a stable amplitude. The ability of DCPA to invert the polarity of the photoresponse in 10 nM Ca^{++} Ringer's was observed in 9 of the 11 OS-IS examined. In a low-Ca^{++} medium, the action of DCPA on the light-regulated current was very different from that observed at normal Ca^{++} levels, which suggests that the blocking site for DCPA or the conductance state of the light-regulated channel may be modified when the Ca^{++} concentration is lowered.

The inverted photoresponse described above was not blocked by the addition of 50 μM diltiazem to the 10 nM Ca^{++} Ringer's containing 50 μM DCPA (in three OS-IS; data not shown). Diltiazem is the only other known antagonist of the cGMP-activated Ca^{++} efflux (Koch and Kaupp, 1985) and conductance (Stern et al., 1986).

In view of the different actions of DCPA at different Ca^{++} concentrations, we repeated the suction electrode experiments with 50 μM diltiazem at 1 mM and 10 nM Ca^{++}. Addition of diltiazem to the perfusate reduced the photoresponse three- to fourfold for both high and low Ca^{++} concentrations (see Fig. 7B for the 10 nM Ca^{++} condition). Thus, DCPA was a more potent inhibitor of the light-regulated current in the intact cell under the 1 mM Ca^{++} condition because diltiazem never completely suppressed the rod photoresponse. Also, the effects of diltiazem were the same regardless of the Ca^{++} concentration, which suggests that the site of action for DCPA may be different from that of diltiazem.

DISCUSSION

We have investigated the inhibitory effects of a derivative of amiloride, DCPA, on the Ca^{++} flux mechanisms in ROS as well as the cGMP-activated conductance in excised patches and the light-regulated current from intact OS-IS. DCPA appears to be a potent inhibitor of the cGMP-activated conductance, the cGMP-induced Ca^{++} release, and the light-regulated current in the intact OS-IS, completely blocking these actions at 10, 20, and 25 μM, respectively. Higher concentrations of DCPA are required to significantly inhibit the Na/Ca exchange mechanism (apparent K_i of ~50 μM).

Our results concerning the ability of DCPA to block the fluxes or conductances in these different preparations are consistent with the notion that the cGMP-induced Ca^{++} transport mechanism of rod disk membranes and the cGMP-activated conductance observed in excised patches of rod plasma membrane may be similar to the light-regulated conductance recorded from the intact OS-IS. (See Matthews, 1987, for a comparison of the light-regulated and cGMP-activated channels.) However, we also demonstrate conditions, i.e., low Ca^{++}, where the ability of DCPA to block the cGMP-activated current and the light-regulated current are different. In the excised-patch experiments of the type shown in Fig. 4A, the cytoplasmic face of the patch is bathed in a low-Ca^{++} medium and DCPA consistently blocks the cGMP-activated current (regardless of the Ca^{++} at the external face of the patch; data not shown). In the OS-IS, depending on the Ca^{++} concentration, the results can be very different. At 1 mM Ca^{++}, DCPA behaves as it does in the patch experiments, but at 10 nM Ca^{++}, the normal photoresponse is blocked with the appearance of the inverted response. Under the low-Ca^{++}

conditions, the observations in the patch are different from those in the OS-IS, which suggests that the cGMP-activated conductance and the light-regulated current may be dissimilar with respect to their pharmacology, or that in the intact OS-IS there may be physiological modulators of the channel whose activity is not observed in the excised membrane patch. The fact that diltiazem does not block the light-regulated current in the intact photoreceptor as it does in the excised patch also argues that the cGMP-activated conductance and the light-regulated channel may be dissimilar in their pharmacology. Pharmacological experiments of this sort might be important in resolving the current debate over the identity of the cGMP-dependent cation channel (see Cook et al., 1987; Matesic and Liebman, 1987).

It is interesting that DCPA, an Na/Ca exchange inhibitor, and diltiazem, whose d-stereoisomer is a Ca^{++} channel blocker in other cell types, have similar actions on the cGMP-activated conductance in excised patches of membrane. Perhaps the site for cGMP activation or gating at the cytoplasmic surface has structural properties that are common to the Ca^{++}-binding site of the Na/Ca exchanger or the Ca^{++} channel.

The different behaviors of DCPA in high and low Ca^{++} raise the possibility that the conformational state of the light-regulated channel is modulated by different concentrations of Ca^{++} (also see the discussion in Borsellino et al., 1986; Owen, 1986). Our results obtained with DCPA may be interpreted most straightforwardly as indicating two different conductance states: one is blocked by DCPA, while another, induced in low Ca^{++}, is modified but not blocked by DCPA. This notion is consistent with several experimental observations. First, in Ringer's solution with no Na^+ and with micromolar levels of Ca^{++} and Mg^{++}, the rod photoresponse becomes inverted, and under these conditions a light-dependent efflux of ^{42}K has been observed (Capovilla et al., 1983). Second, two different conductance levels have been observed in single channel recordings from excised membrane patches of amphibian ROS (Haynes et al., 1986; Zimmerman and Baylor, 1986). Thus, it is possible that in low Ca^{++}, one conductance state is favored over the other. Another possible explanation of the data is that there are two different light-regulated channels in the ROS membrane, one carrying an inward Na^+ current and another carrying an outward K^+ current. This notion is consistent with the finding that two different conductances were observed when ion channels from bovine ROS were reconstituted into lipid bilayers, although the cGMP dependence of these channels was not determined (Hanke and Kaupp, 1984). It is interesting to note that the cGMP-induced Ca^{++} flux across bovine disk membranes showed two components that could be distinguished by their capacity to be blocked or not by DCPA (see Fig. 3C). The activity of the outward conductance might make a small contribution to the total current in 1 mM Ca^{++}, but lowering the Ca^{++} concentration with subsequent addition of DCPA might alter the ionic gradients, enhancing the outward conductance that gives rise to the inverted photoresponse.

The blocking ability of DCPA may prove to be a useful tool to discriminate between these models. This blocker might also be used as a potential label for isolation and purification of the light-regulated channel.

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