

# Blockage of the Light-sensitive Conductance in Hyperpolarizing Photoreceptors of the Scallop

## *Effects of Tetraethylammonium and 4-Aminopyridine*

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**ABSTRACT** The tight-seal whole-cell recording technique was used to examine the effect of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on the photocurrent of hyperpolarizing ciliary photoreceptors isolated from the distal retina of the bay scallop (*Pecten irradians*). In these cells, light causes an increase in a conductance that is highly selective to potassium ions. Extracellular application of TEA at a concentration of 50 mM produced a modest, reversible block ( $\approx 35\%$  at  $-20$  mV holding potential). The blockage was weakly voltage dependent, increasing by  $\sim 20\%$  for a 20-mV hyperpolarization, suggestive of a site of interaction superficially located within the electric field of the membrane. Treatment with TEA produced no significant changes either in the light sensitivity of the photocurrent or in its kinetics. The effects of superfusion with 4-AP were more dramatic: the light-evoked current was nearly abolished ( $> 95\%$ ) at submillimolar concentrations, with a half-maximal dose of  $\sim 0.6 \mu\text{M}$ . The blockage had a rapid onset and was slowly reversible. No significant use or voltage dependency were observed. A number of control experiments indicated that the phototransduction cascade remained functional during treatment with 4-AP: the early receptor current, the prolonged aftercurrent and its suppression, the photoresponse kinetics and the light sensitivity of the cell were little affected by 4-AP, suggesting that the suppression of the photocurrent is due to blockage of the light-sensitive channels, rather than impairment of some of the activation steps. The results are discussed in the light of a possible kinship between the light-activated potassium channels of invertebrate hyperpolarizing photoreceptors and the family of rapidly-inactivating voltage-dependent potassium channels, which typically exhibit high susceptibility to blockage by this drug.

### INTRODUCTION

Pharmacological blockers have played an important role in the study of ion channels for several reasons: (a) to dissect components of the electrical behavior of a cell; (b) to

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help establish categories of ion channels; (c) to provide information on the structure-function of the channel protein; (d) as aids in biochemical isolation of the channels. In photoreceptor cells, particularly from invertebrate organisms, there has been a paucity of candidate agents that may target the light-sensitive channels. The task of identifying them is rendered particularly difficult by the fact that the channel's gating is controlled by a complex sequence of intracellular events that couple rhodopsin isomerization to conductance changes in the plasma membrane. As a consequence, any antagonistic pharmacological action on the photoresponse must be scrutinized to determine whether some element of the activation cascade was compromised, rather than the channel's ability to transport ions.

The bay scallop, *Pecten irradians*, is one of several invertebrate organisms that possess ciliary photoreceptors that hyperpolarize in response to light stimulation (Gorman and McReynolds, 1969; McReynolds and Gorman, 1970*a,b*). In those cells, the photocurrent is mediated by an increase of a potassium-selective conductance in the plasma membrane (Gorman and McReynolds, 1978; Cornwall and Gorman, 1983). However, in one published study using intracellular recording in the intact retina, the photoresponse displayed little sensitivity to TEA and to Cs, two of the most common blockers of K channels (Cornwall and Gorman, 1983*a,b*). This finding is surprising, in view of the many essential similarities between the permeation properties of the light-sensitive conductance of hyperpolarizing photoreceptors and other classes of K conductances (Gorman, Woolum, and Cornwall, 1982). In fact, it had been suggested that the transduction channels in these cells may be Ca-activated K channels, calcium ions serving as the final intracellular messenger in the transduction cascade (Gorman and McReynolds, 1974). TEA is a particularly effective antagonist of Ca-activated K conductances: for example, in molluscan neurons half-maximal blockage has been reported at submillimolar extracellular concentration (Hermann and Gorman 1981*a*). The alleged lack of sensitivity of the scallop photocurrent to some conventional pharmacological blockers of K conductances, if substantiated, may point to a basic dissimilarity between light sensitive K channels and the extensive family of voltage- and calcium-gated K channels. Therefore, we explored the issue further, using enzymatically isolated hyperpolarizing receptors (Gomez and Nasi, 1991, 1994*a*), which can be superfused without the hindrance of the surrounding matrix of tissue, and voltage clamped by the whole-cell technique. We have found that TEA does exert a modest, but significant block. By contrast, 4-AP, a K-channel antagonist particularly effective on the rapidly inactivating K currents ( $I_a$ ) found in many excitable cells (Rudy, 1988), proved to be an extremely powerful blocker of the light-sensitive conductance. Some of these results have been presented in abstract form (Gomez and Nasi, 1994*b*).

#### METHODS

Specimens of *Pecten irradians* were obtained from the Marine Resources Center at the Marine Biological Laboratory (Woods Hole, MA), and used immediately. The protocol for dispersing the retina of these mollusks has been described previously (Nasi and Gomez, 1992; Gomez and Nasi, 1994*a*). Cells were plated in a recording flow chamber pretreated with concanavalin A to promote cell adhesion, as previously described (Nasi, 1991*a*).

Artificial sea water (ASW) contained 480 mM NaCl, 10 mM KCl, 49 mM MgCl<sub>2</sub>, 10 mM

CaCl<sub>2</sub>, 10 mM HEPES, and 5 mM glucose, pH 7.8 (NaOH). Tetraethylammonium (TEA) ASW contained 50 mM TEA-Br (Sigma Chemical Co., St. Louis, MO) replacing NaCl. 4-aminopyridine (>99% pure; Aldrich Chemical Co., Milwaukee, WI) was simply added to ASW. All experiments were conducted at room temperature (22–24°C). Patch electrodes were fabricated with thin-wall (1.5 mm o.d. 1.1 mm i.d.) borosilicate capillary tubing (type 7052, Garner Glass, Claremont, CA) pulled to a 2–3 μm tip diameter and fire-polished immediately before use. Electrode resistance, measured in ASW, was 2–6 MΩ. The “intracellular” filling solution contained 100 mM KCl, 200 mM K-aspartate or K-glutamate, 6 mM Na<sub>2</sub>ATP, 6 mM MgCl<sub>2</sub>, 10 mM HEPES, 1 mM EGTA, 100 μM GTP and 300 mM sucrose, pH 7.3. In all recordings series resistance errors were corrected via a positive feedback circuit in the amplifier (maximal residual error typically < 2 mV). Currents were low-pass filtered with a Bessel 4-pole filter, using a cutoff frequency of 500–2,000 Hz and digitized on-line at 2–5 KHz sampling rate, 12-bit resolution by an analog/digital interface board (model 2821, Data Translation, Marlboro, MA) installed in a microcomputer, and stored on Bernoulli cartridges (Iomega, South Roy, Utah). Voltage- and light-stimuli were applied by a microprocessor-controlled programmable stimulator (Stim 6, Ionoptix, Milton, MA).

Light steps were provided either by a 100 W tungsten-halogen optical stimulator, the beam of which was combined with that of the microscope illuminator via a beam splitter prism, as previously described (Nasi, 1991*a,b*; Nasi and Gomez, 1992), or by a 75 W Xenon arc lamp (PTI, So. Brunswick, NJ) coupled by a fiber optics light guide to the epifluorescence port of the inverted microscope (Gomez and Nasi, 1994*a*). Light intensity was measured with a calibrated radiometer (model 370, UDT Hawthorne, CA). An *in vivo* calibration ( $n = 3$ ) was performed, as previously described (Gomez and Nasi, 1994*a*), to compare the effect of monochromatic stimulation (500-nm peak, 10-nm half width; Ditric Optics, Hudson, MA) with that of white or broadband (515–650 nm) light used in most experiments, in order to provide a quantitative reference. In the experiments described below, light intensity is expressed in terms of equivalent photon flux at 500 nm; when light attenuation was varied in a given protocol, the relative light intensity is expressed as  $\log_{10}(I/I_0)$ , where  $I_0$  is the intensity of the unattenuated beam light. Calibrated neutral-density filters (Melles Griot, Irvine, CA) provided controlled attenuation. During experimental manipulations the cells were viewed with a Newvicon TV camera (model WV-1550; Panasonic, Secaucus, NJ) using a near-IR long-pass filter for illumination ( $\lambda > 780$  nm; Andover Corporation, Salem, NH). The infrared illuminator was turned off for several minutes before testing light responses.

## RESULTS

We first re-examined the effect of TEA on the photocurrent of dissociated cells, to ascertain whether the marginal blockage previously observed in whole retinas may have in part resulted from restricted access of the drug to its target sites when the cells are embedded in a tight matrix of retinal tissue. Fig. 1 shows the result of an experiment in which repetitive light stimuli of moderate, constant intensity were used to monitor the effect of superfusing a voltage-clamped cell with 50 mM TEA. In Fig. 1*A* the peak amplitude of the response is plotted as a function of time during the control recording in ASW, in the presence of TEA and upon washing the drug. The onset of the blockade was relatively rapid, the delay of the effect reflecting essentially the exchange time of the perfusion chamber (1–2 min); the photocurrent remained substantially depressed throughout the TEA treatment. Removal of the drug promptly restored the response amplitude to control levels. Fig. 1*B* shows three superimposed current traces obtained in ASW, in the presence of 50 mM TEA and

after wash. The magnitude of the blocking effect, calculated as  $1-(C_d/C_c)$  ( $C_d$  being the current in the presence of the drug and  $C_c$  the control current amplitude), was 36% ( $\pm 12$  SD,  $n = 7$ ) for 50 mM TEA at  $V_h -20$  mV. A lower concentration of TEA (10 mM) was also tested ( $n = 2$ ) and produced a marginal blockage of  $\approx 10\%$  (not shown). The response reduction was not accompanied by any substantial alteration of the photocurrent shape. In Fig. 1 C, a response before TEA and during steady state block were normalized and superimposed, and exhibit similar time course.

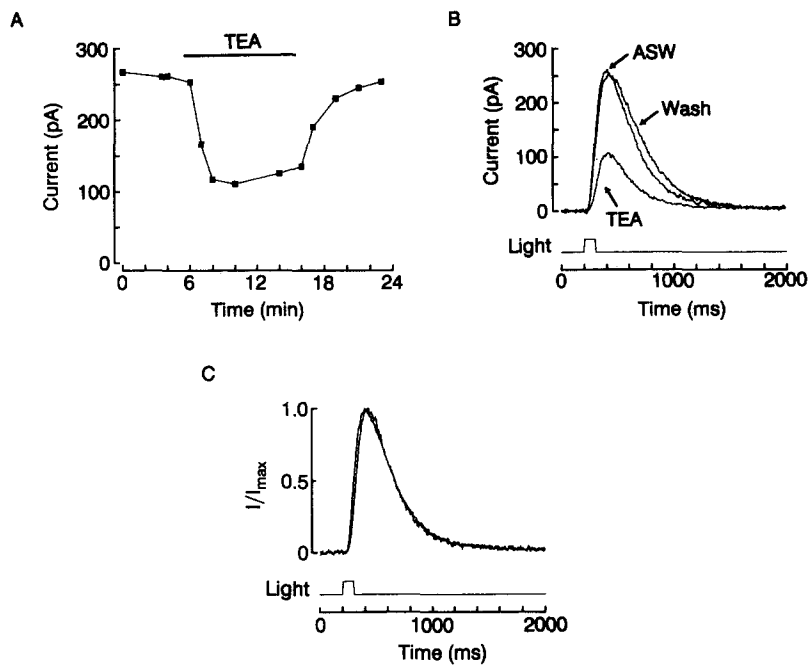


FIGURE 1. Effect of TEA on the photocurrent. A ciliary scallop photoreceptor was voltage clamped at  $-20$  mV and repetitively stimulated with a 100-ms light of fixed intensity ( $6.3 \cdot 10^{13}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ). (A) Peak amplitude of the photocurrent plotted as a function of time. The cell was initially superfused with ASW, then the solution was switched to 50 mM TEA for  $\sim 10$  min, and finally returned to ASW. (B) Superimposed photocurrent traces recorded in the three phases of the experiment. (C) Normalized light-evoked currents before and during treatment with TEA, to illustrate the lack of kinetic changes induced by the blocker.

The treatment with TEA does not induce any significant shift in the light intensity range over which the photoreceptors operate. Fig. 2 A shows two families of photocurrents in response to a flash of increasing intensity spanning a range of nearly 5 log, delivered at a steady holding voltage in ASW (*left*) and during superfusion with 50 mM TEA (*right*). In Fig. 2 B the peak amplitude of the photoresponse at each light intensity was plotted for the two conditions, and a sigmoidal function was fitted to the data by the method of the least squares; only a very small displacement of the curve along the abscissa is observed after exposure to

TEA. The average shift in light intensity producing half-maximal activation was 0.3 log ( $\pm 0.18$  SD,  $n = 7$ ).

TEA is a cation, and in several classes of channels it exerts its blocking action when applied extracellularly, by entering the permeation pore and binding to a site within the dielectric layer. As a result, transmembrane voltage can affect the binding, giving

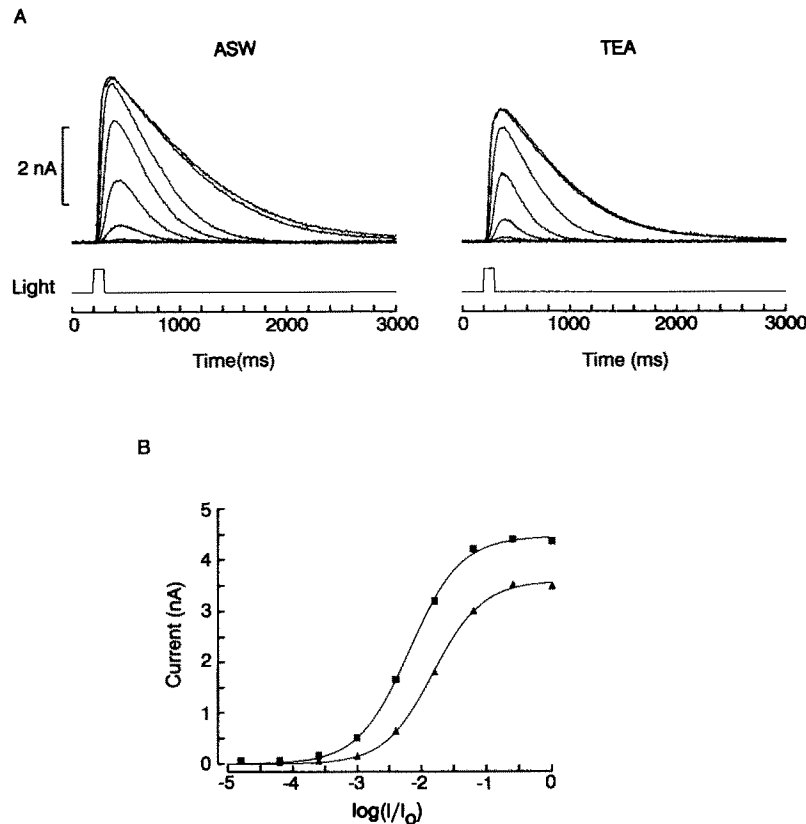


FIGURE 2. Lack of effects of TEA on light sensitivity. (A) Intensity series obtained in a cell bathed in ASW (*left*) or 50 mM TEA (*right*). Light flashes (100 ms, unattenuated intensity  $11 \cdot 10^{16}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ) were presented every 60 s at  $V_h = -40$  mV, increasing the intensity at 0.6 log increments. (B) Plot of the peak amplitude of the photocurrent as a function of light attenuation. (Squares) ASW; (triangles) TEA. Data points were empirically fitted by the least-squares criterion, with the smooth sigmoidal curves of the form  $y = A / (1 + \exp [B \cdot (K_{1/2} - x)])$ , where  $x = \log(I/I_0)$ ,  $K_{1/2}$  is the half-saturating light intensity,  $A$  is the asymptotic response, and  $B$  is a positive constant.

rise to a voltage dependency of the block (Hermann and Gorman, 1981a). In Fig. 3A, a constant light stimulus was applied at different holding potentials ( $-60$  to  $0$  mV at 20-mV increments) in ASW (*left*) or in the presence of 50 mM TEA (*right*). The amplitude of the photocurrent diminished as the membrane was hyperpolarized, and the drug reduced the current at all potentials. In Fig. 3B, the peak photocurrent in

the two conditions was normalized to the maximum current in ASW, and plotted as a function of membrane potential (average of four cells). The extent of the blockage at each holding potential is shown in Fig. 3 C. The resulting graph exhibits a modest, though consistent voltage dependence, increasing by ~20% for a 20-mV hyperpolar-

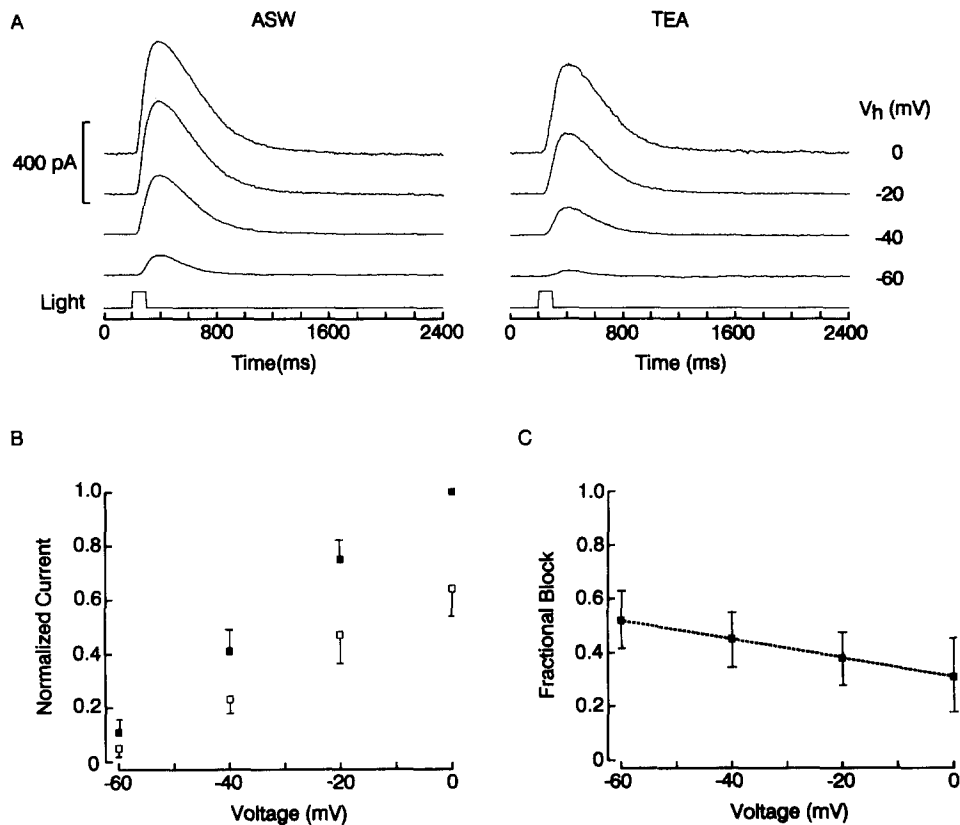


FIGURE 3. Voltage dependence of the block by TEA. (A) Families of photocurrent records in response to flashes of fixed intensity ( $2.4 \cdot 10^{14}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ) delivered at different holding potentials ( $-60$  to  $0$  mV, at  $20$ -mV increments) in control solution (*left*) and during superfusion with  $50$  mM TEA (*right*). (B) Relative amplitude of the photocurrent as a function of the holding voltage in ASW (*filled squares*) and TEA (*open squares*). The data points represent the average of four cells tested under conditions identical to A; for each cell the photocurrents were normalized with respect to the value obtained in ASW at  $0$  mV. Standard deviations are indicated by the error bars. (C) Fractional block as a function of membrane potential. Blockage of the photocurrent, averaged for the same four cells of B, is plotted for each value of the holding potential. A consistent reduction of block with membrane depolarization is clearly visible.

ization. The effect is statistically significant ( $P < .05$ ), as demonstrated by Friedman's nonparametric analysis of variance (Hollander and Wolfe, 1973). This is suggestive of the notion that the binding site for the blocker is located somewhat within the electrical field of the membrane.

The results described above do provide evidence for an inhibitory action of TEA on the outward potassium photocurrent of scallop ciliary cells; the effect, however, was rather modest and required a substantial concentration of the drug. Another well-known blocker of potassium selective channels is 4-aminopyridine (4-AP). Although at concentrations of several millimolar this substance has been shown to block a variety of K channels, it has a particularly high affinity for transient or rapidly inactivating channels ( $I_a$ ), where half-maximal blockage has been observed at submillimolar concentrations (e.g., Howe and Ritchie, 1991; Choquet and Korn, 1992; Castle and Slawsky, 1992). Fig. 4 shows the effect of superfusing a ciliary *Pecten*

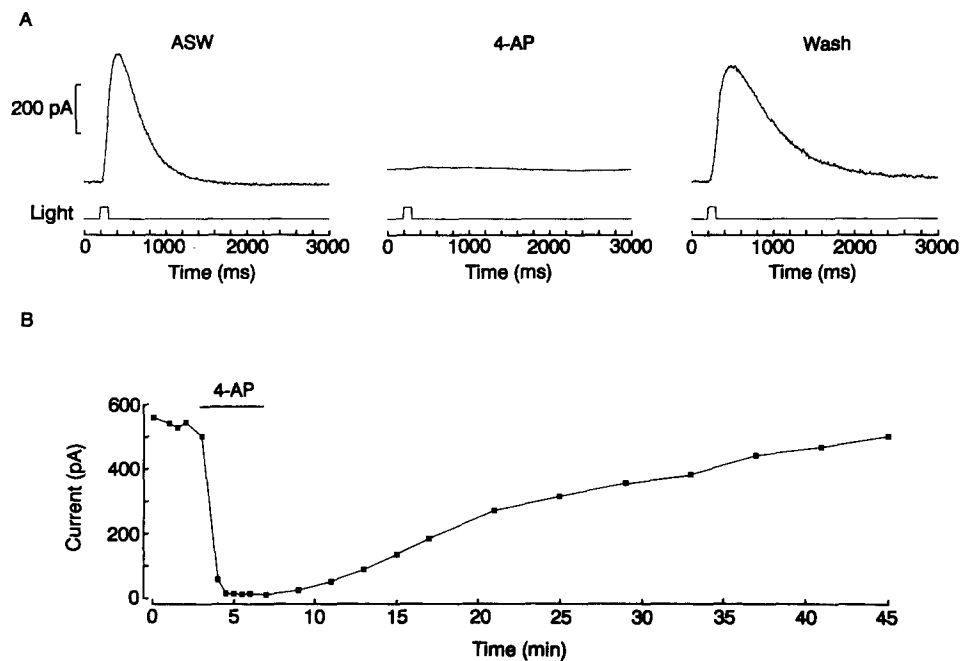


FIGURE 4. Suppression of the light-induced current by 4-AP. (A) Photocurrents elicited by 100-ms flashes ( $6.3 \cdot 10^{13}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ) at a holding voltage of  $-25$  mV in ASW (left), in the presence of 0.5 mM 4-AP (middle), and after prolonged wash with ASW (right). (B) Peak amplitude of the current evoked by repetitive light stimulation, plotted as a function of time. The photoresponse was abruptly suppressed shortly after superfusing with 4-AP (solid bar), and slowly recovered by washing extensively in ASW.

photoreceptor with 500  $\mu$ M 4-AP. The current traces in Fig. 4 A illustrate the response to 100-ms flashes of moderate intensity by a cell voltage clamped at  $-25$  mV. The cell was bathed initially in ASW, then 4-AP was applied for  $\sim 4$  min, and finally the drug was washed during a prolonged interval. 4-AP caused almost complete suppression of the photocurrent ( $>98\%$ ), the effect being largely reversible. In Fig. 4 B the peak amplitude of the light-activated current elicited by repetitive light stimuli is plotted as a function of time during the three phases of the experiment. The blockage occurred very shortly after the introduction of the drug.

By contrast, the washout of the effect was quite slow, and recovery ( $\approx 95\%$ ) required over 35 min ( $n = 2$ ).

The drastic effect of 4-AP at submillimolar concentration was rather surprising, because there was little reason to expect much similarity between the rapidly inactivating voltage-gated K currents that the drug preferentially targets, and the scallop light-sensitive K current, which is not gated by voltage. Because, as previously pointed out, light-dependent channels are activated via a complex biochemical cascade, a first issue to address is the possibility that such dramatic antagonistic effect may in part result from interference with some step of the transduction process, rather than solely from an effect on the channels themselves (see Fain and Lisman, 1981 for a discussion). In the specific case of 4-AP, such possibility is rendered particularly plausible by the fact that the drug is a weak organic base ( $pK \approx 9.2$ ; Albert, 1963) which therefore exists in a charged and uncharged form. The latter, which at pH 7.8 constitutes  $\sim 4\%$  of the total, can partition into the cell membrane and is readily capable of permeating the cell. As a result, superfusion with 4-AP could conceivably impair other elements of the phototransduction cascade, both intracellular (e.g., the cytosolic enzymes) and membrane-imbedded (e.g., photopigment).

As a first approach to ascertain possible indirect effects on the photocurrent we examined whether the cell sensitivity to light was affected by exposure to 4-AP; a substantial shift in the operating range of the cell, for example, could account for the greatly reduced response to a stimulus of moderate amplitude. Such a possibility can be readily assessed by testing with light-intensity series, because the saturating response amplitude under each condition reveals the maximum current that can be elicited by light, irrespective of potential shifts in the stimulus-response relation. Fig. 5 *A* illustrates an example in which a photoreceptor was tested under voltage clamp with light flashes of increasing intensity while bathed in ASW or in the presence of two concentrations of the drug (0.8 and 100  $\mu\text{M}$ ). The response amplitude range was greatly compressed after superfusion with 4-AP, in a concentration-dependent way. To estimate the extent of blockage, the peak photocurrent was plotted as a function of light attenuation for each of the three conditions (Fig. 5 *C*), and the saturating amplitude was determined from the asymptote of sigmoidal functions fitted to the data points. The values obtained in this way indicated a blockage of 55 and 96% in the presence of 0.8 and 100  $\mu\text{M}$  4-AP, respectively. By contrast, the peak response to a light stimulus of moderate, fixed intensity (Fig. 5 *B*) showed a suppression of 72% in 0.8  $\mu\text{M}$  4-AP and 97% in 100  $\mu\text{M}$  4-AP. There is a small discrepancy in the two measures of blockage, due to a minor shift in sensitivity during treatment with the blocker; this is indicated in Fig. 6 *C* by the arrows pointing to the light intensity eliciting half-maximal responses in each condition ( $-2.2$ ,  $-2$ , and  $-1.9$  log at 0, 0.8, and 100  $\mu\text{M}$  4-AP, respectively). Similar observations were obtained from four other cells. Because the shift of the response curve along the ordinate is quite modest, whereas the reduction in the saturating photocurrent amplitude is very pronounced, we can conclude that the inhibitory effect induced by 4-AP on the light response cannot be accounted for by a displacement of the operating range of the cell, but results primarily from a reduction of the membrane conductance that can be activated by light stimulation.

Such reduction in the amplitude of the saturating light-activated current, however,



does not rule out an effect of the drug on some transduction step, leading to a decrease in the maximal output of the internal transmitter, rather than direct blockage of the light-sensitive channels. There are no conclusive physiological tests that can address all of the many conceivable ways of inducing such an effect. A first

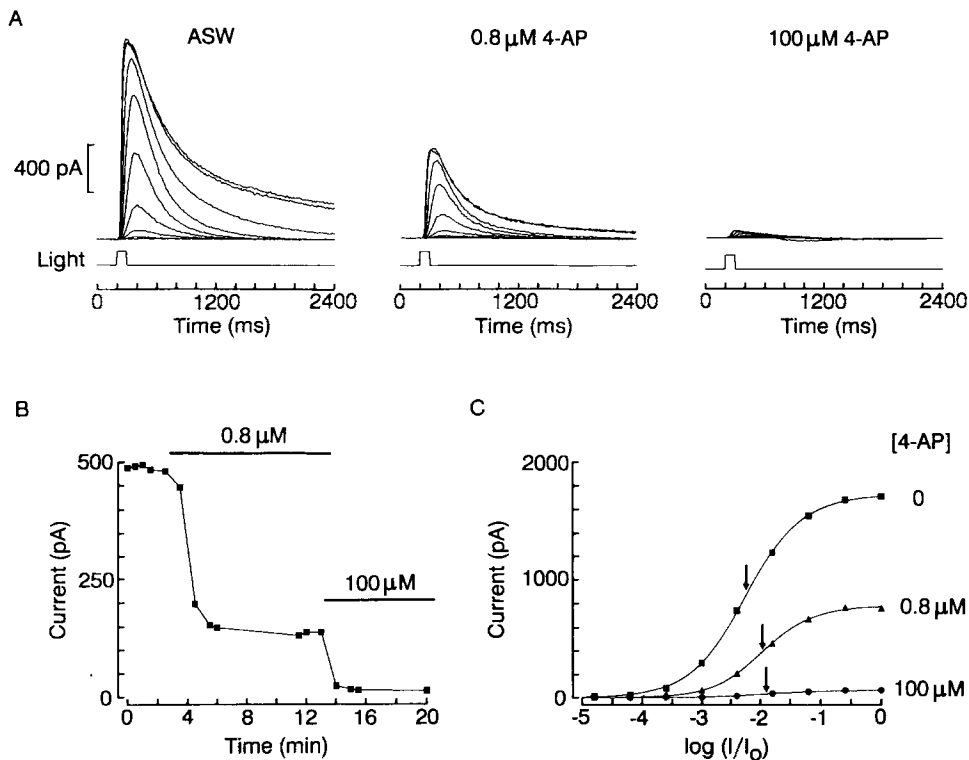


FIGURE 5. Light sensitivity and blockage of the light-induced current by 4-AP. (A) Families of superimposed photocurrents elicited by light stimuli of increasing intensity while the cell was bathed in ASW (*left*), in 0.8 μM 4-AP (*center*) or in 100 μM 4-AP (*right*). Unattenuated light intensity  $11 \cdot 10^{16}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ , light intensity increments 0.6 log, holding potential  $-30$  mV. (B) Dose-dependent suppression of the photocurrent elicited by a repetitive light stimulus of fixed intensity ( $3.7 \cdot 10^{13}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ), in ASW and after switching to 0.8 μM 4-AP and subsequently to 100 μM 4-AP. (C) Peak amplitude of the response plotted as a function of log light attenuation in the three phases of the experiment. The data points obtained in the three conditions were fitted with a sigmoidal function by a least-squares minimization algorithm. The arrows point at the light-intensity producing a half-maximal response, according to the fitted function.

possibility to consider is that the initial step of the transduction cascade is compromised by 4-AP. One indirect approach to examine this entails determining whether any change occurred in the electrical manifestation of rhodopsin isomerization (Brown and Murakami, 1964), namely the early receptor current (ERC). This current

precedes the onset of the photocurrent, is largely independent of membrane potential, and can be altered by manipulations that affect the pigment population available for photoconversion. Fig. 6 *A* illustrates the ERC in a ciliary photoreceptor of scallop, elicited by a brief (4 ms) bright white Xenon flash; the initial portion of the "late" photocurrent is also visible in the right portion of the figure. In the presence of 4  $\mu$ M 4-AP (that caused >75% suppression of the photocurrent) the ERC was unaltered ( $n = 2$ ). This indicates that at least the pool of rhodopsin undergoing photo-isomerization had not diminished. Of course, normal light-induced charge displacement in the pigment does not necessarily imply that its competence to

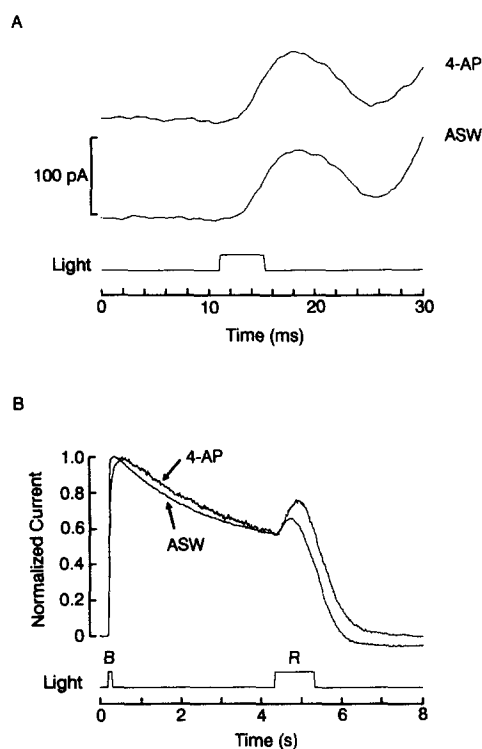


FIGURE 6. Lack of effect of 4-AP on the early receptor current. A brief (4 ms) intense ( $15 \cdot 10^{17}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ) flash was delivered in ASW and in the presence of 4  $\mu$ M 4-AP. The rapid, voltage-insensitive current due to charge displacement during rhodopsin isomerization appears unaffected by the drug treatment (which caused a reduction of the late receptor current by more than 75%). The rising phase of the late receptor current is visible on the right. (*B*) Prolonged after-current and its termination in ASW and during superfusion with 4-AP. A blue flash from the Xenon epi-illuminator was used to elicit a sustained outward current (100 ms, 380–500 nm, equivalent intensity  $3 \cdot 10^{19}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ). 4 s later, a red light (1 s, 620–650 nm, delivering  $2.9 \cdot 10^{16}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$  at 635 nm) was presented to photoconvert the metarhodopsin back to rhodopsin and terminate the prolonged after-current. The two traces were recorded in ASW and in the presence of

4  $\mu$ M 4-AP (which reduced the photoresponse by  $\sim$ 80%), and were normalized with respect to their peak amplitude.

activate the transduction cascade was unaffected. To address this issue we examined the prolonged aftercurrent (PA) which can be elicited by intense narrowband stimulation inducing a substantial net shift of rhodopsin to metarhodopsin (R  $\rightarrow$  M). The PA is thought to reflect saturation of the capacity of the system normally responsible for response termination, resulting from the presence of a large excess of either activated rhodopsin (Minke, 1986), or incompletely deactivated metarhodopsin which can undergo a backward reaction to the active state (Lisman, 1985). Reverse photoconversion M  $\rightarrow$  R, using a spectrally different stimulus, turns off the PA (see review by Hillman, Hochstein, and Minke, 1983). In the scallop, rhodopsin absorbs

maximally near 500 nm, and metarhodopsin at 575 nm, as determined by early receptor potential measurements by Cornwall and Gorman (1983*b*). As a result, blue light can convert a large fraction of R to M inducing a sustained outward photocurrent, which can last for minutes and can be terminated at any time by delivery of a red light (Cornwall and Gorman, 1983*b*). Fig. 6 *B* shows normalized recordings of the PA obtained in ASW and during superfusion with 4  $\mu$ M 4-AP, and demonstrates that the PA and its suppression take place normally in the presence of the drug (albeit with a much reduced amplitude). The implication is that a large amount of active rhodopsin can be generated by light stimulation even though the photocurrent is greatly depressed in the presence of 4-AP.

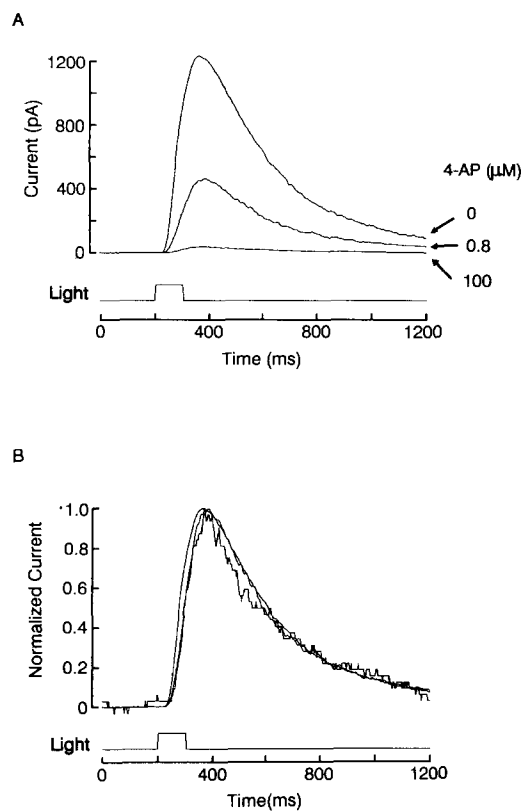


FIGURE 7. Lack of kinetic changes in the photocurrent in the presence of 4-AP. (A) Superimposed photocurrent records elicited by a stimulus of fixed intensity ( $3.7 \cdot 10^{13}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ , 100 ms,  $V_h$  -20 mV) in ASW and in 0.8 or 100  $\mu$ M 4-AP, which caused a reduction in the response amplitude by 64 and 96%, respectively. (B) Superimposed normalized traces from part A. No significant effect on photocurrent kinetics is visible.

It is also possible that an impairment occurs in some of the enzymatic reactions downstream in the transduction cascade. By altering some reaction rate, an effect of this nature would be likely to affect the kinetics of the visual response (see Fain and Lisman, 1981). Therefore, an examination of possible changes in the time course of the photocurrent that may accompany 4-AP-induced blockage can provide further clues on the mode of action of the drug. In Fig. 7 *A*, a cell was tested in ASW and after superfusion with 0.8  $\mu$ M and 100  $\mu$ M 4-AP, which caused a reduction in response amplitude by  $\approx 60$  and  $\approx 97\%$ , respectively. Fig. 7 *B* shows the same three

current records after normalization with respect to the peak amplitude. It can be readily appreciated that the kinetics of the three responses are virtually identical ( $n = 8$ ). Taken together, the results presented in Figs. 5–8 indicate that the transduction cascade remains functional in the presence of 4-AP, and support the view that the antagonistic effects of the drug on the photocurrent in ciliary photoreceptors are chiefly due to blockage of the light-activated channels.

The dose dependency of the 4-AP effect was investigated. Eight cells were used, each of them exposed to two or three different concentrations of the blocker. The concentrations used were 0.8, 4, 20, and 100  $\mu\text{M}$ . At least one wash in normal ASW was interspersed among the treatments to ensure that the photoresponse had not deteriorated. A fixed holding voltage was used for all the tests. Fig. 8 shows a plot of the extent of blockage as a function of 4-AP concentration. Because of the above-mentioned small changes in light sensitivity that may result from exposure to 4-AP, blockage was measured both as suppression of the response to a fixed light of

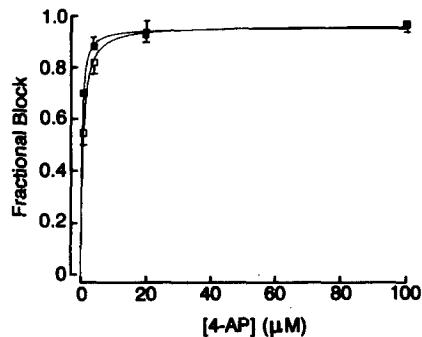


FIGURE 8. Dose-response relation for 4-AP blockage of the light-induced current. Cells were tested in ASW and in the presence of various concentrations of 4-AP (0.8, 4, 20, and 100  $\mu\text{M}$ ). Each cell was exposed to either two or three concentrations of the blocker ( $n = 4$  for 4 and 20  $\mu\text{M}$ ;  $n = 3$  for 0.8 and 100  $\mu\text{M}$ ). The standard deviation is indicated by the error bars; where not marked, the size of the error bar was smaller than that of the symbol. (*Open symbols*)

Blockage measured as suppression of the response to a fixed intensity flash ( $2.5 \cdot 10^{14}$  photons  $\cdot$  s $^{-1}$   $\cdot$  cm $^{-2}$ ). (*Closed symbols*) Blockage measured as reduction of the saturating response, determined from the asymptote of sigmoidal curves least-squares fitted to the data points obtained from intensity series. The apparent  $K_{1/2}$  obtained by the two methods were 0.4 and 0.6  $\mu\text{M}$ , respectively.

moderate intensity (*filled symbols*) or as reduction of the maximal photocurrent amplitude estimated from the asymptote of a sigmoidal function fitted to the data points of light-intensity series (*open symbols*). The  $K_{1/2}$  was empirically obtained by least-square fitting a Langmuir isotherm function to the two sets of data points; the resulting estimates were only slightly different, having values of 0.4 and 0.6,  $\mu\text{M}$ , respectively. The asymptote of the hyperbola approaches the value of 1 in each case, indicating that all the light-sensitive channels are susceptible to blockage by 4-AP.

The mechanisms by which 4-AP blocks various types of voltage-dependent potassium currents are often complex. Because it has been shown that, at least in some instances, 4-AP is active in the cationic form (Kirsh and Narahashi, 1983; Howe and Ritchie, 1991; Choquet and Korn, 1992) the blockade can be voltage-dependent, if the binding site is located within the membrane electrical field. In fact, a common finding is that 4-AP block is relieved by membrane depolarization (Yeh, Oxford, and Narahashi, 1976; Hermann and Gorman, 1981b; Thompson, 1982). The possibility

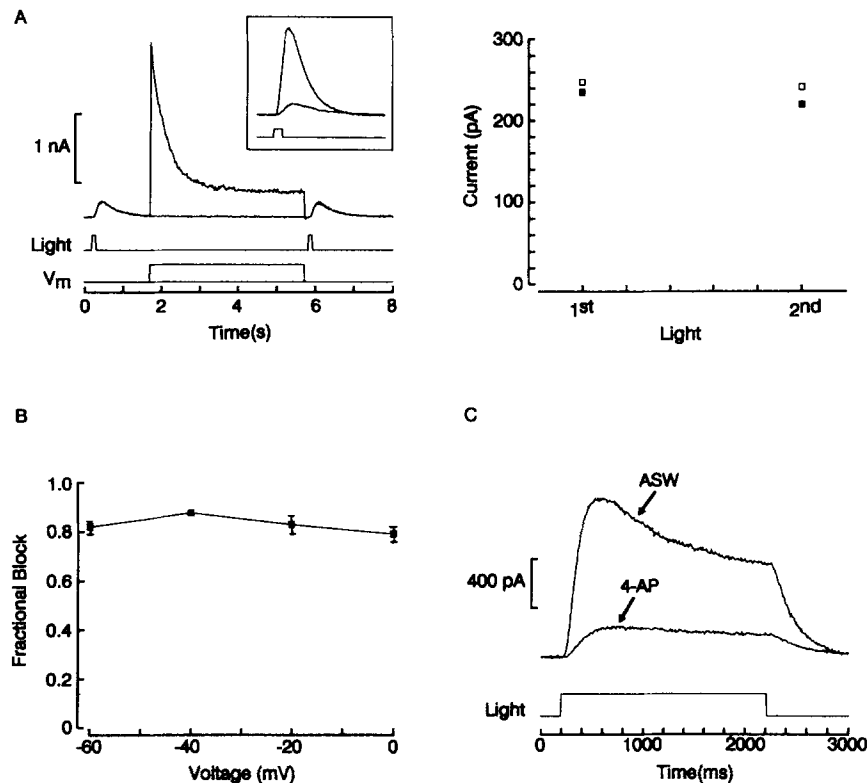


FIGURE 9. Lack of voltage- and use-dependency in the blockade of the photocurrent by 4-AP. (A, left) Double-flash protocol with identical light stimuli ( $100\text{ ms}$ ,  $3.7 \cdot 10^{13}\text{ photons} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$ ) delivered  $5.8\text{ s}$  apart (middle trace). The light stimulation paradigm was repeated with or without an intervening  $4\text{-s}$  depolarizing voltage step to  $+20\text{ mV}$  from the holding potential of  $-20\text{ mV}$  (bottom trace). (Inset) Photoresponses in ASW and in the presence of  $4\text{ }\mu\text{M}$  4-AP, measured before the administration of the double-flash protocol (light stimuli as in A,  $V_h = -20\text{ mV}$ , block  $86\%$ ). (Right) Peak amplitude of the photocurrent elicited by the two successive flashes with (filled squares) or without the conditioning depolarizing step (empty squares). The depolarization did not relieve the blockage of the photocurrent by 4-AP. (B) Average block of the photocurrent evoked by a fixed-intensity flash ( $100\text{ ms}$ ,  $3.7 \cdot 10^{13}\text{ photons} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$ ) at different steady holding voltages ( $n = 4$ ). Error bars indicate the standard deviation; where not visible, the error bars were smaller than the symbols. (C) Photocurrent evoked by a long step of light ( $2\text{ s}$ ) in ASW and after  $7\text{ min}$  of superfusion with  $4\text{ }\mu\text{M}$  4-AP in the dark.

of such an effect on the scallop photocurrent was examined in two ways. Fig. 9A illustrates the results obtained with a protocol in which pairs of flashes were delivered in the presence of  $4\text{ }\mu\text{M}$  4-AP, either with or without an intervening step of membrane depolarization. Similar paradigms have been utilized to investigate the dynamics of 4-AP block in voltage-gated K currents (Yeh, Oxford, and Narahashi, 1976; Simurda, Simurdová, and Christé, 1989; Castle and Slawsky, 1992), and to demonstrate that the time constant for depolarization-induced relief is much faster than the re-block upon repolarization. In the left portion of Fig. 9A the two light

stimuli were presented 5.8 s apart, while the membrane was either held at a steady holding potential ( $-20$  mV) or depolarized with a 4-s voltage step (40 mV in amplitude), which terminated 100 ms before the delivery of the second flash. The intensity of the light stimuli and their temporal separation were chosen to ensure a complete recovery of sensitivity in the intervening interval. It can be seen that the photocurrents elicited by the two stimuli are similar in amplitude, regardless of whether a conditioning depolarizing step was administered ( $n = 2$ ). This can be appreciated more clearly in the right part of the figure, where the peak amplitude of the response to the pre- and the post-flashes in the two conditions are plotted. We also examined the blockage by  $4 \mu\text{M}$  4-AP under steady holding potential in the range  $-60$  to  $0$  mV (at 20-mV increments), with a protocol similar to that used to the test voltage-dependency of TEA block (see Fig. 3). Fig. 9 B summarizes the results: the fractional block of the photocurrent elicited by a light stimulus of fixed intensity is plotted as a function of membrane voltage (average of four cells). The amplitude of the photocurrent was greatly reduced at all potentials, with no systematic voltage dependency. Taken together, the results do not indicate any marked voltage-dependency of blockage of the photocurrent by 4-AP, suggesting that either the site of interaction lies outside the membrane field or that the active form of 4-AP that blocks the light-sensitive channels in these cells is uncharged.

In various preparations, 4-AP seems to interact most strongly with channels in the open conformation; as a consequence, the blocker can alter the kinetics of the current and the resulting block displays use dependency. For example, with some voltage-gated K channels the first response elicited in the presence of the drug attains nearly normal amplitude (regardless of the time of exposure to the blocker), but is followed by a dramatic inactivation; subsequent stimuli evoke much attenuated responses (Wagoner and Oxford, 1990; Choquet and Korn, 1992). All of the experiments described above refer to steady state block, because repetitive stimuli were continuously delivered at regular intervals during the various solution changes, to provide a criterion for response stability under the different conditions. To evaluate the possibility that light stimulation (and the consequent opening of light-dependent channels) aids the onset of the blocking action, we also used a protocol in which photostimulation was interrupted before switching to the solution containing the drug, and the cells were exposed for several minutes to 4-AP before testing. Prolonged steps of light were used instead of flashes, to maximize the possibility of observing accelerated decay kinetics of the first light response in the presence of the blocker. In Fig. 9 C the response to a step of light lasting for 2 s is shown in both ASW and after 7 min of exposure to  $4 \mu\text{M}$  4-AP. Two features should be noted: (a) the peak amplitude of the first photocurrent elicited in the presence of 4-AP is greatly reduced (by the typical amount at this concentration of the blocker, i.e., nearly 80%. See also Figs. 7 and 9 B); (b) there is no accelerated shutting off of the current once it is elicited ( $n = 2$ ). This observation indicates that 4-AP does not require the light-activated channel to open in order to access a blocking site.

#### DISCUSSION

Light-dependent channels of photoreceptors have been notoriously impervious to blockage by a wide variety of pharmacological agents that have been tested. In the

case of vertebrate rods, one of the few effective substances identified to date is *l-cis*-diltiazem (Stern, Kaupp, and McLeish, 1986), a stereo-isomer of a blocker of voltage-dependent Ca channels. In vivo *l-cis*-diltiazem blocks the light suppressible current at a half-maximal concentration of  $\approx 150 \mu\text{M}$  (Rispoli and Menini, 1988), whereas in excised outer segment patches it inhibits the cGMP-activated current with much higher affinity,  $K_{1/2} \approx 1\text{--}5 \mu\text{M}$  (Haynes, 1992; Stern et al., 1986; McLatchie and Matthews, 1992). The mechanism of inhibition, however, does not seem to involve occlusion of the permeation pore, but rather an allosteric action, probably affecting the gating function (Haynes, 1992), perhaps through competition for occupancy of the cGMP binding sites (Rispoli and Menini, 1988). In depolarizing invertebrate photoreceptors, many substances have been tested with no success (briefly reviewed by Fain and Lisman, 1981). The effect of extracellular TEA on the photoresponse of hyperpolarizing scallop photoreceptors had been previously examined in the intact retina by Cornwall and Gorman (1983a). No inhibition on the physiologic photocurrent (i.e., in the outward direction) was seen, whereas when cells were hyperpolarized below the reversal voltage after shifting  $E_k$  by raising  $[K]_o$ , partial blockage of the inwardly directed photocurrent was observed. The dose of TEA employed in those experiments was very high (200 mM). In the present study, a significant effect of extracellular TEA was demonstrated on the outward photocurrent under physiological ionic conditions. Like in voltage-dependent potassium channels of other cells, the blockage of the light-sensitive channels by extracellular TEA exhibited a weak dependency on membrane potential (Hermann and Gorman, 1981a; Yellen, 1984), suggesting that the target site(s) with which the drug interacts are superficially located in the cell membrane. Although the effect of TEA in scallop photoreceptors was consistent, its magnitude was relatively modest and required rather high concentrations of the drug (50 mM). By comparison, extracellular TEA has been reported to suppress calcium-activated K currents with a  $K_{1/2}$  as low as 200–300  $\mu\text{M}$  (Hermann and Gorman, 1983a; Yellen, 1984).

The effect of 4-AP here reported, by contrast, was extremely powerful, producing half-maximal blockage at concentrations that are at least two orders of magnitude lower than for the most susceptible A-currents reported to date, such as those of mouse lymphocytes (Choquet and Korn, 1992) and rabbit Schwann cells (Howe and Ritchie, 1991), and than the effective doses for other K currents that also exhibit high sensitivity to 4-AP (Storm, 1988). One must consider, however, that the latter estimates were determined at physiological external pH for mammals ( $\approx 7.3$ ), which is substantially lower than that of sea water (pH = 7.8). The same authors observed that in more alkaline extracellular media the blocking potency of 4-AP increases: after raising the pH to 8, half-maximal blockage could be attained  $\approx 20 \mu\text{M}$  (Howe and Ritchie, 1991; Choquet and Korn, 1992). This effect is attributable to the fact that, at least in several preparations, the site of action of 4-AP is presumed to be intracellular (Hermann and Gorman, 1981b; Kirsch and Narahashi, 1983; Howe and Ritchie, 1991; Choquet and Korn, 1992), and alkalization increases the fraction of the drug found in the uncharged, lipophilic form that can penetrate the cell membrane (Gillespie and Hutter, 1975). One can estimate that, other things being equal, the  $K_{1/2}$  for the effect of 4-AP on the photocurrent of scallop ciliary cells at external pH 7.3 would increase to nearly 2  $\mu\text{M}$ . Although this argument reduces the

apparent discrepancy between the sensitivity to the drug of  $I_a$  and that of the photocurrent of scallop ciliary cells, the latter still remains extraordinarily susceptible to blockage by 4-AP.

The mechanism of blockage of the light-activated K channels of scallop photoreceptors by 4-AP cannot be determined on the basis of the present information. In several other preparations it has been established that the drug is active in the cationic form (Kirsh and Narahashi, 1983; Howe and Ritchie, 1991; Choquet and Korn, 1992), but the way it interacts with the channel proteins may vary: 4-AP has been reported to bind to channels in the open state (Wagoner and Oxford, 1990; Choquet and Korn, 1992) or the closed state (Thompson, 1982; Yeh et al., 1976; Castle and Slawsky, 1992). In the present case, 4-AP failed to display the depolarization-induced relief of block, which is characteristically found in many classes of K currents, and is similar in this respect to the simpler interaction reported in  $I_{K1}$  in GH<sub>3</sub> cells (Wagoner and Oxford, 1990). The lack of use dependency and of kinetic changes in the photocurrent seem also to indicate that opening of the light-sensitive channels is not necessary for blockage to occur. The absence of significant voltage dependency of the block is consistent with the notion that, if the ionized form of 4-AP is active, the site of interaction is not deeply located into the dielectric core of the membrane.

High sensitivity to blockage by aminopyridines is a widespread feature of rapidly-inactivating K channels, to the point that it has become one of the taxonomic criteria for classification of a potassium current in the  $I_a$  category (see review by Rudy, 1988). It is tempting, therefore, to speculate that a genetic or structural kinship may exist between light-activated K channels and the channels that underlie  $I_a$ , found in many excitable cells. A relationship has previously been suggested between voltage-activated potassium channels and two sensory-transduction ion channels that have been recently cloned: the cyclic nucleotide-activated channel of vertebrate photoreceptors (Kaupp, Niidome, Tanabe, Terada, Bönigk, Stühmer, Cook, Kangawa, Matsuo, Hirose, Miyata, and Numa, 1989) and that of the sensory cilia of olfactory neurons (Dhallan, Yau, Schrader, and Reed, 1990). In both cases, the amino acid sequence and the predicted channel topology lack any similarity to any of the known ligand-gated channels, but are quite reminiscent of voltage-gated K channels (Kaupp, 1991; Guy, Durell, Warmke, Drysdale, and Ganetzky, 1991), including the presence of an S4 segment. This resemblance may seem surprising, in view of the obvious functional differences between voltage-gated K channels and nucleotide-activated channels of sensory cells. Some of these differences, however, may stem from minor structural alterations: for example, the cationic nonselectivity vs the high potassium selectivity of the permeation process may only entail a single amino acid substitution, as suggested by recent mutagenesis experiments in *Shaker* (Heginbotham, Abramson, and MacKinnon, 1992). Interestingly, hyperpolarizing photoreceptors of *Pecten* are homologous to vertebrate rods and cones (Eakin, 1965), in that in both cases the transducing structure derives from modified cilia (Tokuyasu and Yamada, 1959; Miller, 1958; Barber, Evans, and Land, 1967). It is presently not known whether light-sensitive K channels of scallop photoreceptors are also gated by cyclic nucleotides; other remarkable similarities to the vertebrate light-sensitive channel, however, have been shown, including the outward rectification resulting from voltage-depen-



dent blockage by divalent cations and the size of the unitary conductance (Gomez and Nasi, 1994a).

The present results constitute, to the best of our knowledge, the first demonstration of pharmacological blockage of the light-sensitive conductance in an invertebrate photoreceptor. The availability of a high-affinity blocker is not only a useful experimental tool and a informative element for the classification of the channels; one could envision it also as a potentially useful biochemical tool to isolate the channel protein. The light-sensitive potassium channel of the scallop may prove of considerable interest for channel study because unitary currents can be examined in physiological conditions (i.e., normal concentrations of Ca and Mg) and may permit to investigate the evolution of transduction systems from more basic classes of channels (namely voltage-gated K channels) in sensory receptors cells of the ciliary type.

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