Effect of Blocking the Na^+/K^+ATPase on Ca^{2+} Extrusion and Light Adaptation in Mammalian Retinal Rods

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ABSTRACT Membrane current and light response were recorded from rods of monkey and guinea pig by means of suction electrodes. The correlation between adaptation and the Na^+/K^+ pump was investigated by measuring light-dependent changes in sensitivity with and without inhibition of Na^+/K^+ ATPase by strophanthidin. Strophanthidin was found to reduce the dark current, to slow the time course of the photoresponse, and to increase light sensitivity. At concentrations between 20 and 500 nM, the pump inhibitor suppressed in a reversible way the current re-activation occurring during prolonged illumination and modified the light-dependent decrease in sensitivity, which in control conditions approximates to a Weber-Fechner function. The effects of the pump inhibitor on the adaptive properties of rods are associated with an increased time constant of the membrane current attributed to the operation of the Na^+:Ca^{2+},K^+ exchanger. The effects of rapid application of the pump inhibitor on the current re-activation are consistent with the idea that significant changes in the internal sodium occur in rods of mammals during background illumination and that they play an important role in the process of light adaptation.

INTRODUCTION

Retinal receptors adapt to increasing levels of ambient illumination with a light-dependent decrease in sensitivity (Baylor and Hodgkin, 1974; Fain, 1976). The change in sensitivity associated with rod adaptation is initiated by a light-induced fall of internal free calcium ($[Ca]_i$), which results from the blockage of calcium entry through the channels closed by light and continued Ca^{2+} extrusion by an ionic exchanger (Yau and Nakatani, 1985; McNaughton et al., 1986; Matthews et al., 1988; Nakatani and Yau, 1988; Ratto et al., 1988; Pugh and Lamb, 1990; Matthews, 1991). The extrusion of 1 Ca^{2+} is coupled with the co-transport of 1 K^+ and the counter-transport of 4 Na^+ (Cervetto et al., 1989; Schnetkamp et al., 1989; Lagnado et al., 1992; Perry and McNaughton, 1993). Because of this stoichiometry, the equilibrium level of $[Ca]_i$ must be critically dependent on small changes in the transmembrane Na^+ gradient. Furthermore, as the affinity of the exchanger for Ca^{2+} is lowered by increased levels of internal sodium ($[Na]_i$) (Hodgkin and Nunn, 1987; Lagnado et al., 1992), it is expected that conditions leading to changes in $[Na]_i$ will modify the rate of Ca^{2+} extrusion and hence affect rod adaptation.

The purpose of the present paper is to verify this prediction for mammalian rods and to determine how critically light-adaptation depends on the Na_i^+ turn-over. We shall show that light-adaptation in rods of mammals depends on the activity of the Na^+/K^+ ATPase. From the analysis of these results, we conclude that the sensitivity changes occurring in rods during background adaptation are associated with a decrease in internal sodium, which in turn accelerates the rate of Ca_i^{2+} extrusion. The dependence of background adaptation on the cell metabolism is perhaps a special feature of mammalian rods and may offer an explanation for the variability in the adapting properties previously reported for rods of mammals (Baylor et al., 1984; Tamura et al., 1991; Kraft et al., 1993). A partial account of the present results has been presented in abstract form (Demontis et al., 1993).

Received for publication 30 December 1994 and in final form 23 March 1995.

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0006-3495/95/08/439/12 $2.00

MATERIALS AND METHODS

Monkeys (*Macaca menestrina*) and albino guinea pigs (200–400 g) were reared according to institutional guidelines for animal care. The monkeys used in this study had participated previously in other acute physiological experiments, and the eyes were removed from all animals after administration of a lethal dose of anesthetic (monkey: 60 mg Kg^{-1} of ketamine, and 30 mg Kg^{-1} thiopental) (guinea pig 300 mg Kg^{-1} ketamine and 80 mg Kg^{-1} thiopental).

Dark-adapted retinae (2 h) were isolated and chopped under infrared illumination ($\lambda > 900$ nm) with the aid of an infrared converter. The preparation was then placed in a temperature-regulated chamber (volume 350 μl) and perfused with an oxygenated buffered Locke solution (temperature 37°C, pH 7.6) whose composition in mM was: NaCl 145, KCl 3.6, $MgCl_2$ 2.4, $CaCl_2$ 1.2, HEPES 5, Na-ascorbate 0.05, glucose 10. In the experiment of Fig. 6, the low sodium solution was obtained by complete substitution of NaCl for equimolar amounts of choline chloride. Ketamine (*Ketalar*) was obtained from Parke-Davis (Milan, Italy), and thiopental (*Farmotal*) was from Farmitalia Carlo Erba (Milan, Italy). All of the remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

The rod volume was routinely estimated by geometrical determination, assuming an internal free space of 50% of total volume. Accordingly, the average value of the free volume corresponds to 0.023 pl. Membrane current was recorded by means of suction electrodes from rod outer segments attached to small pieces of retina (see Baylor et al., 1984). The suction pipette, filled with the perfusing saline, typically had a resistance of 2–3 MΩ when empty, and of 5–6 MΩ with the cell in place. The average

fraction of membrane current collected by the suction electrode was estimated to be ~50%. The values of the current reported in the results have not been corrected for imperfect collection. The recording apparatus and light stimulation were as reported previously (see Demontis et al., 1993). In all of the experiments, the wavelength used was $\lambda = 510$ nm and the flash duration was 20 ms. On-line data acquisition and processing were performed by using software developed by G. M. Ratto.

Ouabain or strophanthidin (Sigma) were dissolved in Locke solution by stirring for 2 h in the dark at 30°C. The data presented here were obtained by using strophanthidin, whose effects are more promptly reversible upon washing than those of ouabain (Sachs, 1974). Prolonged exposure to strophanthidin (>5 min) was avoided to prevent the accumulative effect of the drug. Hence, all measurements reported in the present study were taken within the interval 2–5 min after inhibitor application. In the experiments of Figs. 1–5, strophanthidin was bath applied at a perfusion rate of 2 ml min^{-1}. Rapid application of strophanthidin was obtained by ejection from a pipette with an inner diameter of about 1 μm, connected to an injector (PLI-100 Medical System Corporation), whose pressure was regulated at ~100–150 mmHg (2–3 psi). The pipette mouth was positioned ~100 μm from the inner segment of the recorded rod. In this way, the concentration of the inhibitor reaching the cell was somewhat lower than in the ejecting system. By comparing the effects obtained in this way and with the drug applied at a known concentration in the superfusing medium, we estimate a dilution factor of ~10.

Strophanthidin in the range 10 nM to 10 μM is unlikely to have a direct action on the Na:Ca exchanger, whose operation has been shown to be unaffected by similar or higher concentrations of the same drug in salamander outer segments (Cervetto et al., 1989) and guinea pig myocytes (Hilgemann, 1990).

Measurements of light sensitivity

The absolute sensitivity or quantum sensitivity (S_f^d) was measured in darkness as the peak amplitude elicited by photoexcitation of a single rhodopsin molecule (Rh*) (Baylor et al., 1979). This value was obtained by extrapolation from light responses in the linear range (established by the superposition principle); the dimensions are pA/Rh*. In the presence of a steady background, the effect of a single Rh* is reduced: the sensitivity measured in these conditions is called flash sensitivity (S_f). The desensitizing effect of background illumination is expressed in the normalized form as the incremental flash sensitivity (S_f/S_f^d). Over a certain range of intensities, the relation between the incremental flash sensitivity and background illumination is described by the Weber-Fechner function: $S_f/S_f^d = I_0/I_0 + I$, where I_0 is the background intensity in photons μm^{-2} s^{-1} that halves the flash sensitivity. The absolute and the flash sensitivity are both defined for a narrow range of test flashes, when the corresponding response amplitude is linearly related to the intensity of the stimulus. In the present study, we shall use the term *light sensitivity* in a sense broader than absolute and flash sensitivity, such as to include conditions in which linearity does not apply. Accordingly, light sensitivity is defined as the reciprocal of the light intensity in photons μm^{-2} necessary to produce a half-saturating response ($I_{0.5}$). $I_{0.5}$ was obtained by fitting the response-intensity relation with a saturating exponential of the form $r/J_D = 1 - e^{-KI}$, where r is the response amplitude, J_D is the dark current in pA, and I is the light intensity in photons μm^{-2} $flash^{-1}$; hence, $(I_{0.5})^{-1} = K/\ln 2$. The quantity K affects the position of the response intensity function on the intensity axis. Specifically, the larger the value of the half-saturating intensity, the more the curve is shifted to the right. Shifts of the response intensity curves to the right correspond to a decrease in light sensitivity. Note that the dimensions of both absolute and flash sensitivity are pA/Rh*, whereas the dimensions of light sensitivity are μm^2 $photon^{-1}$.

Definitions of the principal symbols and constant values used in the text

F = Faraday's constant 96,480 Coulombs mol^{-1}; 23,062 cal V^{-1} mol^{-1}.
R = 8.316 V Coulomb K^{-1} mol^{-1}; 1.98 cal mol^{-1} K^{-1}.
T = absolute temperature in K
v = free rod volume; 0.023 pl
r = response amplitude
J_D = dark current
J_{Na} = the sodium component of the dark current
J_{Ex} = the exchange current
J_{Sat} = saturated exchanger current
$[Na]_i$ (0) = internal sodium in darkness; 10 mM
$[Ca]_i$ (0) = internal calcium in darkness; 240 nM
τ_{Ex} = time constant of the Na^+:Ca^{2+},K^+ exchanger
τ_P = time constant of the Na^+/K^+ pump; 600 ms
K_p = Na^+/K^+ pump rate constant; 1.7 s^{-1}
K_{Ca} = exchanger equilibrium dissociation constant for Ca^{2+}; 0.00017$[Na]_i$
K_{Buff} = buffer dissociation constant for calcium; 2.7 μM
B = buffer capacity for calcium; 300 μM
$[Ca_{Eq}]_i$ = equilibrium concentration of internal calcium for the exchanger
$[Na_{Eq}]_i$ = Na^+/K^+ pump equilibrium concentration of internal sodium
ΔV = transmembrane potential during background illumination; 60 mV
ΔG_{ATP} = change in free energy associated with ATP hydrolysis; −14,300 cal M^{-1}
[ATP] = internal concentration of adenosine trisphosphate; 5 mM
[ADP] = internal concentration of adenosine diphosphate; 100 μM
$[P_i]$ = internal concentration of inorganic phosphate; 1.5 mM

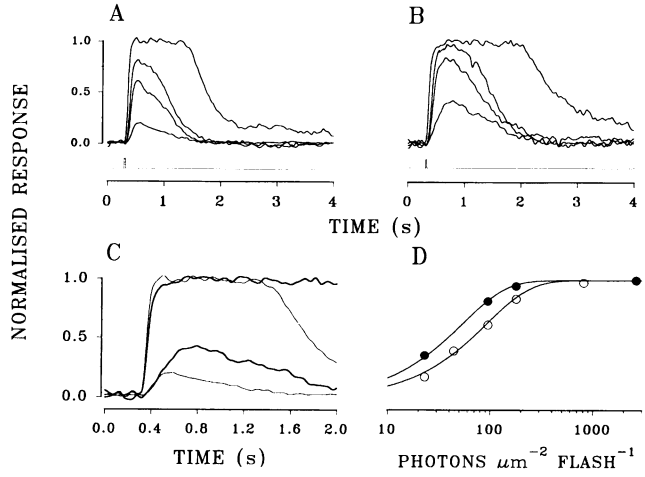

FIGURE 1 *A* shows the normalized response of a guinea pig rod to light stimuli of increasing intensity (23, 95, 179, 2643 photons μm^{-2} $flash^{-1}$) in control conditions; the traces are the average of 24, 4, 4, and 3 sweeps, respectively. The records in *B* show the normalized response, of the same guinea pig rod 2 min after the application of 500 nM strophanthidin, to light flashes of the same intensity as in *A*. Traces are the average of 5, 4, 4, and 3 sweeps. Responses to 23 and 2643 photons μm^{-2} $flash^{-1}$ are reported in *C* for controls (*thin line*) and strophanthidin (*thick line*). In *D* the peak amplitude of the response, for both controls (○) and strophanthidin (●), is reported as a function of the stimulating light intensity in photons μm^{-2} $flash^{-1}$. The solid lines are the best-fitting curves from the equation $r = J_D(1 - e^{-KI})$, where r is the response amplitude, J_D is the dark current, and I is the light intensity in photons μm^{-2} $flash^{-1}$. K is a constant (see Lamb et al., 1981), whose value in controls and strophanthidin is 0.015 and 0.027 μm^2 $photon^{-1}$, respectively, J_D is 7.81 and 5.63 pA in controls and strophanthidin, respectively. For the relation between K and light sensitivity, see Measurement of Light Sensitivity in Materials and Methods. Two additional measurements obtained from responses to flashes of 813 and 44 photons μm^{-2}, in controls (not shown in *A* for clarity), are plotted in *D*. All responses are normalized to the dark current. Data were sampled at 250 Hz, filtered at 25 Hz, and digitally smoothed by a 20-ms-wide window.

RESULTS

Effects of strophanthidin on the dark current, light-response, and sensitivity

Exposure of mammalian rods to a selective Na^+/K^+-ATPase inhibitor, such as ouabain or strophanthidin at micromolar concentrations (>20 μM), rapidly suppressed the dark current and blocked the light response. When the inhibitor was applied at a concentration sufficiently low to induce only a partial reduction of the dark current within the first 5 min (see Materials and Methods), the light response was modified in the way shown in Fig. 1.

Normalized responses to flashes of increasing intensity from a guinea pig rod, in control conditions and in the presence of 500 nM strophanthidin, are compared in Fig. 1, *A* and *B*, respectively. In strophanthidin, the time course of current suppression by light was prolonged at all light intensities of the stimulating flash. The time to the peak of the dim flash response increased from 250 to 400 ms. Furthermore, the relative amplitude of the dim flash response was larger in strophanthidin (*thick trace*) than in controls (*C*), indicating that light sensitivity was increased by the inhibitor (see Materials and Methods for definition of light sensitivity). The normalized peak response amplitude of the same rod, plotted as a function of the log of the average number of photons delivered by the test flash, is shown in Fig. 1 *D*. The experimental measures are well fitted by a saturating exponential (Baylor et al., 1984) with a half-saturating intensity of 64 and 37 photons μm^{-2} $flash^{-1}$ for controls (*open circles*) and strophanthidin (*filled circles*), respectively. This indicates that light sensitivity in the presence of the pump inhibitor is increased by a factor of nearly two.

The effects of 20 nM strophanthidin on the light response of a monkey rod are illustrated in Fig. 2. Responses to dim and bright flashes are shown in *A* for controls and in *B* for

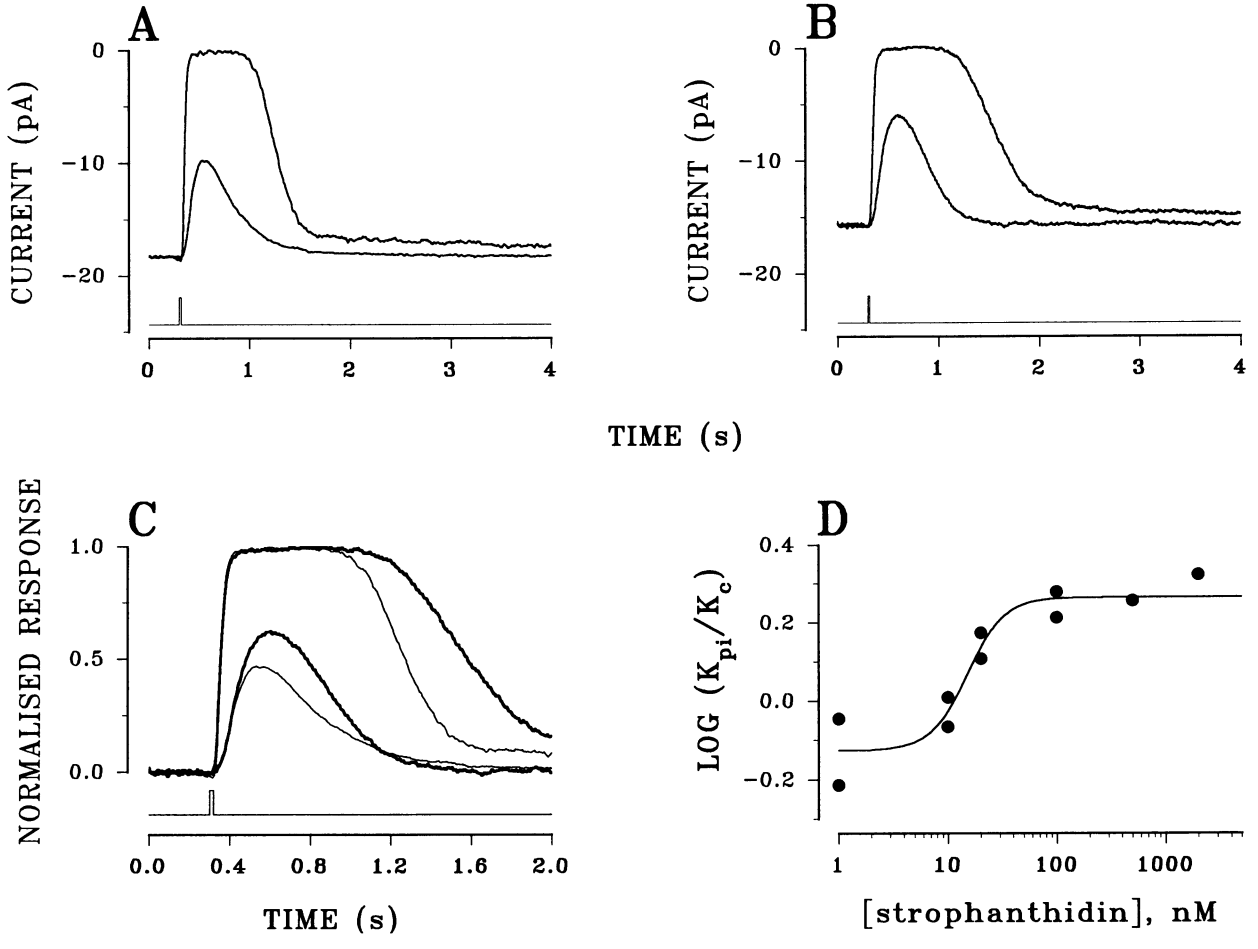

FIGURE 2 Light response of a monkey rod to subsaturating (179 photons μm^{-2} $flash^{-1}$) and saturating flashes (2643 photons μm^{-2} $flash^{-1}$) in control conditions (*A*) and in 20 nM strophanthidin (*B*). The records in controls are the average of 17 (dim flash response) and 3 sweeps (bright flash response) and in strophanthidin of 8 and 6 sweeps for the dim and bright flash, respectively. In strophanthidin the dark current decreases from −18.7 to −16.2 pA and the response amplitude for the dim flash increases from 8.8 to 9.9 pA. (*C*) The responses from *A* (*thin traces*) and *B* (*thick traces*) are shown normalized and superimposed on an enlarged time scale in *C*. (*D*) Log of the ratio between light sensitivity in the presence of the pump inhibitor (K_{pi}/ln 2) and in controls (K_c/ln 2) as a function of strophanthidin concentration. The curve through the experimental points is drawn according to the equation: $K_{pi}/K_c = Y_0 + Y_{max}[strophantidin]^n/[strophantidin]^n + H^n$ where H is the strophanthidin concentration that causes a half-maximum increase in sensitivity. $n = 2.57$, $H = 15$ nM, $Y_{max}n = 0.39$, $Y_0 = -0.12$. Data were acquired at a sampling frequency of 250 Hz and filtered at 30 Hz.

strophanthidin. The same responses normalized to the dark current are shown superimposed in *C*. In this experiment, the drug caused a 13% reduction in the dark current and a 12% increase in the absolute amplitude of the response to the dim flash, corresponding to an increase in light sensitivity of 0.17 log units. The change in sensitivity caused by the pump inhibitor is illustrated in *D*, where the logarithm of the ratio of the light sensitivity measured in strophanthidin and in controls is plotted as a function of the drug concentration for 10 rods. The experimental points are fitted by a function with a Hill coefficient of 2.5, which suggests a marked dependence of the rod light sensitivity on the Na^+/K^+ ATPase activity.

Effects of strophanthidin on background desensitization

The ability of retinal rods to light adapt is recognized mainly by a light-dependent decrease in sensitivity, conforming to a first approximation to the Weber-Fechner law (Baylor et al., 1984; Matthews et al., 1988; Nakatani and Yau, 1988; Tamura et al., 1991; Matthews, 1991; Kraft et al., 1993) and by a time-dependent partial recovery of the photo current during prolonged bright illumination (Cervetto et al., 1985; Demontis et al., 1993). The correlation between pump activity and adaptation was investigated by measuring the light-dependent changes in sensitivity with and without inhibition of Na^+/K^+ ATPase. Averaged responses to flashes and to steps of light in normal saline and with strophanthidin are illustrated in Fig. 3. Fig. 3, *A* and *B* show that the time-dependent partial recovery of the current, which normally occurs during prolonged illumination in light adapting rods (*thin trace*), is suppressed by the drug (*thick trace*). In this experiment, current recovery reverted to normal upon the washing out of strophanthidin (Fig. 3 *B*, *dotted line*).

Reduction in current reactivation by strophanthidin occurred at both bright and intermediate levels of background illumination as illustrated by the plot in Fig. 3 *C*, where the fractional recovery is reported as a function of the peak response to a step of light for eight rods.

The effects of strophanthidin on current reactivation are associated with modifications in the light-dependent changes in sensitivity, as shown in Fig. 4. Responses to steps of light of different intensities, obtained from a rod in control saline and during exposure to 20 nM strophanthidin are illustrated in Fig. 4, *A* and *B*, respectively. The steady-state response, taken from two rods 3 s after the onset of light, is plotted as a function of the steps intensity for controls (*open symbols*) and strophanthidin (*filled symbols*) in Fig. 4 *C*. It is seen that control responses deviate significantly from the theoretical function (*solid line*) obtained assuming response compression with no adaptation. By contrast, a much smaller deviation from a saturating exponential occurs in the presence of the inhibitor, thus indicating reduced adaptation. These properties are further documented in Fig. 4 *D*, where open symbols are values for the incremental flash sensitivity of the same rods in control conditions, plotted as a function of the background illumination, with the dashed line fitted to these data showing a Weber-Fechner relation. Filled symbols are data from rods exposed to strophanthidin. As in Fig. 4 *C*, the solid line shows a function based on the assumption that background illumination causes an exponential compression of the response with no adaptation (Baylor et al., 1984; Matthews et al., 1988; Nakatani and Yau, 1988). A more definite con-

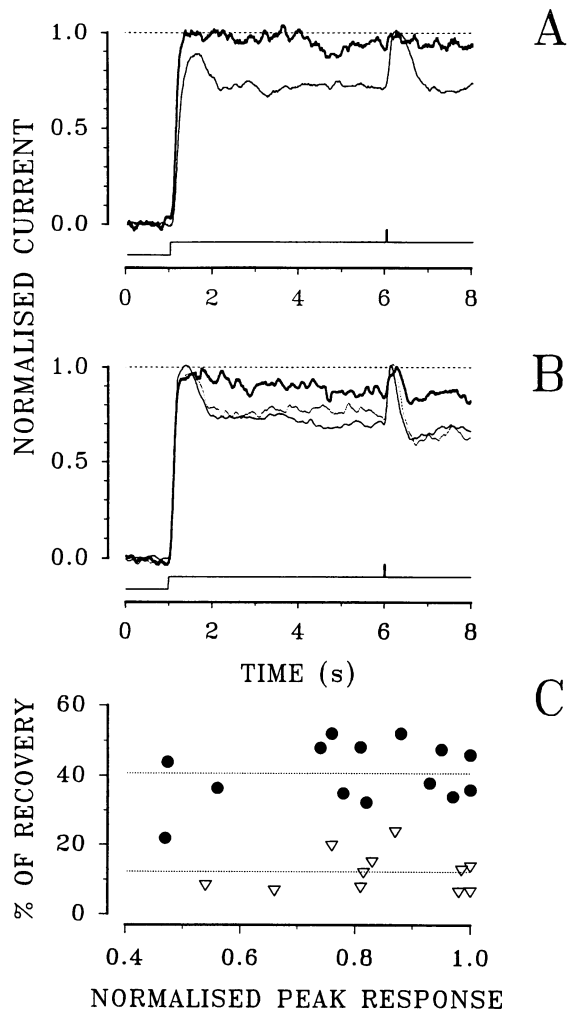

FIGURE 3 Effects of 20 nM strophanthidin on the response of a monkey (*A*) and guinea pig (*B*) rod to a bright step of light (715 photons $\mu m^{-2} s^{-1}$) and superimposed flash (810 photons μm^{-2} $flash^{-1}$). (*thin trace*) Control; (*thick trace*) strophanthidin; (*dotted trace* in *B*) recovery after washing out the pump inhibitor. In *A* the dark current was 5.8 pA (average of 9 sweeps) in controls and 3.8 pA (average of 4 records) in strophanthidin. In *B* the dark current was 8.2 pA (average of 6 sweeps) in controls; 5.1 pA (average of 4 responses) in strophanthidin, and 6.9 pA (average of four sweeps) after washing out the pump inhibitor. (*C*) The % of recovery measured 3 s after the onset of background illumination is plotted as a function of the normalized peak amplitude for controls (*filled symbols*) and strophanthidin (*open symbols*). The horizontal lines through the points give the mean recovery in controls (40.7%, SD 8.3%) and in strophanthidin (12.6% SD 6%). Data were sampled at 200 Hz, filtered at 30 Hz, and digitally smoothed by a 25-ms-wide window.

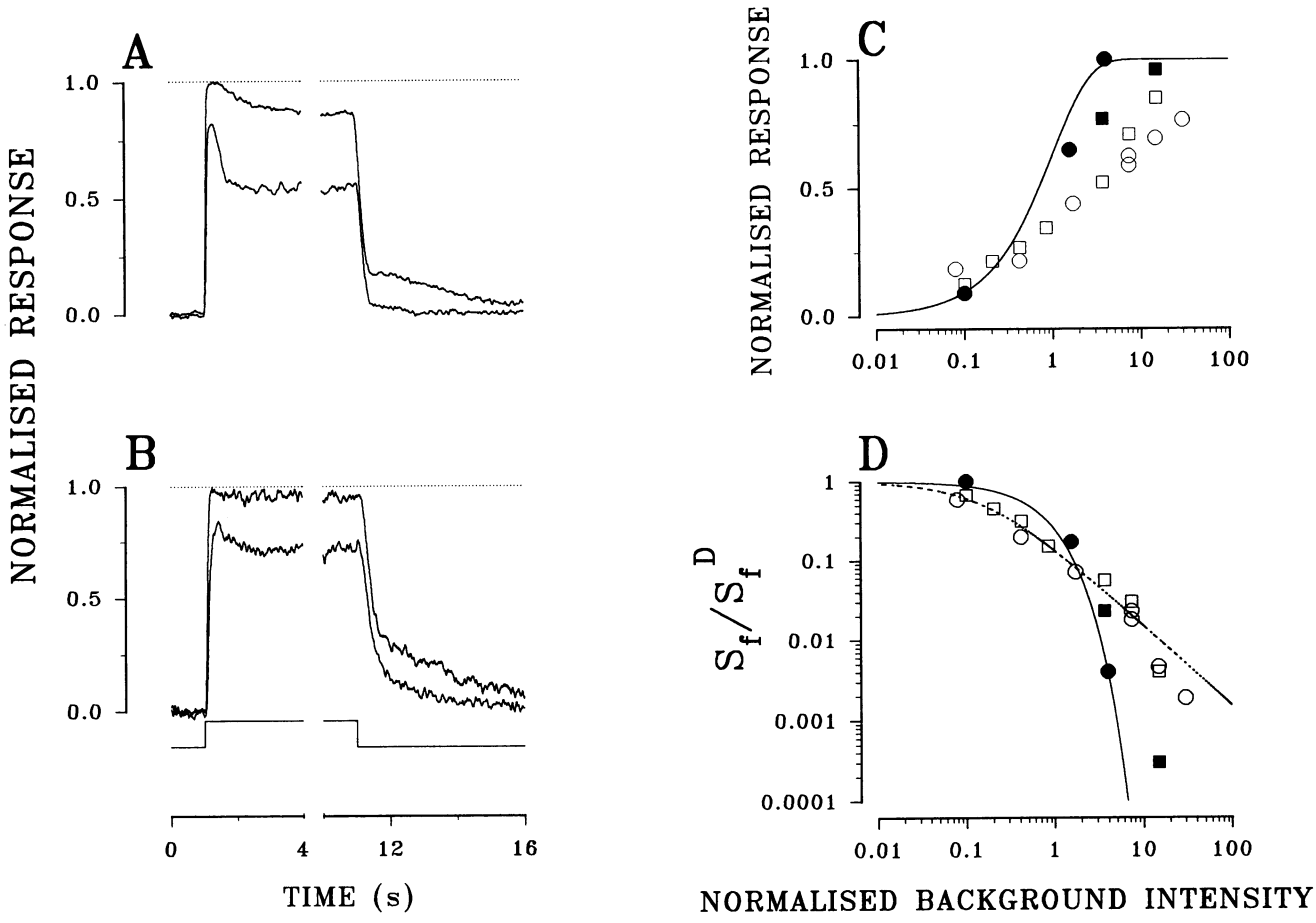

FIGURE 4 (*A*) Responses to 10-s steps of light (3051 and 12544 photons μm^{-2} s^{-1}) in control saline. For illustration purposes, a temporal gap of 6 s was omitted. (*B*) Responses from the same rod to the same light stimuli in the presence of 20 nM strophanthidin. Responses in *A* and *B* are the average of four sweeps for the two control responses and the dimmer response in strophanthidin, whereas the bright response in strophanthidin is the average of two sweeps. J_D was 10.8 pA in controls and 5.3 pA in strophanthidin. The light stimulation protocol is indicated in the lower part of *B*. Data were sampled at 250 Hz, filtered at 30 Hz, and digitally smoothed by a 20-ms-wide window. (*C*) Normalized amplitude of step responses measured 3 s after the onset of light, for two rods, plotted as a function of the light intensity in controls (*open symbols*) and in strophanthidin (*filled symbols*). The continuous curve is drawn from the equation: $r/J_D = 1 - e^{-K_s I}$ where K_s is a constant that reflects the integration time of the dim flash response (see Tamura et al., 1989). The light intensity scale is normalized in units of K_s. (*D*) Incremental flash sensitivity (S_f/S_f^d), as a function of background intensity in controls (*open symbols*) and with strophanthidin (*filled symbols*) in two guinea pig rods. S_f and S_f^d are flash sensitivity and absolute sensitivity, respectively. The dashed line is drawn according to the Weber-Fechner relation: $S_f/S_f^d = (1 + I/I_0)^{-1}$, where I is the intensity of the background in photons μm^{-2} s^{-1} and I_0 is the background intensity that halves the flash sensitivity. The light intensity scale is normalized in units of K_s. The absolute values of I_0 were 73 (○) and 147 photons μm^{-2} s^{-1} (□). The solid line is drawn according to the relation $S_f/S_f^d = e^{-K_S I}$, where I is the light intensity in photons μm^{-2} s^{-1} and K_S is a constant (see Baylor et al., 1984; Tamura et al., 1989). Data were normalized in units of K_S. The values of K_S for the two cells in *C* and *D* were 0.0012 (*squares*) and 0.00218 μm^2 s photon^{-1} (*circles*).

clusion on this point would require more experiments to provide a sufficiently large number of measurements at sub-saturating level of illumination. Because of difficulties in maintaining recording stability with prolonged exposure to strophanthidin, the experiments in Fig. 4, *C* and D, however, represent the only acceptable measurements of this kind.

Both step response and background desensitization in rods exposed to strophanthidin bear a close resemblance to those observed in nonadapting monkey rods by Baylor et al. (1984). Out of eight monkey rods we studied in detail, two adapted like Fig. 3 *A*, one showed no signs of adaptation, and the remaining five displayed an intermediate behavior. The fraction of adapting rods in guinea pigs was >90%.

Effects of Na^+/K^+ pump inhibition on the Na^+:Ca^{2+},K^+ exchanger

To maintain internal sodium within values compatible with efficient calcium extrusion, a normally functioning cell, with well developed adapting properties, must be able to extrude Na^+ out of the cytoplasm at a high rate. Assuming a dark current of 30 pA, of which ~80% is carried by sodium, the influx of this ion in a typical mammalian rod is estimated to be 15 mM s^{-1} (see also Appendix), two orders of magnitude higher than the value reported for amphibian rods (Torre, 1982). To keep pace with the massive influx of sodium in darkness, the Na^+/K^+ pump of mammalian rods must operate at an equivalent rate. The effect of strophan-

thidin, therefore, can be explained assuming that the consequent build-up of internal sodium slows the rate of calcium extrusion by the cell. This idea was tested by analyzing the light-insensitive membrane current associated with the exchanger (Yau and Nakatani, 1984; Cervetto et al., 1989; Perry and McNaughton, 1993).

Fig. 5 shows the relaxation of the exchange current during the response to a saturating step of light in both controls and 500 nM strophanthidin. In the presence of the pump inhibitor (*A*), the light-dependent current suppression is prolonged and the time course of the exchange current becomes slower than in controls. The kinetics of the exchange current in both controls and strophanthidin is better illustrated by the expanded records in Fig. 5 *B*. In both conditions, a single exponential provides a good fit to the initial decay (see, however, Tamura et al., 1991; Gray-Keller and Detwiler, 1994), but the time constant with strophanthidin is nearly twice as long as in controls, which is consistent with a decreased turnover of the exchanger. Collected values of the time constant of the exchange current in controls and after application of various concentrations of strophanthidin are reported in Table 1.

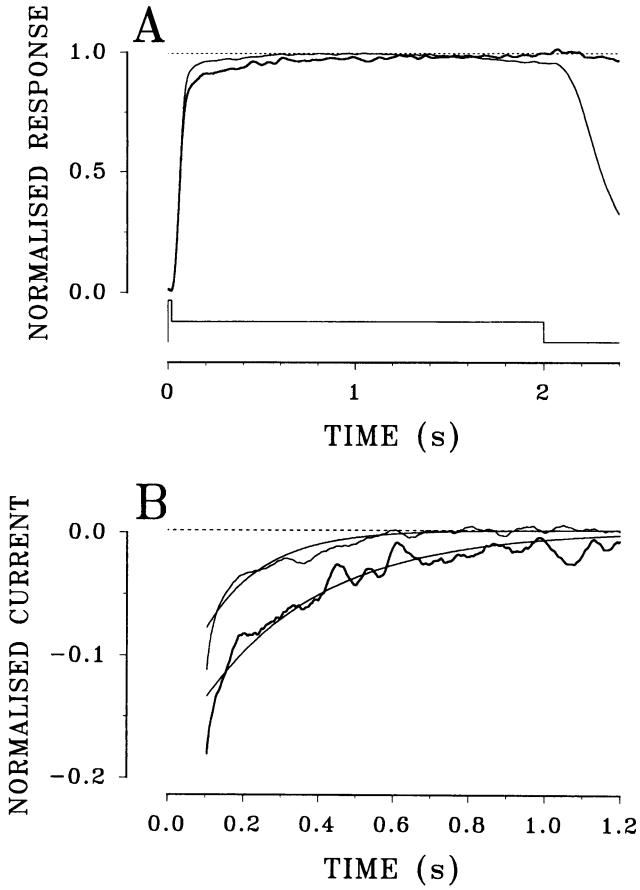

FIGURE 5 (*A*) Response of a guinea pig rod to a bright saturating light in control conditions (*thin trace*) and in the presence of 500 nM strophanthidin (*thick line*). The raised bar in the bottom trace is the light stimulus, consisting of a 20-ms bright flash (813 photons μm^{-2} $flash^{-1}$) simultaneous with the onset of a 2-s step (3051 photons μm^{-2} s^{-1}). (*B*) The transient current associated with the exchanger operation in both controls (*thin trace*) and 500 nM strophanthidin (*thick trace*) is shown on expanded scales. The smooth lines drawn to fit the experimental recordings are obtained assuming a single first-order process, with a time constant of decay (τ_{Ex}) of 152 ms in controls and 313 ms in strophanthidin. Control and strophanthidin records are the average of 18 and 17 sweeps, respectively, sampled at 250 Hz and filtered at 25 Hz. The dark current was −18.2 pA in controls and −5.5 pA in strophanthidin.

Somewhat surprisingly, the normalized amplitude of the light-insensitive component of the membrane current is higher in strophanthidin than in controls, which indicates a change in the relative membrane permeability to Ca^{2+}. This may be due to a direct effect of the pump inhibitor on channel selectivity. The experiment illustrated in Fig. 6 *B*, however, shows that the change in the apparent Ca^{2+} permeability, as well as other properties of light response, are in fact related to changes in $[Na]_i$.

A rod was first primed in low external sodium, until the light response was suppressed. Despite the fact that the outer segment sits inside the recording pipette, this procedure is apparently effective in lowering the sodium concentration around the outer segment membrane (see Materials and Methods). This usually required several minutes, after which the rod was returned to normal saline. Under these conditions, in which the Na^+ influx is reduced, the continued activity by the pump should appreciably lower $[Na]_i$ so that, upon rapidly returning to normal external saline, the transmembrane gradient of this ion should be transiently increased (see also Borsellino et al., 1985). Responses to flashes and steps of light from three guinea pig rods, taken shortly after returning to normal saline, consistently showed signs of a reduced effectiveness of light (i.e., the response duration to the saturating flash was shortened, the amplitude of the response to a step of light was reduced, and the time constant of the exchange current was accelerated), which may be taken as an indication of reduced light sensitivity. Similar phenomena are illustrated in the example of Fig. 6, where responses to a bright flash presented in darkness and during background illumination in normal saline, before and after priming the rod in low external Na^+, are compared. In Fig. 6 *A*, the rising phase of the flash response is the same in both conditions, the duration of the flash response and the initial current suppression by the background, however, are

TABLE 1

(1) Cell	(2) [Stroph]	(3) J_D^c	(4) J_D^s	(5) τ_{Ex}^c	(6) τ_{Ex}^s	(7) τ_{Ex}^c/τ_{Ex}^s
#1	2000	5.9	5.1	64.2	116.5	1.81
#2	500	18.2	5.5	152.4	313.7	2.06
#3	100	5.3	3.9	41.5	56.3	1.36
#4	20	18.7	16.2	66.2	89.1	1.35

Column (2) is the strophanthidin concentration in nM. Columns (3) and (4) are dark current amplitudes, in pA, before (J_D^c) and after strophanthidin (J_D^s). Columns (5) and (6) show the time constants of the exchanger, in ms, in control conditions (τ_{Ex}^c) and in strophanthidin (τ_{Ex}^s). Column (7) gives the ratio for the values in columns (5) and (6) (τ_{Ex}^s/τ_{Ex}^c). The average of the values in column (7) is significantly different from 1 ($p < 0.05$, $n = 4$, *t*-test).

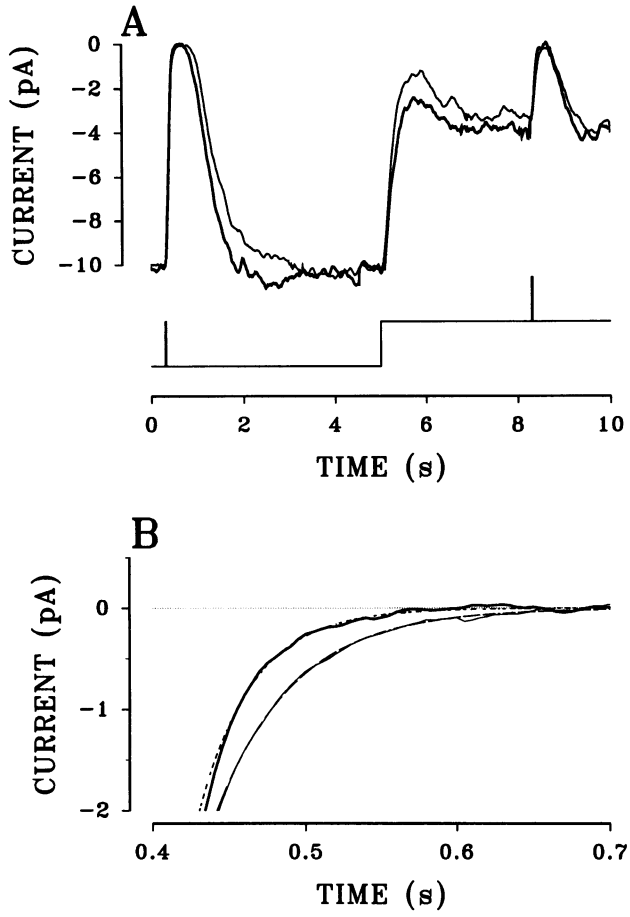

FIGURE 6 (*A*) The light response of a guinea pig rod is shown in control conditions (*thin trace*) and immediately after returning to normal saline (*thick trace*), after a 4-min exposure to a solution with NaCl substituted by choline chloride. During the first 3 min of priming in low sodium, the light response was nearly abolished. The suppression of the light response was taken as an indication that the sodium concentration inside the recording pipette was drastically reduced. This reduction is likely to be due to the imperfect seal between pipette and rod membrane (see also Materials and Methods). The light protocol, shown below the records, consisted in a saturating flash (813 photons μm^{-2} $flash^{-1}$), followed by a bright background (715 photons μm^{-2} s^{-1}) with the saturating flash superimposed. The control record is the average of 10 sweeps; the thick trace is the average of 8 sweeps recorded after exposing the rod twice to the low Na solution. The dark current was −10 and −9.7 pA in controls and low internal sodium, respectively. (*B*) The exchange current in controls (*thin trace*) and in low Na_i^+ (*thick trace*) of the same rod as in *A* are shown on an enlarged time scale. The smooth dotted lines drawn to fit the experimental recordings are obtained assuming a first-order process with a time constant of decay (τ_{ex}) of 51 ms in controls and 35 ms in low Na_i^+. Data were sampled at 200 Hz, filtered at 20 Hz, and digitally smoothed by a 50-ms-wide window.

reduced in conditions of low $[Na]_i$ (*thick trace*). These observations suggest that a decrease in $[Na]_i$ from the resting level is effective in changing internal Ca^{2+} and, therefore, the rod sensitivity, in a direction that is opposite to that observed when the $[Na]_i$ is made to increase by strophanthidin. A comparison of the relaxation of the exchange current in controls and low $[Na]_i$ supports this interpretation. As shown in Fig. 6 *B*, the time course of the exchange transient is significantly more rapid in low Na_i^+ (*thick trace*) than in controls (*thin trace*). The average change observed in three experiments was from 70 to 45 ms. Inspection of the normalized amplitude of the exchange transients indicates that in low Na_i^+ the apparent Ca^{2+} permeability is reduced. There is no simple explanation for this effect, and several factors may be involved: one could be the change in Na^+ driving force, and another could be the non-steady-state conditions of the exchanger.

A more rigorous approach to solve this specific point requires, however, a more precise control of the solution bathing the outer segment, which is lacking in the experiment described here.

Effects of the rapid application of strophanthidin

As pointed out in the preceding paragraph, the Na_i^+-turnover of mammalian rods is 15 mM s^{-1}, a value that is sufficiently rapid to set significant changes in $[Na]_i$ within the time scale of the response to a step of light. To evaluate whether the current reactivation observed during constant illumination is associated with a reduction in $[Na]_i$, the pump inhibitor was rapidly applied at the onset of a bright step of light. The results of this experiment are illustrated in Fig. 7. A puff of strophanthidin was delivered to the rod at the onset of illumination (see Materials and Methods). At this time, the internal Ca^{2+} and Na^+ regime must be the same as with the control medium; because of the drug action, however, the drop in $[Na]_i$ and the associated increase in the transmembrane gradient are now prevented. Blocking these changes is sufficient to suppress current re-activation. The dependence of the suppression of current reactivation on the drug concentration, as well as the fact that the kinetics of the flash responses before the application of strophanthidin is the same in all recordings, irrespective of the initial level of the dark current, support the idea that the effect observed is the consequence of the pump inhibition rather than the aspecific result of the partial loss of dark current occurring during the cell manipulations. The effects of inhibiting Na^+/K^+ ATPase on current reactivation are very rapid and suggest that in mammals the pump can control rod sensitivity on the same time scale as that of the light response.

DISCUSSION

The main finding reported in this paper is that the light-dependent decrease in sensitivity associated with light-adaptation in mammalian rods is suppressed by partial inhibition of Na^+/K^+ ATPase. This observation is consistent with a large body of evidence showing that any manipulation that lowers the Na^+ transmembrane gradient will necessarily reduce the efficiency of the Na^+:Ca^{2+}, K^+ exchanger and hence will interfere with adaptation (Matthews et al., 1988; Nakatani et al., 1988). The present results also suggest that a significant decrease in internal sodium occurs

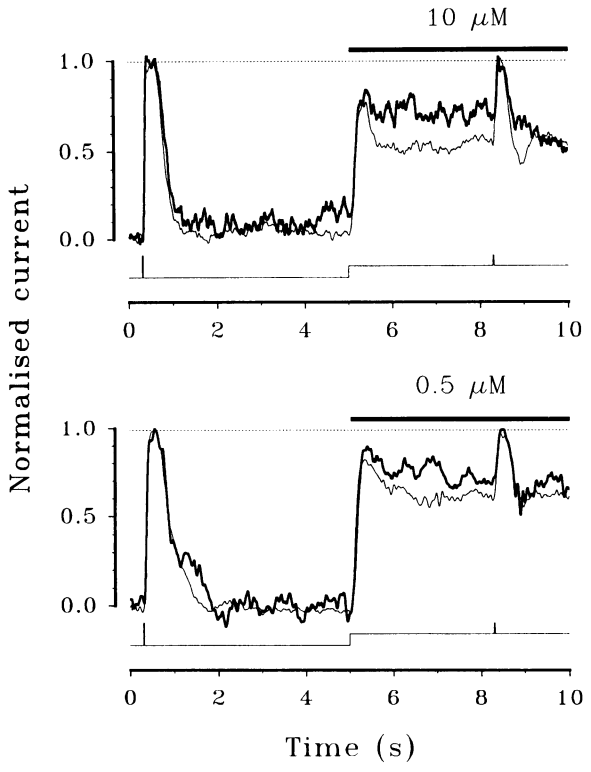

FIGURE 7 The effects of the rapid application of two different doses of strophanthidin on current reactivation during a bright steady light, in two guinea pig rods. Bars at the top indicate the onset of exposure to the drug. The drug was delivered by pressure ejection through a pipette placed at about 100 μm from the tip of the recording electrode. (*thin trace*) Control response; (*thick trace*) in strophanthidin. Numbers on top of the bars indicate the drug concentration within the ejecting pipette. The light stimulus, shown at the bottom of the lower panel, consisted of a saturating flash of 810 photons μm^{-2} $flash^{-1}$ followed by a bright steady light of 715 photons μm^{-2} s^{-1}. The traces are all normalized to the same amplitude. The dark currents for control and test traces are −15.2 and −9.1 pA for (*A*) and are the averages of 3 and 1 sweeps, respectively. For *B* the dark current of −20.3 and −16.3 pA are the average of 4 and 2 sweeps for controls and strophanthidin, respectively. Data were sampled at 250 Hz, filtered at 30 Hz, and digitally smoothed by a 20-ms-wide window.

during illumination and the change in $[Na]_i$ is sufficiently rapid and large to affect the exchanger on the time scale of the light response. Na_i^+ may be viewed, therefore, as an internal messenger regulating rod sensitivity.

Changes of $[Ca]_i$ and $[Na]_i$ during constant illumination

The results of the experiments in Fig. 7 support the idea that a significant fall in $[Na]_i$ does occur during background adaptation. The extent and the time course of this fall have been computed assuming that the main contributions to $[Na]_i$ come from the light-sensitive channel and Na^+/K^+-ATPase (see Eq. 7 in the Appendix). The results are illustrated in Fig. 8 *B* (*thick line*). For a typical mammalian rod (see definition in the Appendix), whose sodium turnover is 15 mM s^{-1}, a light background that completely stops the Na^+ influx is expected to bring $[Na]_i$ from 10 down to 7.5 mM in 200 ms.

Assuming that internal Ca^{2+} depends only on the properties of the exchanger, in a rod with a τ_{Ca} of 81 ms, in which $[Na]_i$ drops from 10 to 7.5 mM, $[Ca]_i$ is estimated to fall from a dark level of 240 to 13 nM within 200 ms of channel closure (*thick curve* in Fig. 8 *C*). With the same initial conditions, but with $[Na]_i$ clamped to 10 mM, $[Ca]_i$

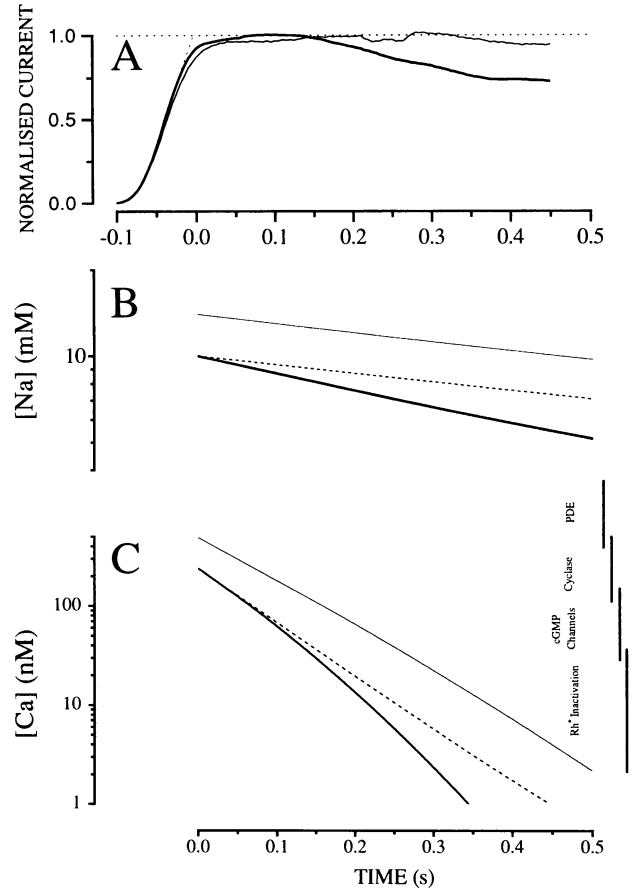

FIGURE 8 A) The responses of the guinea pig rod of Fig. 3 *B* to background illumination, in controls and in strophanthidin, are shown on an expanded time scale. (*B*) The thick line plots the change in $[Na]_i$ during a steady bright light, computed from Eq. 7, with K_p = 1.7 s^{-1}. The dashed line is computed by halving the ATP content of the rod, with a stoichiometric increase in ADP and P_i, which corresponds to an increase of $[Na_{eq}]_i$ from 1.4 to 7 mM. The thin solid line is computed from Eq. 7 when half of the pumping sites have been blocked by a prolonged exposure to a dose of strophanthidin, which causes $[Na]_i$ to increase from 10 to 14 mM. (*C*) The computed decrease in internal calcium, obtained by numerical integration of Eq. 14, is displayed for different initial conditions. The thick line shows how $[Ca]_i$ decays in time when associated with the fall of $[Na]_i$ shown by the thick line in *B*. The dotted line plots the $[Ca]_i$ change when the fall in internal sodium is abolished (e.g., by an instantaneous exposure to strophanthidin). The thin trace on the right illustrates the computed changes in internal calcium, during background illumination, after prolonged exposure to strophanthidin, when $[Na]_i$ has changed from 10 to 14 mM. Vertical bars at the right-hand side indicate the range of $[Ca]_i$ dependence for various processes known to occur in the rod outer segment. The vertical bar labeled as cGMP channels indicates the modulation of the light-sensitive channels affinity for cGMP by calmodulin (Hsu and Molday, 1993). The remaining bars indicate mechanisms described in the text.

will drop to about 20 nM within 200 ms (*dashed curve*, Fig. 8 *C* from Eq. 14 in the Appendix).

A $[Ca]_i$ decrease in these ranges would be especially effective if coupled to a process bearing a steep dependence on $[Ca]_i$. An event with similar properties may be identified in the formation of catalytically active rhodopsin, which has been shown to depend on calcium with a half-activation of 35 nM and a Hill coefficient of ~2 (Lagnado and Baylor, 1994). From these data, it can be estimated that a $[Ca]_i$ drop from 20 to 13 nM would cause a decrease in the formation of active rhodopsin of 15%.

In discussing the significance of the contribution of the Na^+/K^+-pump in further lowering $[Ca]_i$, one should consider that this action also depends on the starting level of $[Ca]_i$ (i.e., the $[Ca]_i$ dark level). In our analysis, we have used for $[Ca]_i$ in controls a starting value of 240 nM. This figure has been derived from measurements of the exchanger current in the rod of Fig. 2 (see Appendix). With an initial $[Ca]_i$ of 500 nM, after 200 ms of bright illumination, with constant Na_i, $[Ca]_i$ will approach 36 nM. Allowing then Na_i to change, $[Ca]_i$ will drop to 25 nM. The corresponding decrease in catalytically active rhodopsin would be in this case ~30%. It should be noted that the dark level of $[Ca]_i$ in amphibian rods has been estimated to be 273 nM (Korenbrot and Miller, 1989), 240 nM (Ratto et al., 1988), 410 nM (Lagnado et al., 1992), and 550 nM (Gray-Keller and Detwiler, 1994).

In our analysis, we also assumed that during prolonged illumination, in the absence of Ca^{2+} influx, $[Ca]_i$ will approach the equilibrium level of the exchanger (100–200 pM). This assumption is supported by recent measurements of $[Ca]_i$ in the whole retina, showing that prolonged illumination with saturating light lowers $[Ca]_i$ down to values close to the equilibrium level of the exchanger (McCarthy et al., 1994). Other results obtained from isolated rod outer segments, however, give values of $[Ca]_i$ during illumination that are two orders of magnitude higher (50–100 nM) (Schnetkamp et al., 1991; Schnetkamp and Szerencsei, 1993; Gray-Keller and Detwiler, 1994).

The variability in the values of $[Ca]_i$ obtained from these measurements may in part reflect differences in methodology but, possibly, it also reflects different functional conditions of the visual cells. As suggested by the present results, a rod with a high $[Na]_i$, caused by a sluggish pumping, will also set $[Ca]_i$ to high levels.

$[Na]_i$ and $[Ca]_i$ changes during constant illumination in the presence of strophanthidin

An estimate of the change in $[Na]_i$ caused by the application of strophanthidin is obtained in the Appendix from a relation based on the dependence of the Ca^{2+}-dissociation constant of the exchanger on $[Na]_i$. Considering the data illustrated in Fig. 2, where the time constant of the exchange current is increased by the pump inhibitor from the control value of 66 to 89 ms (cell #4 in Table 1), $[Na]_i$ is estimated to increase from 10 to 14 mM. Applying the same procedure to the rod of Fig. 4 treated with a higher concentration of the inhibitor (cell #2 in Table 1), we obtain a larger change in $[Na]_i$ (from 10 to 20 mM). The change in $[Ca]_i$, associated with an increased $[Na]_i$, can be evaluated by the relationship between the fractional activation of the exchanger and its dissociation constant for calcium (K_{Ca}). In the rod of Fig. 2, we estimate that strophanthidin causes a $[Ca]_i$ increase from a control value of ~240 to 490 nM. Thus, increasing $[Na]_i$ from 10 to 14 mM will double the dark level of internal free calcium.

The fall in $[Ca]_i$ in strophanthidin, computed starting from initial values of 14 and 490 nM for $[Na]_i$ and $[Ca]_i$, respectively, is plotted in Fig. 8 *C* (*thin curve*). It should be noted that in strophanthidin the value of $[Ca]_i$ is still expected to decrease, but at a rate slower than in controls. As a consequence, adaptation will not be abolished, but reduced. In evaluating the consequences of strophanthidin application on the processes taking place at the outer segment, it is interesting to note that maximal inhibition of guanylate cyclase (GC) occurs for $[Ca]_i$ values of ~400 nM (Koch and Stryer, 1988; Dizhoor et al., 1991; Gorczyca et al., 1994) (see Kaupp and Koch (1992) for a review). Therefore, one may conclude that the sensitivity changes observed in strophanthidin are brought about mainly by a decreased synthesis of cGMP. This conclusion is also supported by the observation that the ratio of the light sensitivity in strophanthidin and in controls is steeply dependent on the inhibitor concentration (Hill coefficient of 2.5, Fig. 2 *D*). A similar steepness has been reported for the relation between GC inhibition and $[Ca]_i$ (Koch and Stryer, 1988, Dizhoor et al., 1991; Gorczyca et al., 1994). Activation of phosphodiesterase, which occurs in a range of $[Ca]_i$ of 400-1000 nM (Kawamura, 1993), may also play some role in the sensitivity changes caused by strophanthidin.

Light-adaptation and rod metabolism

The dependence of adaptation on the Na_i^+ turnover is likely to be critical in mammalian rods where the metabolic load is particularly heavy, both because of temperature conditions and the large Na^+ current relative to small outer segment cytoplasm volume. An estimate of the surface/volume ratio for an average mammalian rod indicates a value which is about 5 times larger than in amphibians. The marked dependence of light adaptation on the Na^+/K^+ ATPase activity in mammalian rods suggests that any fluctuation in the rod energetic metabolism will affect adaptation. The dashed curve in Fig. 8 *B* shows the rate of sodium fall occurring during illumination, when halving the concentration of internal ATP (see Eq. 10 in the Appendix). Considering the effect of the pumping rate on the response to background illumination, it seems reasonable to conclude that a small decrease in ATP synthesis will interfere effectively with rod adaptation. This may offer a simple explanation for the variability in the adapting properties often

reported for mammalian rods (Baylor et al., 1984; Kraft et al., 1993). Another intriguing implication of the dependence of light adaptation on the metabolic rate of rods is that minor fluctuations in oxygen tension or metabolites in the retinal and/or choroidal circulation may produce rapid changes in the rod adapting behavior. It is perhaps important to note that similar changes may easily be detected in human subjects by simply testing visual adaptation.

This work was supported by European Community (SC*-0224-C) and by MURST (40%) grants to L.C.

APPENDIX

Estimates of Na_i^+ changes during a saturating step of light

From Eq. 15 of Lagnado et al. (1992), we have

$$\tau_{Ca} = \frac{F\,\nu\,B\,K_{Ca}}{J_{Sat}\,K_{Buff}}, \tag{1}$$

where v is the rod free volume, B is the capacity of the Ca^{2+}-buffer, K_{Ca} the Michaelis constant of the exchanger activation by internal Ca^{2+}, K_{Buff} the Michaelis constant of the Ca^{2+} buffer, J_{Sat} is the maximum exchange current, and F is the Faraday constant. Assuming that the only parameter that is affected by $[Na]_i$ is K_{Ca}, Eq. 1 can be rewritten as

$$\tau_{Ca} = A\,K_{Ca}, \tag{2}$$

Because K_{Ca} is linearly related to $[Na]_i$ over a range of 10–40 mM (Lagnado et al., 1992) and assuming that τ_{Ca} is approximated by the time constant of the exchange current (τ_{Ex}), we obtain the empirical relation

$$\tau_{Ex} = A'\,[Na]_i, \tag{3}$$

with $A' = 0.00017 \cdot A$ (see Table 2 and Fig. 7 of Lagnado et al. 1992).

The influx of sodium in darkness (J_{Na}), for a typical mammalian rod (v = 0.023 pl; dark current J_D = 30 pA; time constant of the Na^+:Ca^{2+}, K^+ exchanger τ_{Ex} = 81 ms; time constant of the Na^+/K^+ pump (τ_P = 600 ms), is given by the dark current minus the current carried by calcium, minus the electrogenic component of the exchanger (J_{ex}), plus the stoichiometric fraction of the exchange current (4 J_{Ex}). Assuming that the dark current is mainly carried by Na^+ and Ca^{2+} and neglecting possible sources of Na^+ from the inner segment, the change in $[Na]_i$ is given by

$$\frac{d[Na]_i(t)}{dt} = \frac{J_{Na}(t)}{F\nu} - K_P\,([Na]_i(t) - [Na_{Eq}]_i) + \frac{4J_{Ex}(t)}{F\nu}, \tag{4}$$

where $[Na_{Eq}]_i$ is the sodium equilibrium of the Na^+/K^+ pump and K_p is the rate constant (τ_p^{-1} = 1.7 s^{-1}). When all the light-sensitive channels are closed by a saturating step of light (t = 0), the term $J_{Na}(t)\,(F\nu)^{-1}$ approaches zero and Eq. (4) becomes

$$\frac{d[Na]_i(t)}{dt} = -K_P([Na]_i(t) - [Na_{Eq}]_i) + \frac{4J_{Ex}(0)}{F\nu}\,e^{-\frac{t}{\tau_{Ex}}}. \tag{5}$$

The solution of eq. 5 is

$$[Na]_i(t) = \left([Na]_i(0) - [Na_{Eq}]_i - \frac{4J_{Ex}(0)}{F\nu}\,\frac{1}{K_P - 1/\tau_{Ex}}\right)e^{-(K_P t)} + \frac{4J_{Ex}(0)}{F\nu}\,\frac{1}{K_P - 1/\tau_{Ex}}\,e^{-\left(\frac{t}{\tau_{Ex}}\right)} + [Na_{Eq}]_i, \tag{6}$$

where $[Na]_i$ (0) is the internal sodium at t = 0 (which we assume to be 10 mM), $[Na_{Eq}]_i$ is the sodium equilibrium level of Na^+/K^+ ATPase. When t = $2\tau_{Ex}$ the contribution to Na^+ influx by the exchanger is very small, then $[Na]_i$ (t) is given by

$$[Na]_i(t) = ([Na]_i(0) - [Na_{Eq}]_i)e^{-tK_P} + [Na_{Eq}]_i, \tag{7}$$

where $[Na]_i$ (0) is the concentration of intracellular sodium in the dark and K_P is the rate of the sodium-potassium pump. $[Na_{Eq}]_i$ can be obtained by writing the energy balance of the pumping process:

$$\Delta G_{ATP} + \Delta G_{Na} + \Delta G_K = 0, \tag{8}$$

where ΔG_{ATP} is the free energy associated with ATP hydrolysis, and ΔG_{Na} and ΔG_K are those associated with ion transport. With a stoichiometry of the pump of 3 Na^+ and 2 K^+ transported against their electrochemical gradient for each ATP molecule hydrolyzed, we have

$$[Na_{Eq}]_i = [Na]_o e^{\left(\frac{\Delta G_{ATP} + zF\Delta V + 2RT\ln\frac{[K]_i}{[K]_o}}{3RT}\right)}. \tag{9}$$

Taking ΔG_{ATP} = −14,300 cal M^{-1} (Läuger, 1991), $[K]_i$ = 120 mM and assuming, during illumination, a transmembrane potential (ΔV) of 60 mV, $[Na_{Eq}]_i$ = 1.4 mM.

The effect of changing the levels of ATP on $[Na_{Eq}]_i$ can be evaluated from

$$\Delta G_{ATP} = \Delta G_0 + RT\ln\frac{[ADP][P_i]}{[ATP]} \tag{10}$$

where if [ATP] falls from a control level of 5 to 2.5 mM (with a stoichiometric increase in [ADP] and $[P_i]$ from the control values of 100 μM and 1.5 mM to 2.6 and 4 mM, respectively) the value of $[Na_{Eq}]_i$ will increase from 1.4 to 7 mM. The computed changes in $[Na]_i$ in various conditions are illustrated in Fig. 8. It must be emphasized, however, that because of the possible Na^+ influx (not considered here) through voltage-gated channels at the inner segment (Demontis and Cervetto, 1995), the estimate for the fall in $[Na]_i$ during illumination represents an upper limit.

Changes in Ca_i^{2+} during a saturating step of light

From Tamura et al. (1991) we have that the exchange current (J_{ex}) is given by

$$J_{Ex} = J_{Sat}\,\frac{[Ca]_i - [Ca_{Eq}]_i}{[Ca]_i - [Ca_{Eq}]_i + K_{Ca}} \tag{11}$$

where $[Ca_{Eq}]_i$ is the equilibrium concentration for the intracellular free calcium (see below). Considering K_{Ca} linearly related to $[Na]_i$ (see Eq. 3), assuming for J_{Sat} the value of 5.2 pA measured in monkey rods by Tamura et al. (1991) and taking for J_{Ex} the values from the experiment in Fig. 2 in controls (0.65 pA) and in strophanthidin (0.9 pA), then the initial $[Ca]_i$ is 240 nM in controls and 490 nM in strophanthidin.

When all the light-sensitive channels are closed, the influx of calcium is interrupted, whereas extrusion is continued for some time by the operation of the exchanger. Assuming that the only source for Ca_i^{2+} is the influx at the outer segment, it follows that the internal free calcium will reach the equilibrium level in a period of time that depends on the kinetics of the exchanger and on the properties of the buffering systems.

The change in total internal calcium Ca_T, which includes free calcium $[Ca]_i$ and calcium bound, is then given by

$$\frac{dCa_T(t)}{dt} = -\frac{J_{Ex}}{F\nu} \tag{12}$$

Considering for simplicity the properties of a single buffer, which in agreement with Lagnado et al. (1992) is in rapid equilibrium with Ca^{2+} (see, however, Gray-Keller and Detwiler, 1994):

$$Ca_T = [Ca]_i\left(1 + \frac{B}{[Ca]_i + K_{Buff}}\right) \quad (13)$$

where K_{Buff} is the dissociation constant of the buffer and B is the maximum buffering capacity. These constants were chosen so as to have $\tau_{Ca} = 81$ ms (see Eq. 1) and the values obtained ($K_{Buff} = 2.7$ μM and $B = 300$ μM) are in good agreement with those reported by Tamura et al. (1991). Combining Eqs. 11, 12, and 13, we obtain

$$\frac{d[Ca]_i(t)}{dt} = -\frac{([Ca]_i(t) + K_{Buff})^2}{([Ca]_i(t) + K_{Buff})^2 + BK_{Buff}} \frac{J_{Sat}}{F\nu} \frac{[Ca]_i(t) - [Ca_{Eq}]_i}{[Ca]_i(t) - [Ca_{Eq}]_i + K_{Ca}} \quad (14)$$

where $[Ca_{Eq}]_i$ is the calcium equilibrium concentration given by the energetic equilibrium of the exchange (Blaustein and Hodgkin, 1969; Cervetto et al. 1989).

The time course of the intracellular calcium has been computed by numerical integration of Eq. 14, assuming that Eq. 3 holds down to 6 mM $[Na]_i$, and the results are shown in graphical form in Fig. 8 *C*.

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