

Whole-Cell Clamp of Dissociated Photoreceptors from the Eye of *Lima scabra*

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ABSTRACT Voltage-dependent membrane currents were investigated in enzymatically dissociated photoreceptors of *Lima scabra* using the whole-cell clamp technique. Depolarizing steps to voltages more positive than -10 mV elicit a transient inward current followed by a delayed, sustained outward current. The outward current is insensitive to replacement of a large fraction of extracellular Cl^- with the impermeant anion glucuronate. Superfusion with tetraethylammonium and 4-aminopyridine reversibly abolishes the outward current, and internal perfusion with cesium also suppresses it, indicating that it is mediated by potassium channels. Isolation of the inward current reveals a fast activation kinetics, the peak amplitude occurring as early as 4–5 ms after stimulus onset, and a relatively rapid, though incomplete inactivation. Within the range of voltages examined, spanning up to $+90$ mV, reversal was not observed. The inward current is not sensitive to tetrodotoxin at concentrations up to 10 μM , and survives replacement of extracellular Na with tetramethylammonium. On the other hand, it is completely eliminated by calcium removal from the perfusing solution, and it is partially blocked by submillimolar concentrations of cadmium, suggesting that it is entirely due to voltage-dependent calcium channels. Analysis of the kinetics and voltage dependence of the isolated calcium current indicates the presence of two components, possibly reflecting the existence of separate populations of channels. Barium and strontium can pass through these channels, though less easily than calcium. Both the activation and the inactivation become significantly more sluggish when these ions serve as the charge carrier. A large fraction of the outward current is activated by preceding calcium influx. Suppression of this calcium-dependent potassium current shows a small residual component resembling the delayed rectifier. In addition, a transient outward current sensitive to 4-aminopyridine (I_a) could also be identified. The relevance of such conductance mechanisms in the generation of the light response in *Lima* photoreceptors is discussed.

INTRODUCTION

Enzymatic dissociation of the retina of the file clam *Lima scabra* yields isolated, physiologically viable photoreceptors, which are suitable for electrophysiological recording by means of fire-polished patch electrodes. In the preceding article,

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membrane voltage recording from these cells revealed a complex multiphasic photoresponse, which includes the production of action potentials. Such spiking behavior is not unexpected, given the fact that *Lima* photoreceptors do not rely on second-order neurones to transmit light-encoding information centripetally. Rather, they directly project a long axon that runs in the circumpallial nerve bundle. The solitary cells, however, have their axon completely severed during the dissociation procedure. This implies that voltage-dependent ionic conductances are present in the cell body, and may play a significant role in shaping the cell's response to light. To elucidate the ionic mechanisms that participate in the generation of the photoresponse in *Lima* photoreceptor cells, a characterization of voltage-activated conductances was undertaken using the whole-cell clamp technique. A subsequent report will focus on the properties of the photocurrent under voltage clamp.

METHODS

The protocol for obtaining solitary *Lima* photoreceptors has been described in the preceding paper.

Whole-Cell Clamp

The techniques for voltage clamping small cells with a patch electrode have been discussed by numerous authors (Hamill et al., 1981; Marty and Neher, 1983). Patch electrodes were fabricated using either borosilicate glass from Frederick Haer & Co. (Brunswick, ME) or type #7052 from Garner Glass (Claremont, CA) (Rae and Levis, 1984), using a vertical pipette puller (model 700; David Kopf Instruments, Tujunga, CA). Electrodes were fire-polished immediately before use as described by Hamill et al. (1981); their resistance measured in sea water ranged from 2 to 4 M Ω . The current-to-voltage converter was built using a 3523 operational amplifier (Burr-Brown Corp., Tucson, AZ). The feedback resistor had a value of 200 M Ω , which allows injection of currents up to 60 nA, given the amplifier's voltage compliance of ± 12 V. This is sufficient to clamp the active currents observed in these cells, while maintaining a low level of background noise. Since capacitive transient cancellation circuitry was not used, the access resistance to the cell interior in conjunction with the value of the feedback resistor limits the speed of the clamp. Capacitive currents in response to a voltage step typically settled with a time constant of 200–500 μ s. A positive feedback circuit was used to compensate for a large fraction of the series resistance; the maximum residual error was estimated to be no larger than 1–2 mV, given the relatively low resistance electrodes used and the modest size of the currents involved. A larger error could result if, after seal formation and patch rupture, the electrode tip was partially occluded. This appears not to be the case, since adjusting the series resistance compensation to a value exceeding the initial electrode resistance by more than ~ 30 –50% usually induced ringing, indicating overcompensation (see also a discussion by Marty and Neher, 1983).

All data acquisition was performed on-line by an IBM PC microcomputer equipped with a 12-bit analog-to-digital interface board (Data Translation, Inc., Marlboro, MA). Signals were first low-pass filtered using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) with a corner frequency set at 1–2 kHz to avoid aliasing, then digitized at 5 kHz and stored on floppy disks for off-line display and analysis. If needed, additional filtering was performed digitally by means of an algorithm implementing a Blackman-smoothed "brickwall" transfer function with zero-phase characteristics (Antoniou, 1979). Linear leak correction was used on all records. To this end, in each experiment hyperpolarizing voltage-clamp pulses 10 mV in amplitude (eliciting no active responses) were administered, and the resulting steady-state current was

used to scale and subtract a replica of the command signal from the test current records. In some experiments capacitative currents were also subtracted, in which case ensemble averaging was used to reduce the noise on the correction trace. Current–voltage (I - V) relations were determined automatically by a computer routine designed to scan data files to determine signal values at selected points in time, as well as minima and maxima within specified intervals. Voltage stimulation was applied through a programmable, microprocessor-controlled stimulator designed by Dr. Douglas Tillotson (Boston University School of Medicine, Boston, MA).

Artificial sea water (ASW) contained 480 mM NaCl, 10 mM KCl, 49 mM MgCl₂, 10 mM CaCl₂, and 10 mM HEPES, pH 7.8 (NaOH). Whenever the K channel blockers 4-aminopyridine (4-AP) and tetraethylammonium bromide (TEA) were used, they replaced NaCl on an equimolar basis. The recording chamber was perfused with a constant-flow system at a rate of ~1 ml/min. For each solution change, 10 ml of perfusate was allowed to flow before beginning a new set of measurements, which is equivalent to ~20 times the volume of the chamber. Two “intracellular” solutions were used to fill electrodes: the first one contained 200 mM K-aspartate, 100 mM KCl, 6 mM Na₂ATP, 8 mM MgCl₂, 1 mM EGTA, 300 mM sucrose, and 10 mM HEPES, buffered to pH 7.3 (KOH). The other was similar, except that Cs replaced K, and was used in some of the experiments to block outward currents through K channels.

All experiments were conducted at room temperature (22–24°C) in darkness or under dim, deep red illumination through a long-pass filter (50% transmission cut-off at 650 nm; Ditic Optics, Hudson, MA). Cells were visualized with a Newvicon tube TV camera (model 1350A; Panasonic, Secaucus, NJ) attached to a side port of a Nikon Diaphot inverted microscope. As soon as a tight seal (10–200 GΩ) was formed between the electrode and the plasma membrane, the electrode potential was set at the intended holding voltage (–50 or –60 mV), and the patch was ruptured by a brief, intense pulse of negative pressure. Before beginning the recording, 3–5 min were allowed to elapse in order for the electrode solution to equilibrate with the cell's intracellular constituents (see Fernandez et al., 1984).

RESULTS

Ionic Currents under Voltage Clamp

Fig. 1 *A* shows a typical family of current traces recorded in response to 100-ms depolarizing voltage-clamp steps applied from a holding potential of –50 mV at 20-mV increments. The photoreceptor cell was bathed in ASW. An early transient inward current was observed, followed by a larger sustained outward current. The activation threshold was similar for both components, and was slightly more positive than –10 mV (Fig. 1 *B*). The outward current appears to be only marginally affected if Cl[–] in the superfusate is reduced from 608 to 50 mM by replacing NaCl with Na-glucuronate, as can be seen in the current traces shown in Fig. 2. Decreasing extracellular concentration of Cl[–] resulted in a 7-mV change in junction potential, which was not compensated for and probably accounts for the small differences in the two current records.

By contrast, a dramatic reduction in the outward current can be observed if the K channel blockers TEA and 4-AP are present in the bathing solution. The blockage is quite substantial at concentrations of 10 and 1 mM, respectively, and in most cells no outward current is detectable when 50 mM TEA and 5 mM 4-AP are used (Fig. 3, *A* and *B*). This effect is totally reversible upon returning to ASW (Fig. 3 *C*). In Fig. 3 *D* the I - V relation for the peak outward current is plotted for the three phases of the

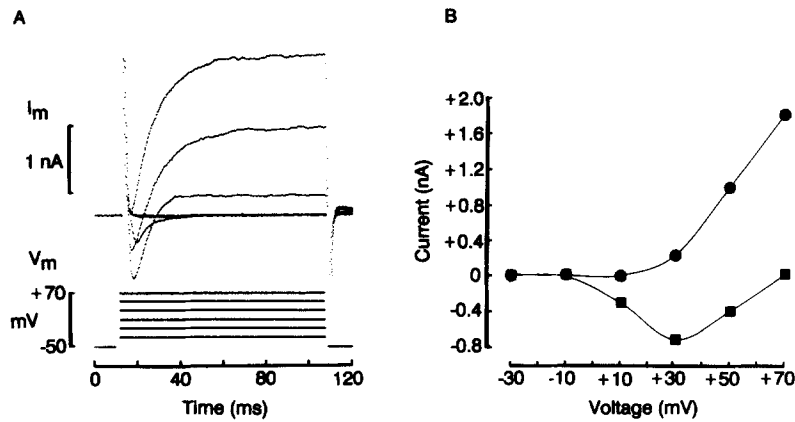


FIGURE 1. (A) Family of current traces elicited by voltage-clamp steps applied to a photoreceptor cell in normal ASW in the dark. The holding potential was -50 mV. Depolarizing steps 100 ms in duration were applied at 20-mV increments every 20 s. Records were low-pass filtered at 1 kHz and corrected for leakage current. (B) I - V relation for the peak inward current (squares) and peak outward current (circles) of the records shown in A.

experiment. It can be concluded that the outward current activated by depolarizing steps is mediated entirely by K channels.

Kinetics and Ion Selectivity of the Inward Current

TEA and 4-AP were used at their maximally effective concentrations of 50 and 5 mM, respectively, to isolate and characterize the early inward current (see Fig. 4 A). Alternatively, in some experiments Cs was used in the solution filling the patch electrode to block K currents, with similar effects (Fig. 4 C). The inward current is largely transient, and its maximum amplitude occurs in the vicinity of 30–35 mV, as can be seen from the I - V relations plotted in Fig. 4 B (filled squares). Within the range of voltages explored (which spanned up to +90 mV in some experiments) no indication of reversal was observed. The absolute magnitude of this current varies across cells, probably reflecting differences in cell size; on the average its peak is in the range of 0.4–1.0 nA. The activation kinetics becomes faster at more positive test potentials, and at maximally activating voltages the peak occurs as early as 4–5 ms

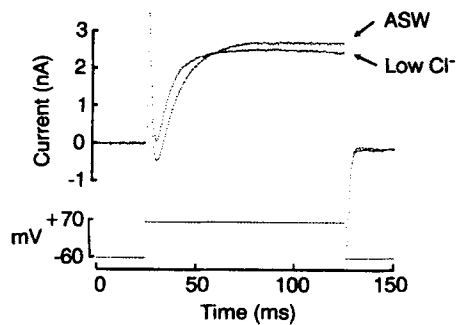


FIGURE 2. Effect of reducing Cl^- concentration in the extracellular solution from 608 to 50 mM by substituting it with glucuronate. Holding potential -60 mV, depolarizing voltage step to $+70$ mV. No correction was applied for the change in junction potential, which was 7 mV.

after the onset of the stimulus. The inward current decays rather rapidly, but the inactivation process is not complete, and a sustained component can be recorded, provided that K currents are properly blocked. As the stimulating voltage is increased beyond the point of maximal activation, the amplitude of the plateau does not decrease monotonically like the peak inward current (Fig. 4 B, *filled squares*). Rather, it starts growing again, and a distinct notch becomes visible in the *I-V* plot for the current measured at the end of the voltage-clamp pulse (Fig. 4 B, *filled circles*).

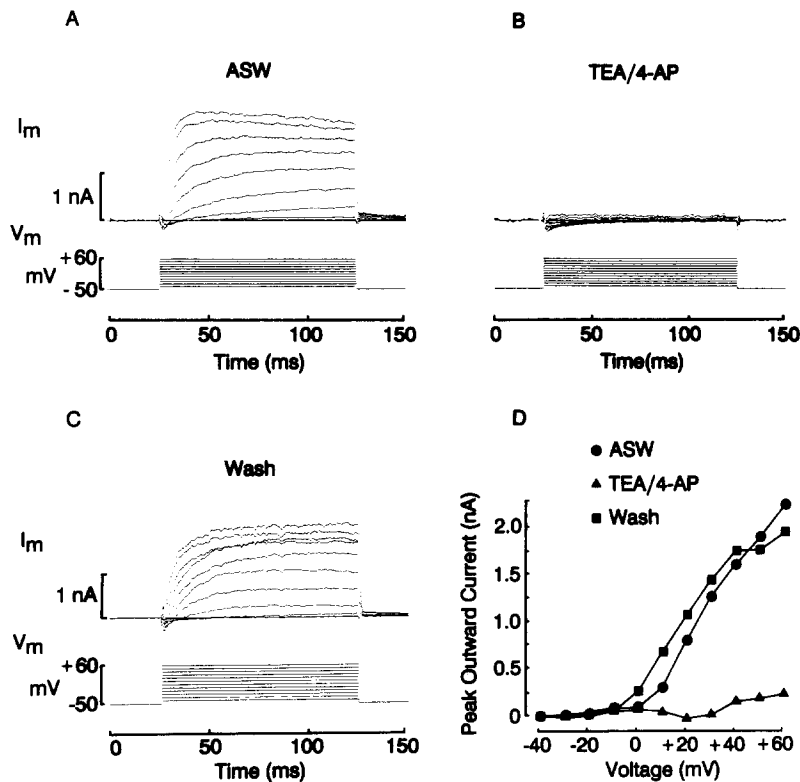


FIGURE 3. Effect of K channel blockers on voltage-dependent outward current. In *A* the cell was bathed in normal sea water; in *B* the superfusate contained 50 mM TEA and 5 mM 4-AP. (*C*) Return to ASW. (*D*) *I-V* relation for the total outward current recorded at the end of the 100-ms depolarization steps in the three phases of the experiment.

The rapid kinetics and inactivation of the early inward current could suggest a contribution by voltage-dependent Na channels. To test this hypothesis, tetrodotoxin (TTX) was added to the bath at concentrations of $5\text{--}10 \times 10^{-6}$ M, but no appreciable blocking effect was observed ($n = 3$) (Fig. 5). TTX-resistant, voltage-dependent Na channels have been described in the literature (Twarog et al., 1972; Kidokoro et al., 1974); furthermore, the trypsin treatment used to isolate the cells could have reduced their sensitivity to TTX, as has been reported for some Na channels (Lee et al.,

1977). Therefore, additional experiments were conducted in which all extracellular Na was replaced with the impermeant ion tetramethylammonium (TMA). Preliminary observations indicated that a small reduction in the amplitude of the inward current resulted from Na removal (Nasi, 1986). This effect, however, was difficult to separate from the progressive wash-out that sometimes occurred during the course of prolonged experiments, and could not be corroborated in later attempts. Fig. 6, *A* and *B*, shows an example in which substitution of all extracellular sodium with TMA

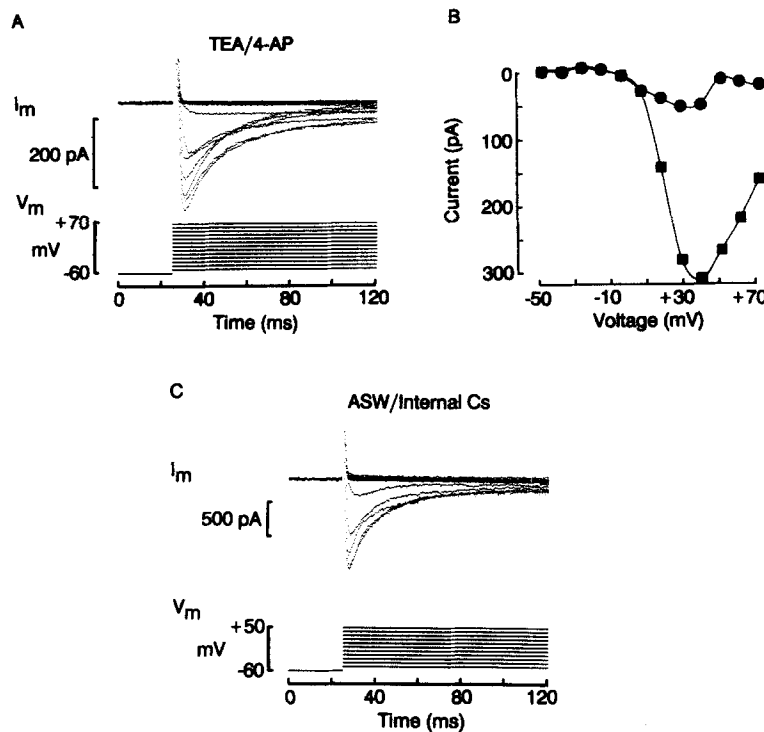


FIGURE 4. Isolation of the inward current elicited by membrane depolarization. In *A* the traces were obtained in the presence of 50 mM TEA and 5 mM 4-AP. The corresponding $I-V$ plot for the peak inward current (*filled squares*), and the current measured 100 ms after the onset of the voltage steps (*filled circles*) is shown in *B*. The family of traces shown in *C* was recorded in normal ASW, but the electrode-filling solution contained 300 mM Cs instead of K. The holding potential was -60 mV in both sets of records.

produces no significant change in the magnitude or the time course of the inward current; only a small shift in the $I-V$ curve is observed (Fig. 6 *C*), probably due in part to a change in junction potential.

Ca ions are the obvious alternative candidate to carry the observed voltage-activated inward current. Voltage-dependent Ca currents in a variety of cell types are blocked by Cd ions at submillimolar concentrations (Hagiwara, 1983). The effect of Cd^{2+} on the behavior of *Lima* photoreceptors was assessed by adding it to the

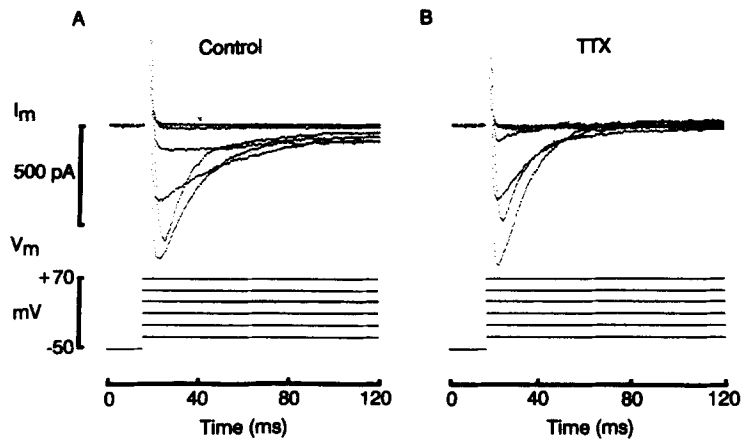


FIGURE 5. Lack of blocking effect of tetrodotoxin on the voltage-dependent inward current of *Lima* photoreceptors. In *A* the cell was bathed in a solution containing 50 mM TEA and 5 mM 4-AP to suppress K currents. The records in *B* were collected after 10 μ M TTX was added to the bath. Holding potential -50 mV.

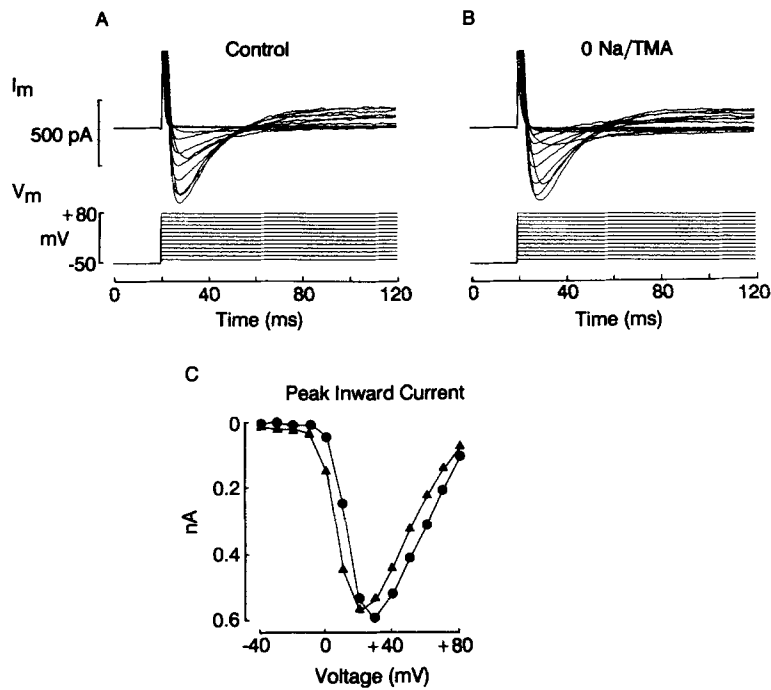


FIGURE 6. Current traces recorded in the presence of TEA and 4-AP before (*A*) and after (*B*) replacing all the Na in the extracellular solution with TMA. The blockage of K channels was incomplete, as revealed by the small residual outward current at very positive membrane potentials. (*C*) I - V plot for the peak inward current with normal ASW (circles) and Na-free solution (triangles).

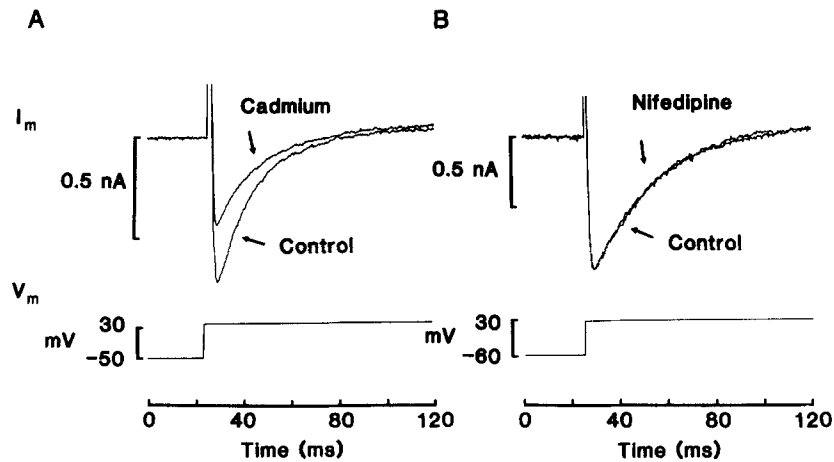


FIGURE 7. (A) Effect of Cd^{2+} on the inward current. The current produced by a voltage-clamp step from -50 to $+30$ mV in the presence of K channel blockers (50 mM TEA and 5 mM 4-AP) is shown before and after adding 500 μM CdCl_2 to the perfusate. (B) Lack of effect of extended superfusion with 10 μM nifedipine. K currents were blocked by internal perfusion with 300 mM Cs.

perfusing solution (containing 50 mM TEA and 5 mM 4-AP) at a concentration of 500 μM . A reduction in the size of the inward current was apparent (Fig. 7 A), its magnitude ranging from 20 to 50% ($n = 3$). The effect was only partially reversible. The sensitivity of the inward current to Ca channel antagonists of the dihydropyridine family was tested by comparing the size of the current elicited by a depolarization to $+30$ mV before and after exposing the cell to 10 μM nifedipine for 15 min. No measurable blockage was observed ($n = 2$), as shown in Fig. 7 B. On the other hand, Fig. 8 shows that elimination of Ca^{2+} from the bath by equimolar substitution with Mg^{2+} resulted in a nearly complete, reversible abolition of the inward current. It was concluded that Ca ions are the major charge carrier involved.

The effect of replacing extracellular Ca with other divalent cations was also investigated. Fig. 9 shows that an inward current could still be recorded when Ca^{2+} (A) was substituted with Ba^{2+} (B). The activation threshold and the voltage producing the maximum current amplitude were not significantly shifted (see the I - V plot in D

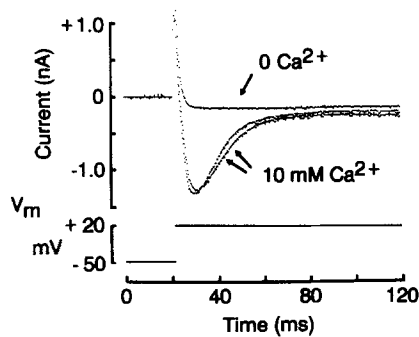


FIGURE 8. Effect of removal of extracellular Ca ions on the voltage-activated inward current. Voltage steps were administered from -50 to $+20$ mV as the cell was superfused with a solution containing 50 mM TEA and 5 mM 4-AP. In the nominally Ca-free solution Ca was replaced with Mg on an equimolar basis.

of the same figure). The amplitude, however, was consistently found to be smaller by a variable factor ranging from 0.8 to 0.15 ($n = 5$). The time course of the current was also altered in the presence of different divalent cations. If records obtained in response to a voltage-clamp step to +20 mV are normalized (Fig. 9 E), it is clear that the activation phase of the inward current is less rapid when Ba serves as the charge carrier. The effect is even more conspicuous in the inactivation process, which becomes much slower and largely incomplete by the end of a 100-ms depolarizing

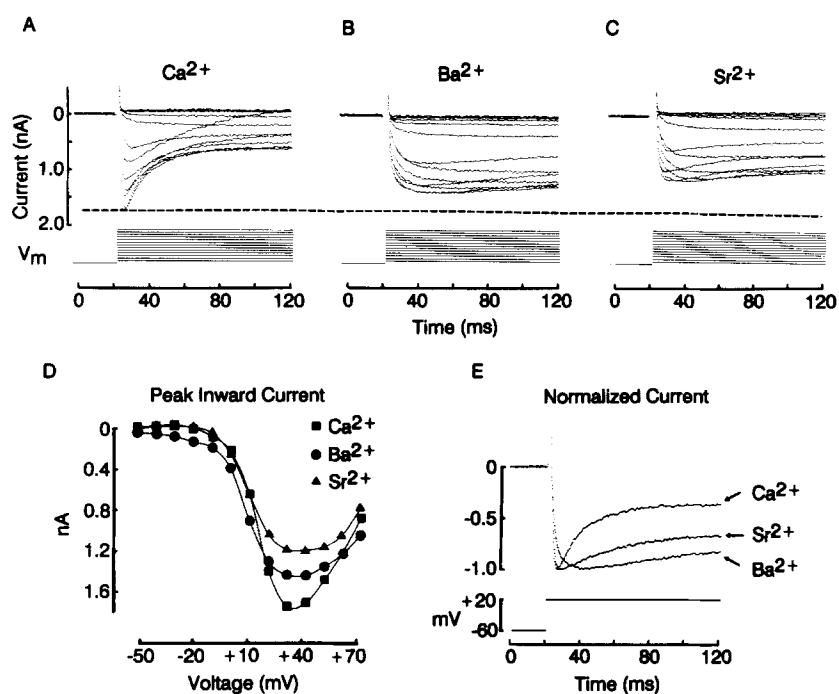


FIGURE 9. Voltage-activated inward currents in the presence of different divalent cations in the extracellular solution. The holding voltage was -60 mV. The patch electrode contained 300 mM Cs, and TEA and 4-AP were present in the bath throughout the experiment. A family of current traces was recorded in the control solution, containing 10 mM Ca^{2+} (A), and after Ca^{2+} was replaced with Ba^{2+} (B) or Sr^{2+} (C). The $I-V$ plots in D show no significant shifts in the voltage dependence of the peak inward current in the three conditions. (E) Superimposed normalized traces elicited by a voltage step from -60 to $+20$ mV in the presence of Ca^{2+} , Ba^{2+} , and Sr^{2+} , showing differences in the kinetics of the inward current.

step. Sr^{2+} was approximately as effective as Ba in carrying the inward current, and the falling kinetics of the current was intermediate between those observed with Ca and with Ba (Fig. 9 C). These observations suggest that Ca influx may in part mediate the inactivation process of the Ca current under physiological conditions (Tillotson, 1979). The fact that the sustained phase of the inward current grows larger as the early peak diminishes at more positive potentials (Fig. 10 A) may also be viewed as consistent with such a hypothesis. Ca-mediated inactivation, however, is unlikely to

provide a full explanation of the relaxation kinetics. In Fig. 10 *B*, depolarizing steps were administered to different voltages, selected to produce a similar peak amplitude of the current and therefore a comparable size of the early Ca influx. The difference in the inactivation kinetics is quite pronounced, suggesting that other factors, in addition to Ca-dependent inactivation of the Ca current, are probably involved.

Analysis of the K Currents

The presence of a prominent voltage-dependent Ca conductance in these photoreceptors provides the conditions for the outward K current to be activated in part by a rise in intracellular free Ca (Meech and Strumwasser, 1970; Meech and Standen, 1975). If this is the case, a correlation should be observed between the magnitude of the Ca current and that of the total outward current. To examine whether such a relation is present, experiments were conducted in normal ASW, extending the amplitude of the depolarizing steps to more positive potentials (up to +110 mV). At

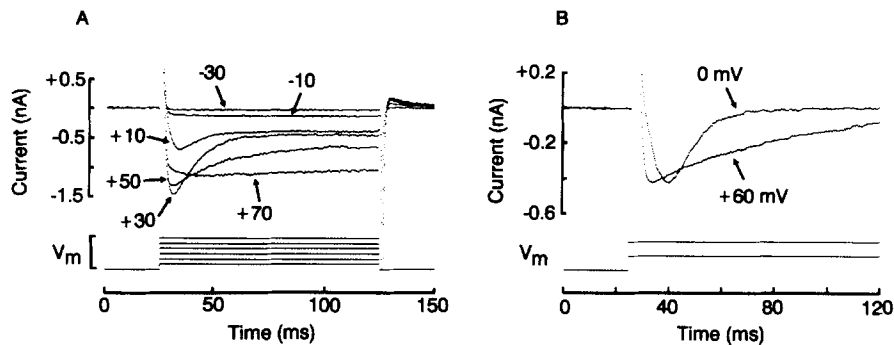


FIGURE 10. (A) Voltage dependence of the transient and sustained phases of the Ca current. With large depolarizations the amplitude of the plateau continues to grow, but the early peak diminishes. (B) Comparison of the time course of the Ca current elicited by depolarizing steps to different voltages, which produced a similar peak amplitude of the inward current.

the high end of this range the decrease of the inward current becomes conspicuous as the equilibrium potential for Ca ions is approached. Fig. 11 shows that the amplitude of outward current also begins to drop above +80 mV, leaving a more slowly developing residual component, reminiscent of the inward rectifier. This behavior is consistent with the suggestion that Ca^{2+} influx plays a role in the activation of the K current.

Further tests for the presence of a Ca-dependent K current ($I_{\text{K(Ca)}}$) were conducted, replacing Ca^{2+} with Ba^{2+} in the extracellular solution. Ba has been shown to be substantially less effective than Ca in eliciting $I_{\text{K(Ca)}}$, either when entering the cell through voltage-dependent Ca channels (Hermann and Gorman, 1979) or when injected ionophoretically into neurones (Gorman and Hermann, 1979). Fig. 12, A and B, shows that superfusion with Ba sea water results in a noticeable reduction in outward current, the effect being fully reversible (Fig. 12 C). Fig. 12 D displays the I - V relationship for the total outward current, plotted for each of the three phases of

the experiment. The observed reduction in the outward current, however, does not necessarily reflect the presence of a Ca-activated K current which Ba^{2+} cannot support as effectively, but could derive instead from a direct blocking effect of Ba^{2+} on other types of K channels, as has been demonstrated for the delayed rectifier of the squid giant axon (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980). To rule out such an explanation a similar test was carried out, substituting Mg^{2+} for Ca^{2+} . Fig. 13 shows that the K current is depressed to an even greater extent, confirming that it is largely dependent on the preceding influx of Ca ions. The outward current in normal sea water sometimes shows a significant decrement during the course of a depolarizing voltage-clamp step. The left panel of Fig. 14 A shows a particularly clear example of such behavior. This decay could indicate a reduction of $I_{K(Ca)}$ due to the transient nature of the Ca current that supports it, or could arise from the slow inactivation of the delayed rectifier channels (Ehrenstein and Gilbert, 1966; Adrian et

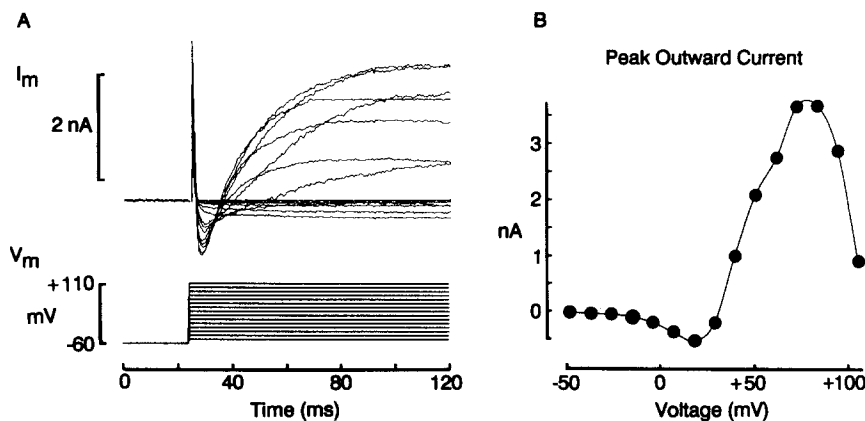


FIGURE 11. Reduction of the total outward current at very positive potentials. (A) Voltage clamp steps were administered to a photoreceptor cell bathed in normal ASW, depolarizing the membrane up to +110 mV. At the high end of the range the amplitude of the outward current decreases, and the residual component displays substantially slower activation kinetics. (B) I - V plot of the peak outward current from the records shown in A.

al., 1970; Aldrich et al., 1979). A further possibility is the presence of a separate, rapidly inactivating, voltage-dependent K current (I_a), as has been documented in neurones (Connor and Stevens, 1971a; Neher, 1971). Since I_a is known to be very sensitive to 4-AP (Thompson, 1977), this blocker was tested at a concentration of 1 mM, which should be largely ineffective in blocking other types of K channels (Thompson, 1977). The middle panel in Fig. 14 A shows that this treatment markedly depressed the fast-activating component of the outward current, producing little change in the total outward current measured 100 ms after the beginning of the step. The traces in the right panel of Fig. 14 A represent the difference of the currents recorded under the two conditions, and reveal a component with fast turn-on kinetics and pronounced inactivation. These observations are consistent with the presence of an I_a -like current in *Lima* photoreceptors. Not all the cells tested

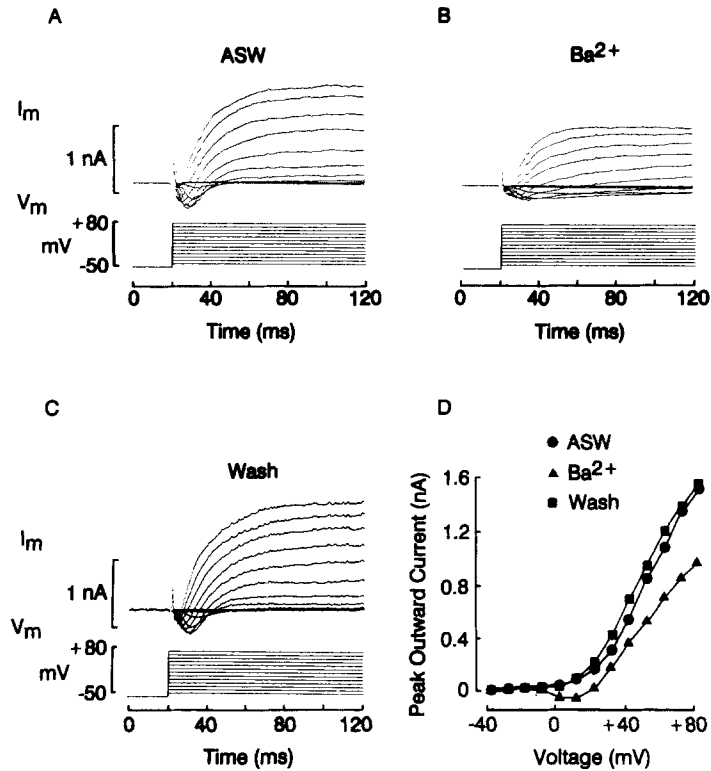


FIGURE 12. Effects of Ba on the amplitude of the K current. The outward current recorded in normal sea water containing 10 mM Ca (A) is reversibly reduced if Ca is replaced by Ba (B and C). (D) I - V plot for the total outward current measured at the end of the 100-ms depolarizing steps. Holding potential -50 mV.

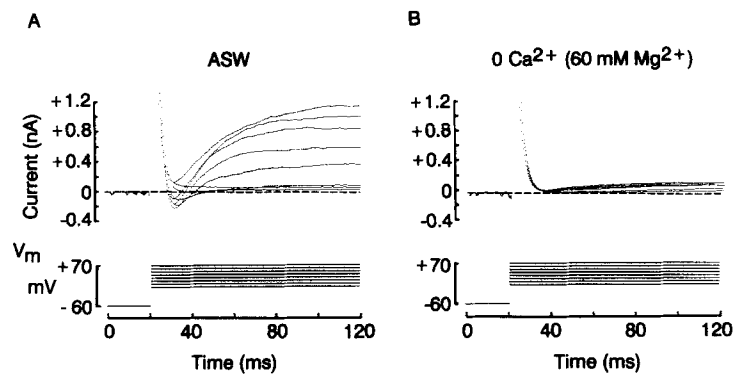


FIGURE 13. Effect of replacing extracellular Ca with Mg. Superfusion with Ca-free ASW causes a marked depression in the outward current, and completely abolishes the inward current. Holding potential -60 mV. Depolarizing steps were delivered from 0 to $+70$ mV, in 10-mV increments.

exhibited a decaying outward current in ASW, however. A possible explanation for this inconsistency could be that I_a is present but largely inactive at the holding potentials typically used (-50 or -60 mV), since in other cells this current exhibits a pronounced steady-state inactivation (Connor and Stevens, 1971a), with a 50%

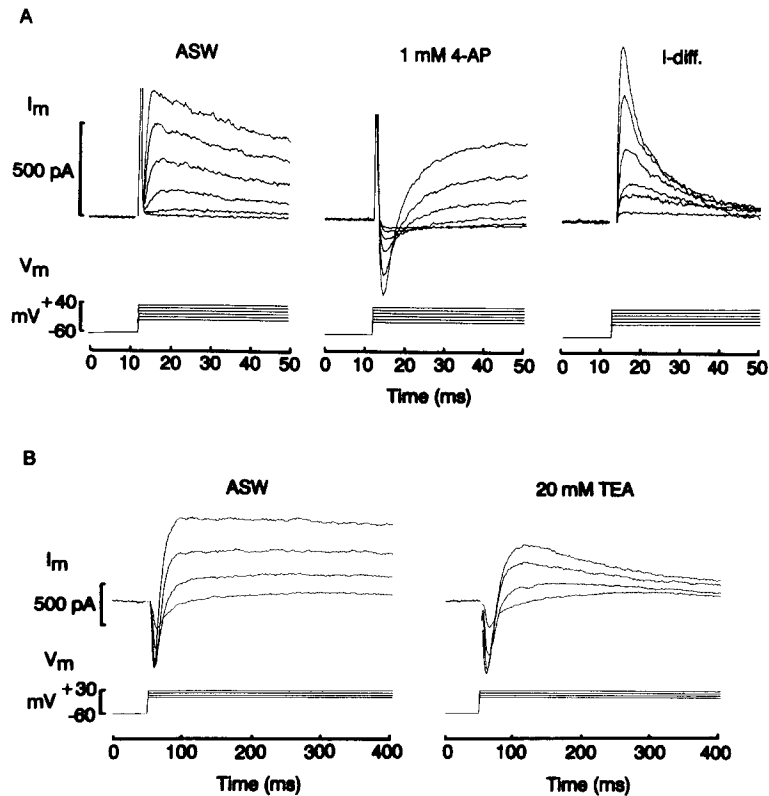


FIGURE 14. Transient outward current in *Lima* photoreceptors. (A) Depolarizing steps administered from a holding potential of -60 mV in ASW, eliciting outward currents that decay in amplitude (*left*). Addition of 1 mM 4-AP to the perfusate (*middle*) removes a phasic component of the total outward current, leaving a slowly activating, sustained component. The right panel shows the difference in the traces recorded in the absence and presence of 4-AP. (B) Effect of superfusion with 20 mM TEA. The outward current in this cell gave no indication of time-dependent inactivation when bathed in ASW (*left*). However, when TEA was introduced the steady-state outward current was substantially reduced, and the residual outward current exhibited a distinct falling kinetics (*right*). Holding potential -60 mV; depolarizing steps from -20 to $+30$ mV.

inactivation voltage in the range -55 to -75 mV (for review, see Rudy, 1988). However, testing several cells with a 1-s hyperpolarizing prestep to -90 mV (designed to remove inactivation) before administering depolarizing test pulses failed to reveal a decaying outward component (not shown). Another possibility is that a

small phasic current often remains undetected because of the concomitant presence of other currents. To facilitate visualization of the transient outward current, a moderate concentration of TEA was used, since in molluscan neurons this substance is effective in suppressing $I_{K(Ca)}$ at lower extracellular concentrations than those required to block I_a (Neher and Lux, 1972; Thompson, 1977). Fig. 14 B shows that the K currents elicited by depolarization in ASW (left family of traces) change significantly in the presence of 20 mM TEA and exhibit a marked falling phase (records on the right).

DISCUSSION

Dissociated photoreceptors from the retina of *Lima* were studied with the whole-cell, patch-clamp technique, which permitted the identification of a number of conductances activated by membrane depolarization in the absence of photostimulation. The characterization of such ionic mechanisms provides important clues for understanding the complex light-induced changes in membrane voltage (which were examined in the preceding article), and is also helpful in interpreting some of the measurements of the photocurrent under voltage clamp (presented in the following paper).

Characteristics of the Ca Current

An inward current is seen as the membrane voltage is stepped to levels more positive than -10 mV. It has a fast turn-on kinetics and displays substantial, though incomplete, inactivation. Experiments with ion substitutions and blockers revealed that under physiological conditions this current is carried by Ca^{2+} ions. A peculiar aspect of the Ca current in these cells is its modest sensitivity to Cd, which is known to exert a powerful blocking effect in many types of Ca-selective channels (Hagiwara, 1983). However, it must be pointed out that the effective K_d for Ca current blockage by Cd is rather variable across preparations: for example, in different classes of molluscan nerve cells the reported values range from the low micromolar range (Kostyuk et al., 1977; Byerly et al., 1985) to millimolar levels (e.g., Akaike et al., 1978). Furthermore, Fukuda and Kawa (1977) and Palade and Almers (1978) have described Ca channels in muscle cells through which Cd^{2+} can actually permeate. *Limulus* ventral photoreceptors also have a class of voltage-dependent channels that are permeable to Ca and only marginally affected by Cd^{2+} at concentrations as high as 10 mM (Lisman et al., 1982).

The inward current measured in these photoreceptors was consistently smaller when Ba replaced Ca in the extracellular solution. While this result is not in line with most reports on ion conduction in Ca channels, there are at least two precedents in the literature: the egg cell of the starfish *Mediaster aequalis* (Hagiwara et al., 1975), and that of the marine polychaete *Neanthes arenaceodentatus* (Fox and Krasne, 1984). Alternatively, it could be argued that the present results perhaps reflect incomplete solution changes due to restricted diffusional access to the channel mouth. If such were the case, a minute residual amount of Ca during superfusion with Ba could cause an anomalous molar fraction effect. This phenomenon can be expected in channels with multiple occupancy sites and single-file permeation (Hille and Schwarz,

1978), and has been documented for Ca channels in cardiac cells by Hess and Tsien (1984) and in skeletal muscle by Almers and McCleskey (1984). Inadequate control of solution changes could also offer a tentative explanation for the puzzling variability in the relative amplitude of the current in Ca and Ba in different cells, but it is difficult to assess to what extent it constitutes a viable account. The molar fraction required to produce an anomalous effect can be quite high (Byerly et al., 1985), a condition that would be unlikely to occur with the large volume of perfusate that was always allowed to flow between tests in different solutions (see Methods). Detailed experiments on permeation through these channels will be carried out to clarify this issue.

The sustained component of the inward current could result either from incomplete inactivation of a single population of Ca channels or from the presence of a second, distinct class of Ca channels, as in many other cell types (for a concise review, see Tsien et al., 1988). Invertebrate Ca channels have not been as amenable to pharmacological separation as the Ca currents of vertebrate cells (Tsien et al., 1988). In the present experiments no evidence of selective blockage was obtained with either Cd^{2+} or nifedipine. Two lines of evidence, however, argue in favor of the existence of separate voltage-activated Ca conductances, albeit indirectly. First, the inactivation phase follows an exponential time course best fitted by two time constants (data not shown). This may be suggestive of the presence of two channel types, but is not a conclusive argument, since Chad et al. (1984) have proposed a model of Ca-dependent inactivation that can account for the complex fall kinetics of the Ca current in *Aplysia* neurons without invoking multiple classes of channels (see also Standen and Stanfield, 1982). Second, the size of the sustained component appeared to increase well beyond the voltage at which the early transient reached its maximum amplitude: the different decay kinetics observed when *Lima* photoreceptors are depolarized to different voltages eliciting a similar initial influx of Ca is difficult to reconcile with a simple notion of Ca-mediated inactivation of a single class of channels. Chad and Eckert (1984) suggested that Ca levels within microscopic domains associated with individual channels may play a relevant role: local Ca accumulation may occur to a greater extent at lower voltages (few open channels, large driving force) than at more positive potentials (more open channels, reduced unitary current; see also Simon and Llinás, 1985). Differences in the local distribution of Ca could conceivably affect the inactivation process, but there is currently no direct evidence for such a mechanism to be operative. The present data are perhaps most parsimoniously explained by postulating two distinct populations of voltage-dependent Ca channels, one phasic and the other sustained, with different voltage sensitivities (Tsien et al., 1988). Conclusive evidence, though, will require single-channel recordings.

The observation that all the depolarization-activated inward current in these cells is mediated by Ca channels indicates that the action potentials elicited by bright lights (see preceding paper) can almost certainly be identified as Ca spikes. Ca action potentials have been reported in other invertebrate visual cells, such as the distal photoreceptors of the scallop *Pecten irradians* (Cornwall and Gorman, 1979). The threshold of activation for the Ca current in *Lima* is more positive than in molluskan neurons (Brown et al., 1981). Such a characteristic, however, is perhaps understand-

able in view of the fact that in unclamped photoreceptors quantum bumps of considerable amplitude (sometimes >20 mV) are recorded even in darkness (see preceding paper). If voltage-dependent Ca channels had a more negative threshold, action potentials would be continuously generated and light stimulation could not be reliably encoded.

Multiple Components of the K Current

Outward current in response to depolarizing voltage-clamp steps is carried by K ions, as determined by its insensitivity to replacement of most of the extracellular Cl^- with glucuronate, and its complete elimination with the use of the K channel blockers TEA and 4-AP or intracellular perfusion with Cs. Several components of the outward current could be identified. A Ca-dependent conductance accounts for a large fraction of the total outward current. Two main lines of evidence support this conclusion.

(a) The outward current diminishes at very positive voltages, where the Ca current is also small. The discrepancy in the turn-around point for the Ca and for the total outward current (+30 mV vs. +80 mV or more) can be accounted for by the monotonic increase in the driving force on K ions as the membrane potential is made more positive, so that the observed current can grow larger although the underlying conductance is actually decreasing. In addition, it is known that $I_{\text{K(Ca)}}$ is also intrinsically voltage dependent (Gorman and Thomas, 1980), and the probability of channel opening increases at positive potentials for a fixed Ca concentration at the inner face of the plasma membrane (Barrett et al., 1982).

(b) The amplitude of the outward current is significantly reduced after replacement of extracellular Ca with Ba, which permeates through Ca channels but is known to be less effective in activating $I_{\text{K(Ca)}}$. Moreover, a more dramatic suppression is observed when Ca is replaced by Mg.

A voltage-dependent component with a slower activation, similar to the delayed rectifier, is also visible under conditions that reduce the amplitude of $I_{\text{K(Ca)}}$. Its contribution to the total current appears to be marginal, as judged by the small size of the residual outward current when Ca is replaced with Mg (Fig. 13), or when the membrane is depolarized to very positive voltages, eliciting a minimal Ca influx (Fig. 11). The latter observation is particularly revealing, because under those conditions (a) the driving force on K ions is quite large, and (b) the conductance should be fully open. Finally, a transient outward current sensitive to 4-AP (I_a) was also recorded, although it was not consistently observed in all cells tested. Channel wash-out could be a factor in such inconsistency. In general, K currents are quite impervious to wash-out, but no information specifically concerning I_a is available. Variability in the falling kinetics of the depolarization-activated outward current has also been reported in *Limulus* ventral photoreceptors (Lisman et al., 1982). In the present case it is probably mostly due to a variable degree of masking by inward currents and by other K currents. Consistent with such a view, superfusion with low concentrations of TEA (designed to preferentially reduce $I_{\text{K(Ca)}}$) resulted in a residual outward current with clearly decaying kinetics. However, I_a was not revealed by removal of extracellular Ca, which eliminates both the inward Ca current and the outward Ca-activated K

current. This result could be due to the fact that in molluskan neurons I_a seems to require the presence of Ca ions in the bathing solution (Junge, 1985).

The presence of a large Ca-dependent K current in these cells may in part account for the hyperpolarizing phase of the photoresponse (see the preceding paper): if the cell depolarizes to a sufficient extent in response to a light stimulus, the Ca-selective conductance is increased and the resulting Ca influx could then trigger $I_{K(Ca)}$, thus contributing to the hyperpolarizing "dip." Furthermore, the fact that the dip becomes more prominent with light adaptation is consistent with the involvement of $I_{K(Ca)}$, assuming that Ca_i increases under such conditions (Brown and Blinks, 1974; Brown et al., 1977; Levy and Fein, 1985). A similar dip has been described by Detwiler (1976) in *Hermisenda* photoreceptors. Grossman et al. (1981) were subsequently able to identify it as an increase in K conductance, and showed that its magnitude is affected by factors that are presumed to control the influx and intracellular accumulation of Ca ions. Voltage-clamp measurements by Alkon et al. (1984) later confirmed the presence of a Ca-dependent K conductance in *Hermisenda* photoreceptor cells. A hyperpolarizing dip with similar characteristics occurs also in the photoresponse of *Balanus* photoreceptors (Hanani and Shaw, 1977). In those cells Bolsover (1981) was able to identify a Ca-dependent K current using voltage clamp.

The interplay between I_{Ca} and $I_{K(Ca)}$, together with the cell's ability to buffer cytosolic calcium transients, have been postulated to play a central role in the generation of oscillatory or bursting behavior in molluskan neurones (Gorman et al., 1981). The transient K current has also been implicated in the production of repetitive action potential firing (Connor and Stevens, 1971*b*). Since these conductances are present in the membrane of *Lima* photoreceptors, similar schemes could account for the trains of action potentials that are observed after stimulation with bright light.

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