Ion Permeation through Light-activated Channels in Rhabdomeric Photoreceptors

Role of Divalent Cations

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ABSTRACT The receptor potential of rhabdomeric photoreceptors is mediated primarily by a Na influx, but other ions must also permeate through light-dependent channels to account for some properties of the photoresponse. We examined ion conduction in macroscopic and single-channel light-induced currents of *Lima* and *Pecten* photoreceptors. In the absence of Na, a fivefold change in extracellular K shifted the reversal voltage of the photocurrent (V_{rev}) by $\approx$ 27 mV. Because the dependency of V_{rev} on $[K]_o$ was sub-Nernstian, and V_{rev} in each condition was more positive than E_K, some other ion(s) with a positive equilibrium potential must be implicated, in addition to K. We assessed the participation of calcium, an important candidate because of its involvement in light adaptation. Three strategies were adopted to minimize the impairments to cytosolic Ca homeostasis and loss of responsiveness that normally result from the required ionic manipulations: (*a*) Internal dialysis with Na-free solutions, to prevent reverse operation of the Na/Ca exchanger. (*b*) Rapid solution changes, temporally limiting exposure to potentially detrimental ionic conditions. (*c*) Single-channel recording, exposing only the cell-attached patch of membrane to the test solutions. An inward whole-cell photocurrent could be measured with Ca as the only extracellular charge carrier. Decreasing the $[Ca]_o$ to 0.5 mM reduced the response by 43% and displaced the reversal potential by -4.3 mV; the shift was larger ($\Delta V_{rev} = -44$ mV) when intracellular permeant cations were also removed. In all cases, however, the current carried by Ca was < 5% of that measured with normal $[Na]_o$. Unitary light-activated currents were reduced in a similar way when the pipette contained only divalent cations, indicating a substantial selectivity for Na over Ca. The fall kinetics of the photoresponse was slower when external Ca was replaced by Ba, or when the membrane was depolarized; however, dialysis with 10 mM BAPTA failed to antagonize this effect, suggesting that mechanisms other than the Ca influx participate in the modulation of the time course of the photocurrent. Key words: light-dependent channels • ion permeation • ionic selectivity • calcium • photoreceptors

INTRODUCTION

Recent advances in invertebrate photoreceptor physiology have shed light on several aspects of the biochemical cascade that controls light-dependent conductances (reviewed by Payne, 1986; Lisman et al., 1992; Minke and Selinger, 1992). The process of ion permeation through light-activated channels, by contrast, has received relatively less attention since the seminal work by Millecchia and Mauro (1969) and Brown and Mote (1974) in *Limulus* ventral photoreceptors, and by Brown et al. (1971) in *Balanus* photoreceptors. Those authors had concluded that, under physiological conditions, the inward current that underlies the depolarizing receptor potential is carried by sodium ions. Several observations, however, indicate that other ions must also be significantly permeant: (*a*) If $[Na]_{out}$ is altered, the photocurrent reversal potential (V_{rev}) changes significantly less than the amount predicted by the Nernst equation; the departure is especially evident in the lower range of Na concentrations. (*b*) V_{rev} is several tens of millivolts more negative than E_{Na}. (*c*) At physiological membrane potentials, replacement of external Na by sucrose leaves a residual light-evoked current that is inwardly directed.

The observation that $V_{rev} < E_{Na}$ implicates some other ion(s) with an equilibrium potential more negative than E_{Na}. In *Limulus*, the reported insensitivity of V_{rev} to large changes in extracellular chloride implies that light-dependent channels are cationic and suggests

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that potassium is likely to be a permeant species (Brown and Mote, 1974). The persistence of an inward current at physiological membrane voltages in the absence of external Na, on the other hand, could implicate calcium ($E_{Ca} > 120$ mV) and/or perhaps magnesium ($E_{Mg} > +30$ mV). However, removal of Ca from the normal extracellular solution had little or no effect on V_{rev} (Millecchia and Mauro, 1969; Brown and Mote, 1974); by contrast, a significant shift was reported in *Balanus* photoreceptors (Brown et al., 1971). A complementary observation in these two species is that the intracellular rise in Ca that accompanies the light response was drastically reduced in low-Ca external solution in *Balanus* but was only attenuated in *Limulus* (Brown and Blinks, 1974), suggestive of differences in the extent to which Ca influx may occur during the photoresponse.

The possibility that light-dependent channels may be permeable to calcium is of considerable importance because of the well-known involvement of calcium in the modulation of light sensitivity (Brown and Lisman, 1975). In fact, recent observations by Hardie (1991) and by Ranganathan et al. (1991) indicate that, in *Drosophila* photoreceptors, calcium influx during the photoresponse is involved in a regulatory feedback loop: At negative membrane potentials the inward photocurrent decays rapidly, whereas the fall kinetics of the outward current is significantly slower if the cell is depolarized above the reversal potential. If extracellular Ca is removed, the time course for both inward and outward photocurrents is similarly slow (Ranganathan et al., 1991). The effect was interpreted in terms of Ca entering the cell during the photoresponse and causing shut-off of the light-dependent channels. Consistent with this view, the reversal potential of the light-induced current was reported to change with manipulations of external calcium, implying a high selectivity (25–40×) of these channels for Ca over Na (Hardie, 1991; Hardie and Minke, 1992). However, no photocurrent could be demonstrated in an extracellular medium containing Ca as the sole putative permeant species (Hardie and Minke, 1992), a result that was attributed to interference with the Na/Ca exchanger and the consequent build-up of cytosolic Ca and desensitization of the response. In *Limulus* ventral photoreceptors, for example, it has been shown that omission of Na_o (in the presence of extracellular calcium) can indeed increase $[Ca]_i$ via reverse operation of the Na/Ca exchanger (O'Day and Gray-Keller, 1989) and lead to loss of light responsiveness (O'Day et al., 1991).

The extent of Ca contribution to the light-dependent current of rhabdomeric photoreceptors is difficult to assess, not only because the required ionic manipulations can affect the transduction cascade, but also because of other complexities, such as the intrinsic voltage dependency of the gating process of light-sensitive channels (Bacigalupo et al., 1986; Nasi and Gomez, 1992). In the present report, ionic permeation was examined in enzymatically isolated photoreceptors from the mollusks *Lima scabra* and *Pecten irradians*. Because these cells are glia-free and present an exposed rhabdomeric lobe, the composition of the extracellular medium can be accurately controlled; their small size also permits efficient dialysis of the cytosol via a patch pipette, so that uncertainties on the intracellular ionic environment are reduced. The structural features and functional properties of retinal cells from the two species are extremely similar (Nasi, 1991*b*, *c*; Nasi and Gomez, 1992; Gomez and Nasi 1994). The advantage of *Pecten* rhabdomeric cells is that light-dependent single-channel currents can be routinely recorded (Nasi and Gomez, 1992), whereas *Lima* photoreceptors prove more resistant for whole-cell voltage-clamp recording and survive prolonged experimental protocols involving multiple solution changes.

METHODS

Enzymatically dissociated rhabdomeric photoreceptors from *Lima scabra* and *Pecten irradians* were obtained using the protocols previously described (Nasi, 1991*a*; Nasi and Gomez, 1992). Macroscopic light-evoked currents recorded under whole-cell voltage clamp (Nasi, 1991*c*) were low-pass filtered at 500–1,500 Hz with a Bessel four-pole filter. For cell-attached recordings of light-dependent single-channel currents (Nasi and Gomez, 1992), the cutoff frequency ranged from 2 to 5 KHz. Recordings were digitized on-line (5–15 KHz sampling rate, 2821 12-bit analog interface board; Data Translation, Marlboro, MA) and stored on Bernoulli cartridges (Iomega, South Roy, UT) for subsequent analysis. An optical stimulator was used to deliver flashes confined to a small circular area (≈150 μm in diameter), as previously described (Nasi and Gomez, 1992). Because near-saturating lights can induce the appearance of a second, slower component of the light response, which in *Lima* has a more negative reversal voltage (Nasi, 1991*c*), particular care was taken to use moderate stimulus intensities, at which the photocurrent displays a unique reversal potential. Light intensity was measured with a radiometer (United Detector Technology, Hawthorne, CA) and is expressed in terms of flux of effective 500-nm photons, as determined by an in vivo calibration, as described elsewhere (Gomez and Nasi, 1994). Voltage and light stimuli were applied by a microprocessor-controlled programmable stimulator (Stim 6; Ionoptix, Milton, MA).

The solution flowing through the recording chamber (≈1 ml/min) could be changed by means of a set of manifolds and reservoirs. Alternatively, a puffer pipette local perfusion technique was used in some experiments to achieve fast solution changes in the zone surrounding a single cell and to permit brief exposure to ionic conditions that may be detrimental to the light response. The small size of molluscan photoreceptors and especially of their rhabdomeric lobe, where the light-activated conductance is localized (5–10 μm diameter; Nasi, 1991*a*; Nasi and Gomez, 1992), makes such an approach particularly effective. Puffer pipettes were constructed from multibarrel capillary glass

(Glass Company of America, Millville, NJ) pulled to a tip OD of ≈4–8 μm. After filling, the pipettes were connected to a multiport valve, which was in turn connected to a solenoid-controlled valve; a pulse of adjustable duration delivered by the stimulator activated the solenoid and permitted the application of pressurized nitrogen (2–5 psi) and the ejection of a stream of solution. To monitor the spatial extent of the local perfusion, a long-wavelength fluorescent dye (Cy5.18; Biological Detection Systems, Inc., Pittsburgh, PA) was included in the test solution (100–200 μM), and, after testing a cell, the fluorescence of the ejection plume was visualized with a charge-coupled device camera (EEV, Chelmsford, UK) contact coupled to the output screen of a red-enhanced microchannel plate image intensifier (DEP, Roden, Holland). The fluorescence excitation light was provided by an arc lamp (Xenon; PTI, So. Brunswick, NJ) filtered at 650 nm (10-nm bandwidth) and was confined to an area ≈120 μm in diameter by means of an adjustable iris serving as a field stop; emission was filtered at 700 nm (40-nm bandwidth). These values are close to the absorption and emission peaks of the dye (652 nm and 667 nm, respectively). Separate measurements were performed to estimate the speed of local solution exchange: To this end, an adjustable mask (model 86968; Nikon, Inc., Melville, NY) was placed in the output beam path to restrict the optical field to a square region, ≈10 × 10 μm, in a position similar to that of a photoreceptor cell in an actual experiment. A photomultiplier tube was connected to the camera port, and the change in fluorescence upon activation of the puffer was measured.

Table I shows the composition of the extracellular solutions used either to superfuse the flow chamber or to fill puffer pipettes for local perfusion. The compositions of the various intracellular solutions used to dialyze the cells via the patch pipette are shown in Table II. The concentration of free Mg in the internal solutions was calculated using the program Chelator, kindly provided by Dr. Theo Schoenmakers (University of Nijmegen, The Netherlands). The values obtained were 0.99 mM for the solutions containing EGTA and 0.94 mM for the solution that contained 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA).[1]

RESULTS

Initial experiments were designed to assess briefly any possible involvement of chloride ions in the generation of the light response. For that purpose, the reversal potential of the photocurrent was measured in voltage-clamped *Lima* rhabdomeric cells, first in standard artificial sea water (ASW) and then after replacing 490 mM of external chloride with gluconate (for the same cell); the calculated displacement in E_{Cl} is 41 mV, in the positive direction. The obtained values of V_{rev} in normal and reduced external Cl were +12.5 mV ±1.9 (SD) and +11.1±1.2 mV (n = 3), respectively. The small discrepancy (which is in the direction opposite to that expected if Cl were permeant) probably reflects a residual error in the correction for the change in junction potential (which was measured at 9±0.2 mV, n = 4). In view of this observation, which confirmed a previous report by Brown and Mote (1974) in *Limulus*, subsequent experiments focused exclusively on cations.

Search for Nonpermeating Cations

Many of the manipulations described below required replacing a given cation with a poorly permeating substitute. When the selectivity of the channels under study is not known beforehand, a nonionic substitute such as sucrose is obviously a safe option (Brown et al., 1971; Brown and Mote, 1974) and was used in some of the experiments. However, straight replacement with sucrose presents the disadvantage that ionic strength cannot be maintained constant (unless additional constituents are concomitantly changed). We therefore examined the effect of several cationic replacements in the external and/or in the intracellular solution: Cs, Tris, *N*-methyl-D-glucamine (NMDG), and tetraethyl ammonium (TEA). Ideally a substitute should fulfill the criteria of (*a*) causing a large change in the photocurrent size and its reversal potential and (*b*) not affecting adversely the transduction cascade.

Internal replacements. Cesium, Tris, TEA, and NMDG were tested as replacements of potassium in the intracellular solution used to fill the patch electrode. Because the pipette was not perfused, comparisons necessarily had to be carried out across cells, and the only reliable information that is not affected by individual variations in current size is the reversal potential measured in the presence of a standard extracellular solution (ASW). Fig. 1 summarizes the data. Panel *A* shows normalized peak amplitude currents obtained at different holding potentials around the reversal point, from representative cells. Nearly all the I-V curves measured with the various K substitutes were shifted in the positive direction, indicating that the permeability of these substances through light-sensitive channels is lower than that of potassium ions. Pronounced quantitative differences were observed: NMDG produced the most positive reversal voltage, and Cs was almost indistinguishable from K. The histogram of Fig. 1 *B* shows the mean values and standard deviation of the reversal potential for each of the conditions: V_{rev} was +19.8±2.8 mV with potassium (n = 4), +20.6±5.6 mV with Cs (n = 8), +63.2±1.1 mV with Tris (n = 3), +77±3 mV with TEA (n = 2), and +88.3±5.6 mV with NMDG (n = 4). Therefore, according to this criterion, the order of selectivity was K ≈ Cs > Tris > TEA > NMDG. From the above results, permeability ratios can be estimated from the differences in observed reversal potentials, using the expression $P_X/P_Y = ([Y]_o/[X]_o)\exp[(V_{rev,X} - V_{rev,Y})zF/RT]$ (Hille, 1971). Because the substitutions are equimolar and the ions in question are monovalent, the expression simplifies to $P_X/P_Y = \exp[(V_{rev,X} - V_{rev,Y})F/RT]$. Taking K as comparison standard, the obtained

[1] *Abbreviations used in this paper*: ASW, artificial sea water; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; NMDG, *N*-methyl-D-glucamine; TEA, tetraethyl ammonium.

TABLE I

Extracellular Solution Composition (mM)

Solution:	NaCl	Na-gluc.	KCl	K-gluc.	$CaCl_2$	$MgCl_2$	$BaCl_2$	HEPES	HEPES/Tris	NMDG-Cl	Tris Cl	Sucrose	Glucose
ASW	480	—	10	—	10	49	—	10	—	—	—	—	5.5
Low-Cl ASW	—	480	—	10	10	49	—	10	—	—	—	—	5.5
30 K/0-Na	—	—	30	—	1	1	—	—	10	550	—	—	—
150 K/0-Na	—	—	150	—	1	1	—	—	10	430	—	—	—
0-Na/Tris	—	—	10	—	10	49	—	—	10	—	480	—	—
0-Na/NMDG	—	—	10	—	10	49	—	—	10	480	—	—	—
60 Ca/0-Na	—	—	—	—	60	—	—	—	10	490	—	—	—
10 Ca/0-Na	—	—	—	—	10	49	—	—	10	490	—	—	—
0.5 Ca/0-Na	—	—	—	—	0.5	59	—	—	10	490	—	—	—
High Ca	—	—	0–10	—	220	—	—	—	10	—	—	0–500	—
High Ba	—	—	—	—	—	—	220	—	10	—	—	—	—
High Mg	—	—	—	—	—	220	—	—	10	—	—	—	—

The pH was adjusted to 7.8 in all solutions.

ratios P_X/P_K were 0.97 for Cs, 0.17 for Tris, 0.10 for TEA, and 0.06 for NMDG.

Examination of the kinetics of the light response, shown in Fig. 1 *C*, revealed that with either Cs or Tris in the pipette, the photocurrent was comparable to that observed with the K-based control internal solution, indicating that these ions are relatively inert in terms of possible interactions with the transduction cascade. Unfortunately, the two least permeant species tested, TEA and NMDG, caused a very pronounced slowing down of the time course of the photocurrent and a substantial loss of sensitivity. As a consequence, intracellular NMDG was used in a limited number of experiments, and, when intracellular K needed to be replaced, Tris was often the ion of choice, in spite of the fact that its permeability is not entirely negligible.

The determination of ionic permeabilities from the reversal voltages measured under various intracellular ionic conditions rests on the premise that the cytosol equilibrates with the pipette solution. To check the validity of this assumption, tests were also conducted with a single permeant cation present both intra- and extracellularly, so that the observed reversal voltage could be compared with the calculated Nernst potential. To this end, cells were dialyzed with 300 mM Cs (in addition to 300 mM sucrose, 10 Tris, and 1 EGTA) via the patch pipette, and superfused with isotonic (580 mM) CsCl (10 mM Tris added to buffer pH). The reversal potential obtained under these conditions was $+18.2\pm0.8$ mV ($n = 2$); when intracellular Cs was reduced to 100 mM, V_{rev} was $+39.1\pm0.9$ mV ($n = 2$). These values compare favorably with the calculated values of E_{Cs} (+16.6 mV and +44.2 mV, respectively), confirming the effectiveness of internal dialysis.

All the intracellular solutions used in the above tests lacked sodium, in anticipation of the measurements to be described below. Lack of internal Na did not cause noticeable effects on either the time course or the light sensitivity of the photocurrent. By contrast, the average reversal potential measured in ASW using Na-free, K-based intracellular solution was ≈6 mV more positive than that measured with normal intracellular solution containing the standard 27 mM Na ($V_{rev} = +14\pm3.1$ mV, $n = 4$); in addition, the amplitude of the saturating photocurrent also tended to be larger. Both effects can be expected from the increased magnitude of the Na gradient, and from the fact that sodium is the predominant ion that moves through the light-dependent channels of rhabdomeric photoreceptors under physiological conditions.

TABLE II

Intracellular Solution Composition (mM)

Solution:	NaCl	K (asp./Cl)	Cs (asp./Cl)	Tris (asp./Cl)	NMDG (asp./Cl)	TEA (asp./Cl)	HEPES	HEPES/Tris	EGTA	BAPTA
Standard	22	200/100	—	—	—	—	10	—	1	—
Ca-buffer	22	200/100	—	—	—	—	10	—	—	10
K/0-Na	—	200/100	—	—	—	—	—	10	1	—
Cs-0-Na	—	—	200/100	—	—	—	—	10	1	—
Tris/0-Na	—	—	—	200/100	—	—	—	10	1	—
NMDG/0-Na	—	—	—	—	200/100	—	—	10	1	—
TEA/0-Na	—	—	—	—	—	200/100	—	10	1	—

All the solutions contained 5 mM MgATP, 300 mM sucrose, and 100 μM GTP, in addition to the constituents listed above. The pH was adjusted to 7.3.

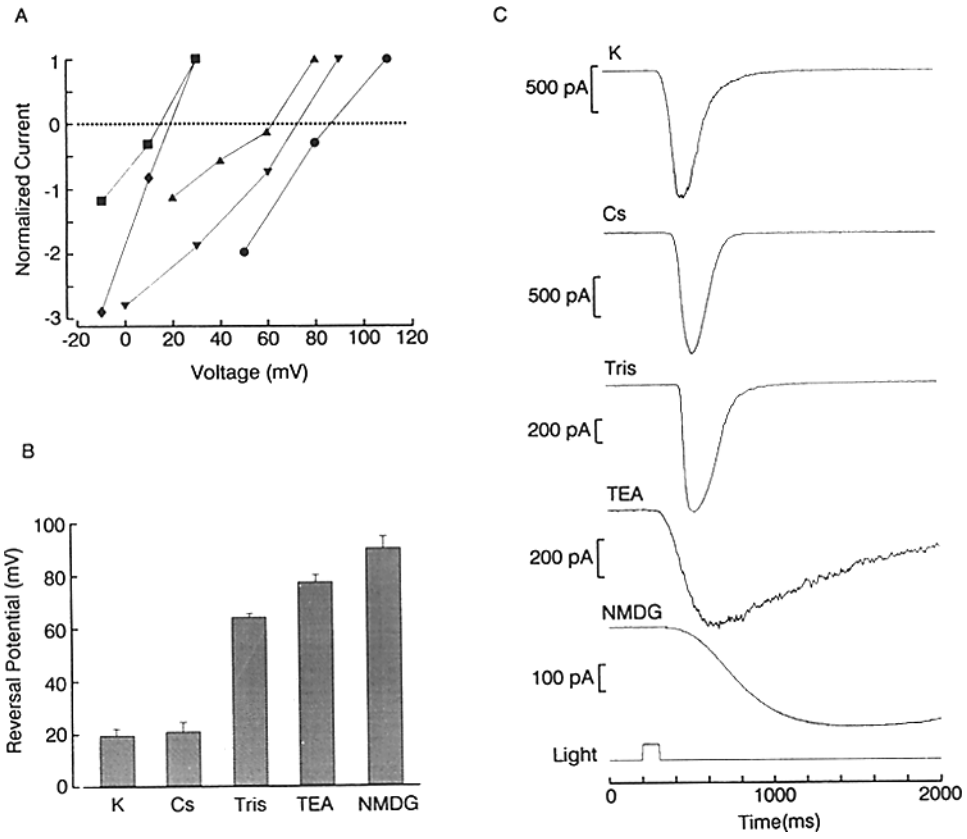

FIGURE 1. Effects of replacing intracellular K on the light-induced current. Different cells were dialyzed via the patch electrode with solutions containing K, Cs, Tris, TEA, or NMDG as the main cation. (*A*) Dependence of the reversal potential on the internal cation. Normalized peak photocurrent amplitudes measured at different holding voltages in representative cells. *Squares*, K; *diamonds*, Cs; *triangles*, Tris; *inverted triangles*, TEA; *circles*, NMDG. The shift of V_{rev} to the right reflects lower permeability of the replacement cation through the light-activated channels. (*B*) Reversal potential values pooled for all the cells in each of the conditions. (*C*) Effects of K replacement on photocurrent kinetics. Whereas Cs and Tris appeared to be inert, so that the time course of the photocurrent was indistinguishable from that observed with K-based internal solution, intracellular dialysis with either TEA or NMDG slowed down both the activation and the decay phase of the light response. For each cell, the flash intensity was adjusted to elicit an approximately half-saturating response (range: $8 \times 10^{10} - 95 \times 10^{11}$ photons $\times$ s^{-1} $\times$ cm^{-2}).

External replacements. The results in the preceding section indicate that Tris is poorly permeant through the light-dependent conductance. As expected, substitution of extracellular Na with Tris, shown in Fig. 2 *A*, reversibly decreased the size of the inward photocurrent elicited by test flashes of fixed intensity at V_h −50 mV. The time course of the effect is plotted in panel *B* of the same figure. The magnitude of the reduction was 83±2.5% (n = 2). The residual response, normalized and superimposed on a control photocurrent, is shown in Fig. 2 *C*; an acceleration of the decay of the current was seen in both cells treated with Tris, an effect reminiscent of the faster kinetics induced by light adaptation and by increasing internal Ca (Brown and Lisman, 1975; Fein and Charlton, 1977; Nasi, 1991*c*).

It is likely that a build-up of cytosolic Ca occurs in Na-free media because of disruption of the activity of the Na/Ca exchanger, as it was documented in *Limulus* (O'Day and Gray-Keller, 1989); this effect is therefore expected to be common to all replacements of external sodium. The optimal replacement for extracellular cations is not necessarily the same as the preferred intracellular substitute: NMDG, which displayed a very low permeability but markedly degraded the light response when applied internally, may be suitable externally, precisely because its influx during the photoresponse could be negligible. This notion was supported by the data in Fig. 3 *A*, which shows that the photocurrent elicited by test flashes in ASW was dramatically reduced in size after switching to Na-free, NMDG-substituted solution, but the time course of the residual photoresponse (see normalized traces in Fig. 3 *C*) was somewhat more rapid than the control, consistent with the results obtained substituting Na with Tris (see Fig. 2), and in sharp contrast with the extremely slow kinetics observed with internal NMDG application (Fig. 1). This measurement was repeated in a total of eight cells; the magnitude of the photocurrent reduction (93±4.5%) was more pronounced than with Tris, corroborating the results obtained with internal dialysis. In six of eight cells, the decay of the photoresponse was faster in the presence of NMDG; a slight lag in the rising phase

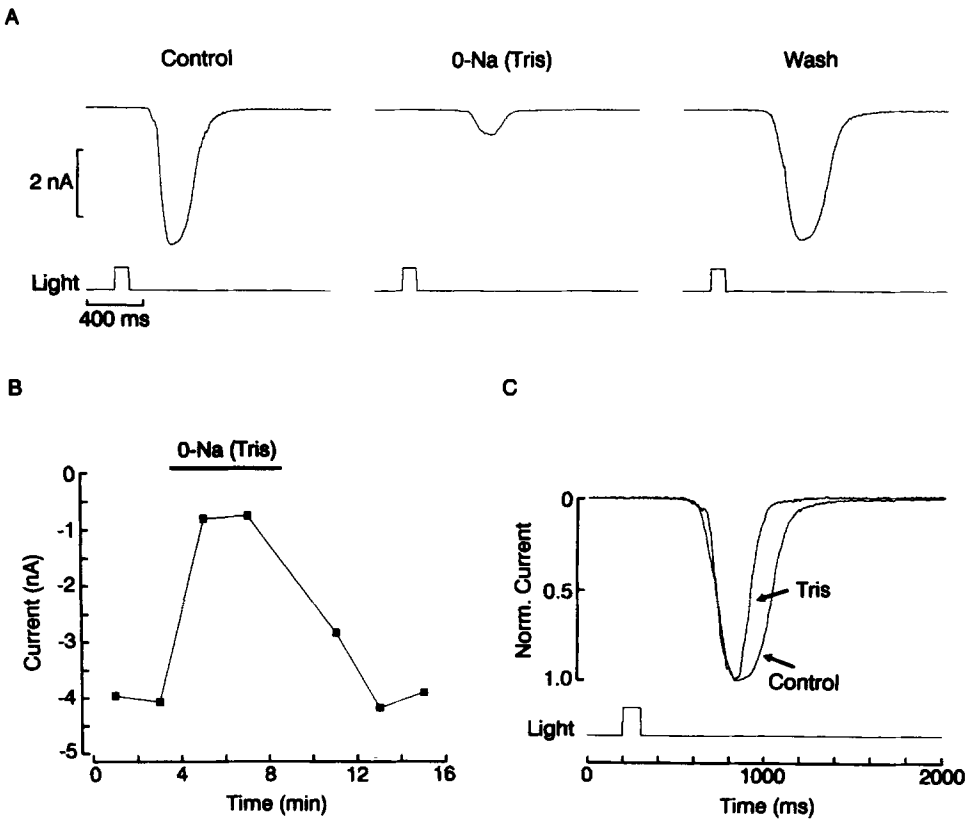

FIGURE 2. Reduction of photocurrent amplitude by replacement of extracellular Na with Tris. (*A*) Light-induced currents elicited by test flash of fixed intensity (8×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) in normal ASW, in the presence of 480 mM Tris, and upon returning to the control condition. (*B*) Time course of the effects of superfusion with Na-free ASW (indicated by the thick bar at the top); peak amplitude of the photocurrent is plotted as a function of time. (*C*) Acceleration of the response kinetics by exposure to Na-free ASW. Traces of light-induced currents before and during superfusion with Tris ASW were normalized and superimposed. The internal solution contained Cs (0-Na), and the holding potential was −50 mV.

was visible in five cases. The residual response reversed at −27.6±1.7 mV ($n = 4$), ~47 mV more negative than V_{rev} measured in ASW, which leads to an estimate of P_{NMDG}/P_{Na} of ≈0.02. These observations make NMDG a good impermeant extracellular cationic replacement. By contrast, no reduction in amplitude was seen in the photoresponse when Cs was the predominant extracellular cation (substituting for both Na and K), in good agreement with the results obtained with intracellular ionic replacements.

Permeation of Potassium

To ascertain the contribution of potassium ions to the photocurrent, we examined the effects of manipulating $[K]_o$; sodium was replaced by NMDG to maximize the sensitivity of the test. Fig. 4 *A* shows photocurrents measured in a cell dialyzed with 300 mM K, held at different holding potentials in the presence of 30 mM $[K]_o$ (*left*) and after increasing it fivefold, to 150 mM (*right*). These elevated extracellular concentrations were chosen so that the photocurrent could be reversed without requiring large hyperpolarizations (as expected in the absence of external Na), which are often detrimental to the cells. In Fig. 4 *B*, the peak amplitude of the photocurrent for this cell and two additional cells is plotted as a function of membrane voltage in the two conditions. It can be readily seen that at higher $[K]_o$ (*open symbols*), V_{rev} is substantially more positive. The average reversal potential measured in 30 vs 150 mM potassium is shown in panel *C* of the same figure (mean displacement 27.3 mV±3.9 mV). The observed shift, although substantial, is still only ≈ 65% of the amount predicted by the Nernst equation for a perfect K electrode (40.5 mV at 25°C). Furthermore, the absolute reversal voltages in 30 and 150 mM $[K]_o$ (−33.9±1.5 mV and −7.6 ±1.96 mV, respectively) are significantly less negative than the calculated values of E_K under the two conditions (−58 and −17.5 mV), indicating that, even in the absence of sodium, potassium ions compete with other ions having a more positive equilibrium voltage. The fact that in both cases the obtained value of V_{rev} was more positive than E_{Cl} (−44.5 mV) strengthens the previous conclusion that such role cannot be attributed to chloride ions. Possible candidates are therefore Mg and Ca, which were present in the extracellular solution at a concentration of 49 and 10 mM, respectively.

Divalent Cation Permeation through Light-dependent Channels

We sought to measure directly light-evoked currents that could be unambiguously ascribed to calcium influx; to minimize the problems that may arise with extracellular ionic conditions in which Ca is the sole permeant species, several strategies were adopted, as described below.

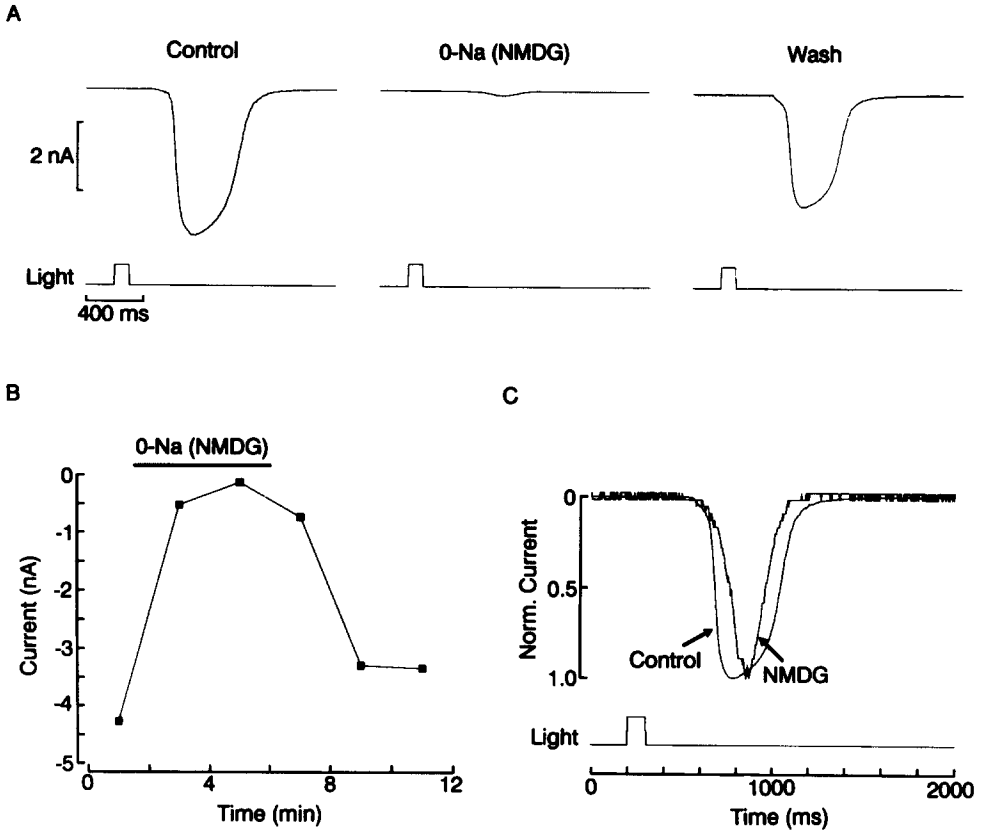

FIGURE 3. Reduction of photocurrent amplitude by replacement of extracellular Na with NMDG. (*A*) Photocurrents in response to a light of constant intensity (4×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) before, during, and after switching the superfusate to Na-free ASW. In the presence of NMDG, the photoresponse was nearly abolished. (*B*) Time course of the effects of superfusion with Na-free ASW (indicated by the thick bar at the top); peak amplitude of the photocurrent is plotted as a function of time. (*C*) Comparison of the light response kinetics in normal ASW and in the presence of 480 mM NMDG. Light-induced currents were normalized and superimposed. The internal solution contained Cs (0-Na), and the holding potential was −50 mV.

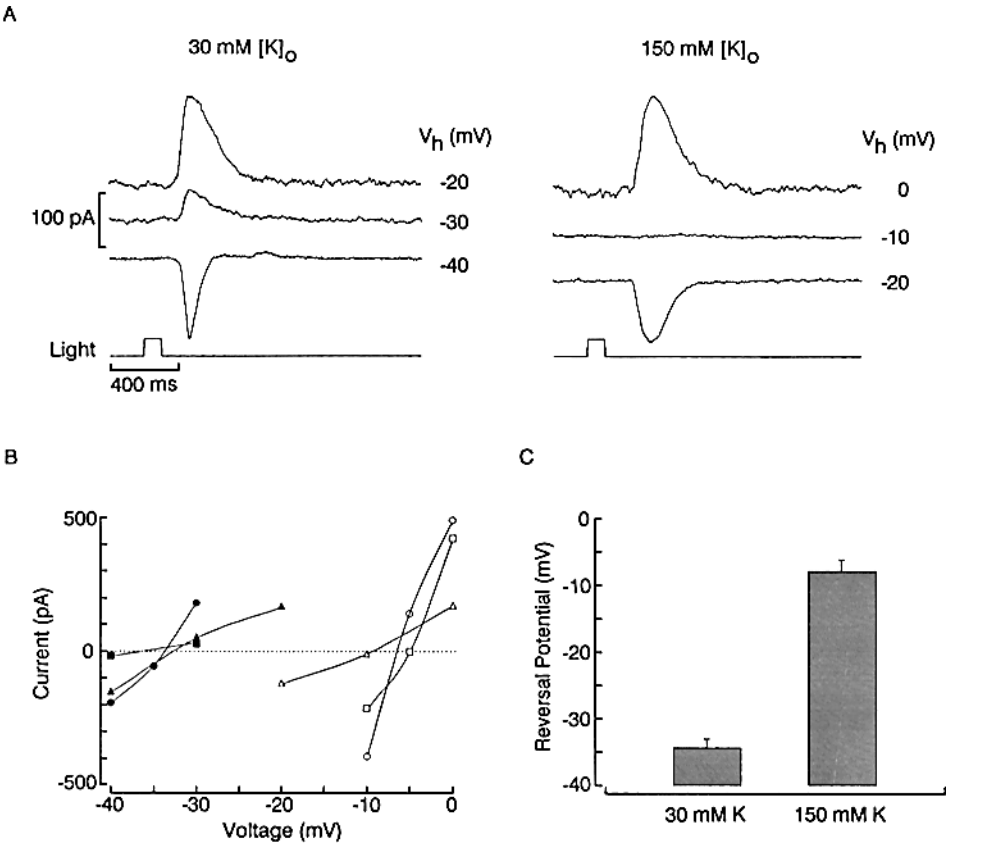

FIGURE 4. Dependence of the photocurrent reversal potential on the concentration of extracellular potassium. (*A*) Recordings were obtained in a photoreceptor superfused with 0-Na ASW (substituted by NMDG); light flashes (100 ms, 7×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) were delivered at different holding potentials, indicated at the right of each trace, during exposure to 30 mM (*left*) or 150 mM K (*right*). (*B*) Peak amplitude of the photocurrent recorded in three different cells each bathed with 30 mM (*solid symbols*) or 150 mM K (*open symbols*). (*C*) Average reversal potential measured in the two conditions in the same three cells of *B*. Error bars indicate standard deviation.

Macroscopic recording with 0-Na internal dialysis. Because reverse operation of the Na/Ca exchanger is a mechanism that could likely lead to Ca loading of the cytosol, a simple precaution entails dialyzing the cytosol with 0-Na solution, in such a way that inversion of the Na gradient is not possible. Such conditions were used in the experiment already described (see Fig. 3), in which replacement of external Na with NMDG caused a dramatic reduction in the amplitude of the photocurrent, although its direction remained inward.

We first examined whether such residual current or part thereof can be attributed to influx of Ca through the light-sensitive conductance rather than to a minute influx of the near-impermeant Na substitutes. Initial tests designed to rule out any contribution by the Na substitute used sucrose as a replacement. To maintain the ionic strength constant, Ca was increased to 220 mM. As shown in Fig. 5, this treatment also drastically reduced the inward photocurrent but did not completely abolish it. There remains a small possibility, however, that the 10 mM Tris, which had to be included both in the external and the internal solutions to buffer pH, may account for the residual inward current at negative potentials, in view of the small but finite permeability of the light-sensitive conductance to Tris (e.g., Fig. 1). Use of a positive holding potential would circumvent this problem, but at the expense of setting up a large driving force for a competing outward current. The following conditions were therefore devised to exclude all ions other than Ca as potential carriers of an inward current, while reducing the risk of masking such a current. Fig. 6 *A* shows the results obtained in a *Lima* photoreceptor internally dialyzed with NMDG (0-Na, 0-K) and 10 mM Tris to buffer pH, and initially superfused with ASW while the holding potential was set at +20 mV (i.e., ~68 mV negative of V_{rev} measured under these conditions; see Fig. 1). A test flash of moderate intensity evoked a response with the characteristic slow time course (see also Fig. 1). The external solution was then switched to one in which both Na and K were replaced by NMDG (490 mM), Ca was raised to 60 mM (omitting Mg), and Tris was 10 mM. The equilibrium potentials were therefore +12 mV for NMDG, 0 mV for Tris, −45 for Cl, >100 mV for Ca and near $-\infty$ for Mg. At $V_h = +20$ mV, only Ca experiences an inwardly directed driving force. A second, identical test flash was delivered after 8 min of superfusion (i.e., allowing ≈40× the volume of the chamber to flow, to ensure complete solution exchange). The resulting response was much attenuated, but still inwardly directed (see inset), and must have been carried by calcium ($n = 2$). To further corroborate this notion, the concentration of extracellular Ca was manipulated in the absence of both extracellular Na and K (replaced with NMDG). Fig. 6 *B* shows the results of an experiment in which a cell was stimulated by a test light delivered at −40 mV in the presence of 10 mM extracellular Ca, then after reducing Ca to 0.5 mM, and finally upon restoring the initial concentration; the internal solution contained Cs.

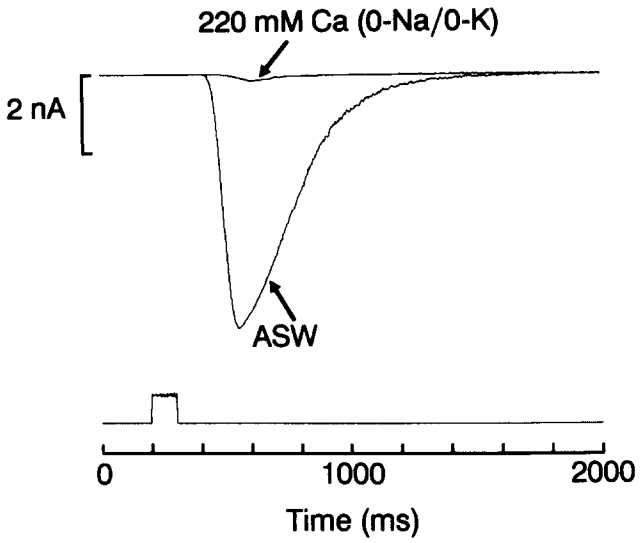

FIGURE 5. Effects of nonionic replacement of all extracellular ions other than Ca. A photoreceptor was voltage clamped at −50 mV with a patch electrode containing the standard intracellular solution and stimulated while superfused with normal ASW. After a period of dark adaptation, the solution was switched to one in which Ca was the only cation; ionic strength was preserved by raising its concentration to 220 mM, whereas sucrose (500 mM) was introduced to maintain osmolarity. The response to the light (100 ms, 9.8×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) was dramatically attenuated in amplitude but remained inwardly directed.

Lowering the level of calcium resulted in a distinct, reversible reduction in the size of the inward photocurrent. The decrease in the response averaged 43±9% ($n = 4$). The reversal voltage was also affected, although in a modest way, shifting by 4.3 mV in the negative direction ($n = 2$). The equilibrium potential of a divalent ion is less sensitive to concentration changes than that of monovalents (≈ 29 mV per decade change in concentration). In an attempt to optimize conditions, additional tests were conducted with 60 vs 0.5 mM $[Ca]_o$, in cells internally dialyzed with Tris. In this case the change from high to low Ca concentration induced a more substantial displacement of V_{rev} (44±1 mV, $n = 2$), as it would be expected from the greater relative change in Ca gradient (120-fold) and the reduced competition by a less permeant internal cation. By contrast, the reduction in response size at a fixed potential was not more pronounced (30±7%, $n = 2$); this observation may suggest that above 10 mM the calcium flux could be approaching saturation, although possible residual contributions by Tris make such a proposition difficult to quantify.

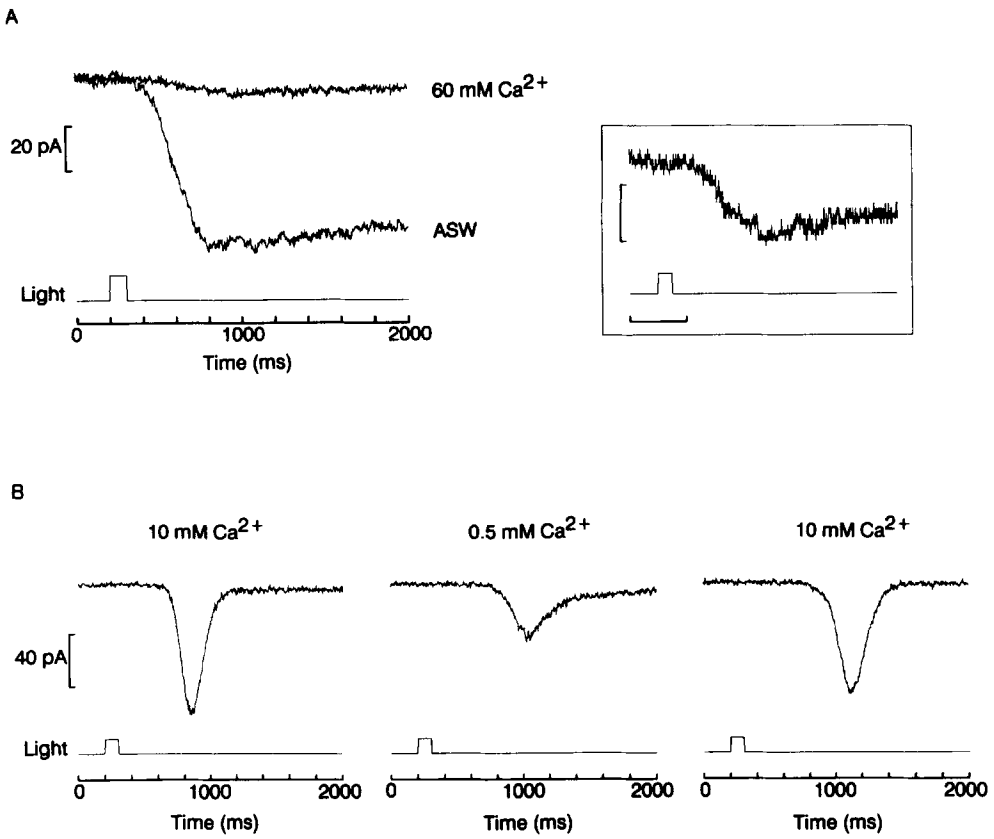

FIGURE 6. (*A*) Inward photocurrent carried by calcium. Responses to a test flash (100 ms, 7×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) in a cell dialyzed with NMDG replacing both K and Na, held at +20 mV. The extracellular solution was initially normal ASW, and the photocurrent displayed a slow time course as typically observed under these conditions. The superfusate was then switched to one containing 480 mM NMDG, 60 mM Ca, and 10 mM Tris. The response was severely attenuated but remained inward. (*Inset*) Expanded response in 60 mM Ca; calibration bars 400 ms, 5 pA. (*B*) Dependence of the photocurrent on the concentration of extracellular Ca, in the absence of both Na and K. The responses were elicited by a flash (100 ms, 16×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$) at a holding voltage of −50 mV. NMDG replaced both Na and K extracellularly, whereas intracellularly the main monovalent cation was Cs. A reversible reduction in the light response was observed upon reducing external Ca 20-fold.

Macroscopic recording with fast solution changes. The above results demonstrate that, under suitable conditions, one can measure an inward current that represents influx of Ca ions through light-dependent channels. The size of this current is small, in spite of the fact that the operation of the Na/Ca exchanger was prevented (in either direction). However, when $[Ca]_o$ is elevated, influx of calcium can occur through other pathways (Gorman et al., 1984; Hochstrate and Juse, 1991); the inability of the Na/Ca exchanger to clear such load could then compromise light responsiveness. We therefore tested brief applications of high-Ca, Na-free external solutions via a rapid local perfusion system. This approach is more cumbersome in that it requires independent verification that the target cell is exposed to the desired test conditions, but it presents the advantage that the light response can be examined shortly after switching solutions, presumably before the intracellular Ca can be significantly perturbed.

We first ascertained the speed and effectiveness of the solution exchange in the vicinity of the tip of the puffer pipette. Fig. 7 *A* shows recordings obtained by pressure ejecting a solution containing the fluorescent dye, and measuring the emission from a small square window defined by the optical mask (see Methods). The top panel shows that when the applied pressure was changed from 4 to 1 psi, the time to achieve 95% of the steady-state signal (t_{95}; *thin dotted line*) increased from 105 to 260 ms, but both traces attained the same asymptote. Similarly, when the distance between the tip and the edge of the window was increased from 25 to 50 μm (*bottom panel*), there was a small delay in the rising phase of the fluorescence signal (t_{95} increased from 260 to 350 ms), but again the final level was the same. The constant amplitude of the asymptotic fluorescence signal as pressure or distance were changed is suggestive that, at least within this range, little or no mixing between the ejected solution and the bath occurred. Similar results were obtained with two additional puffer pipettes. Because the position of the optical window was similar to that of the rhabdomeric lobe of a photoreceptor in actual experiments, and the tip diameters and pressure values in these tests were near the lower end of the range subsequently used, these estimates indicate an upper bound for the time required for solution exchange of < 500 ms. This value dictated the minimum interval that must elapse between initiating the local perfusion and delivering a test flash. We then tested the effect of brief local superfusion with high-calcium solution on the light response of photoreceptors. Fig. 7 *B* shows the results of one of these experiments: The puffer pipette tip was positioned ≈ 35 μm from a

Pecten cell that was voltage clamped at −50 mV. A light stimulus was delivered and a macroscopic response was obtained while ASW was continuously ejected from one of the barrels. After a period of dark adaptation, the superfusion solution was switched to high-Ca (220 mM Ca, 500 mM sucrose, and 10 HEPES/Tris); after ~1 s, another identical light stimulus was delivered and evoked a greatly attenuated response. After this test, the epi-illumination beam was turned on, to corroborate that the ejection plume from the puffer completely engulfed the cell. Similar results were obtained in four more cells; the decrease in response amplitude in the presence of high Ca was 97±2%.

Single channel recordings. A final approach to determine whether a substantial fraction of the photocurrent can be carried by Ca was cell-attached measurements of light-dependent channels. This technique presents the advantage that the test solution is confined to the patch electrode, thus avoiding exposure of the entire cell to unphysiological conditions. Because the surface area of the patch constitutes only a small fraction of the total cell membrane (≪1%), the fluxes that may originate therein are unlikely to affect the ionic concentrations of the cytosol, and hence the transduction cascade. We had previously demonstrated that light-activated single-channel currents are readily recorded in the majority (≈80%) of cell-attached patches in the villous lobe of *Pecten* rhabdomeric photoreceptors (Nasi and Gomez, 1992). These channels are unresponsive to voltage stimuli delivered in the dark, and their properties can account for the macroscopic photocurrent in all salient respects: light sensitivity, ensemble kinetics, density, and reversal voltage (Nasi and Gomez, 1992). The left side of Fig. 8 shows several representative traces obtained in different photoreceptors, using patch electrodes filled with ASW. Prominent bursts of light-dependent channels were elicited by the onset of light stimulation; this channel activity subsided to a low level shortly after stimulus onset, indicating light adaptation. In the right part of the figure, the electrode filling solution contained only 220 mM $CaCl_2$ and 10 HEPES; in this case photostimulation induced no distinct single-channel currents; in some patches (5 of 12), barely detectable fluctuations could be seen shortly after the light onset (e.g., bottom trace); such fluctuations, however, are largely buried in the baseline noise, thus precluding any measurements of unitary current size.

We investigated several possibilities (other than a very low permeability to Ca) that might account for the negligible light-dependent channel activity in high-Ca solution. The first is that inward currents carried by Ca could be masked by a competing outward flux of K

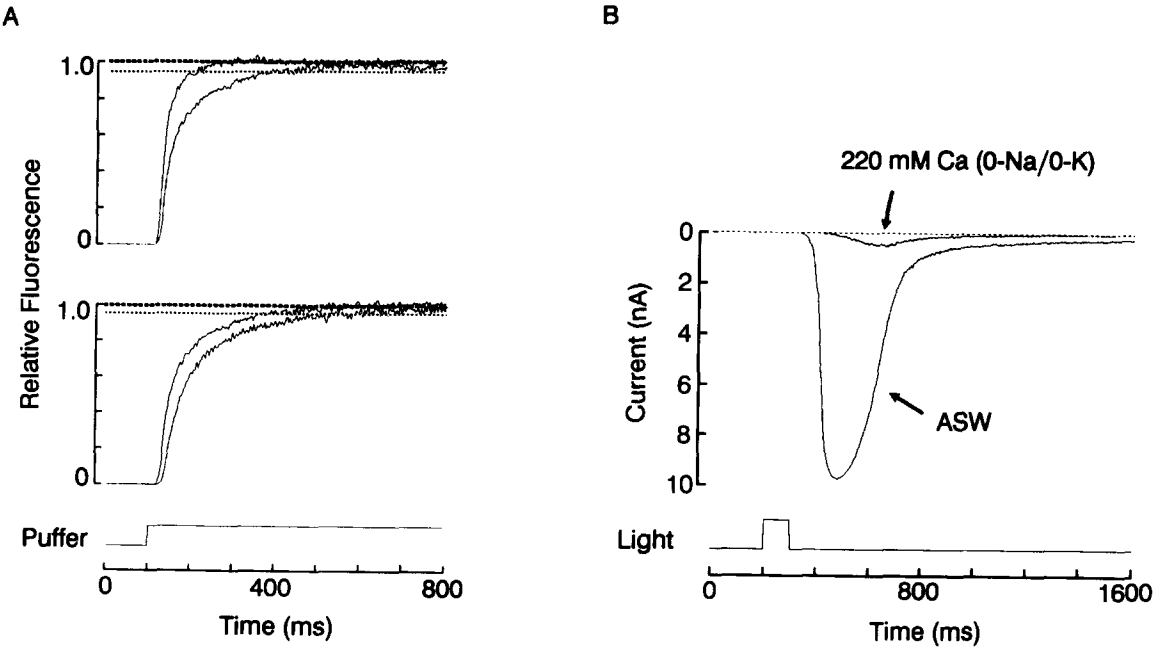

FIGURE 7. (*A*) In vitro test of the effectiveness of local perfusion. The top panel shows the fluorescence measured from a 10 × 10 μm region. A puffer pipette located 25 μm away ejected a solution containing 50 μM fluorescent dye, with a pressure of either 4 or 1 psi. The thin dashed line marks 95% of the mean asymptotic level attained. In the bottom panel a similar test was performed using a constant pressure (1 psi) but changing the spatial separation between the optically sampled region and the tip of the puffer from 25 to 50 μm. (*B*) Reduction of the macroscopic light response during brief, local superfusion of a photoreceptor cell with a high Ca solution. The photocurrent elicited by a test flash of fixed intensity (26×10^{10} photons $\times s^{-1} \times cm^{-2}$) was recorded while puffing ASW, and, after a period of dark adaptation, 220 mM $CaCl_2$. In high calcium, the response was dramatically reduced. The patch pipette contained standard intracellular solution; the holding potential was −50 mV.

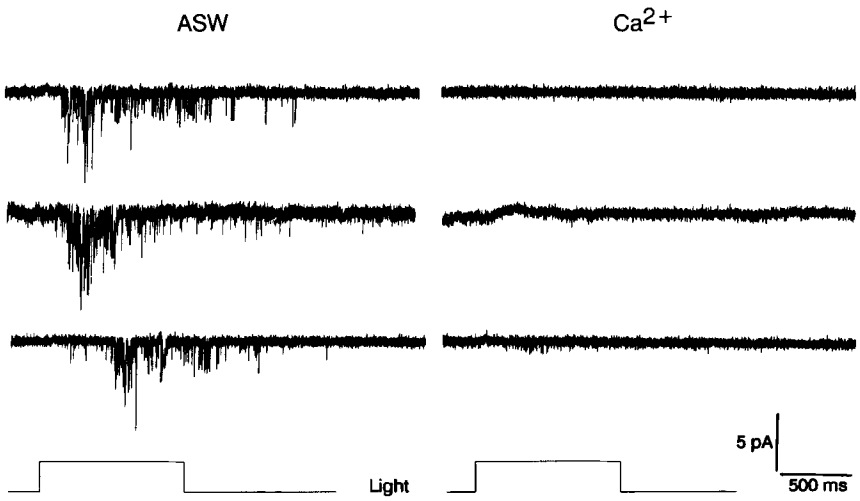

FIGURE 8. Lack of measurable light-dependent unitary currents when Ca is the only extracellular cation. The left part of the figure shows examples of channels specifically activated by light, recorded on the rhabdomeric lobe of three different *Pecten* photoreceptors. The pipette contained ASW, and the potential (V_p) was set at +30 mV. Light stimulation was 1 s in duration, 11–26 × 10^{10} photons × s^{-1} × cm^{-2}. The traces on the right illustrate typical recordings performed in different cells with pipettes containing 220 mM $CaCl_2$ and 10 mM HEPES/Tris. The patches are virtually silent (except for the small capacitatively coupled shunt, indicating that the cells responded to light), with only occasional low-amplitude fluctuations induced by photostimulation (e.g., bottom trace). Light intensity was 26 × 10^{10} photons × s^{-1} × cm^{-2} for the middle trace and was increased to 4 × 10^{14} photons × s^{-1} × cm^{-2} for the other two.

ions. As demonstrated in the previous whole-cell clamp experiments, potassium can readily permeate through light-sensitive channels; furthermore, because K concentration in the recording electrode was zero, the driving force across the patch would be virtually infinite. We repeated the cell-attached measurements, including between 1 and 10 mM potassium in the electrode solution and applying large hyperpolarizing potentials to the patch (40–70 mV), but we were still unable to resolve light-dependent unitary currents (data not shown). Possible adverse consequences of the osmotic gradient (the electrode-filling solution being substantially hypoosmotic) were also controlled by adding mannitol, to maintain constancy of both ionic strength and osmolarity, but no improvement was observed. An additional possible problem is the formation of a vesicle at the tip of the electrode (Milton and Caldwell, 1990), which could uncouple the light-dependent channels from the transduction machinery. The presence of divalents is known to facilitate vesiculation (Hamill et al., 1981), and the very high concentrations used in the filling solution could exacerbate the problem. However, recordings with a pipette solution containing physiological Ca concentration (10 mM) but no Na (replaced by either sucrose or mannitol) also failed to reveal light-dependent channel activity (n = 6). A more conclusive demonstration that light-dependent channels remain viable in patches exposed to high Ca is presented in Fig. 9. Cell-attached patches were stimulated with voltage ramps in the dark and during steady illumination; when the electrode was filled with ASW there was no activity in the dark, whereas in the light inward unitary currents were observed at negative voltages, eventually inverting direction as the patch was depolarized. With a high concentration of $CaCl_2$ alone in the pipette, patches were also silent in the dark, and photostimulation elicited vigorous outward channel activity at depolarized potentials, but no inward currents in the negative range of voltages (n = 3).

This observation is not consistent with the conjecture that failure to record light-dependent channels with high-Ca solutions is due to patch vesiculation. Because reports in *Drosophila* indicate that the light-dependent channels deactivate upon admitting Ca (Hardie, 1991; Ranganathan et al., 1991), it is conceivable that in very high external Ca this shut-off process may occur extremely rapidly, so that individual events escape detection at the recording bandwidth used. We therefore examined other divalent cations that may substitute for Ca in the permeation process but that are less effective agents for other Ca-controlled cellular functions. Barium readily permeates most Ca channels, yet is incompetent in supporting Ca-dependent Ca channel inactivation (Tillotson, 1979), which is most relevant to the present context, as well as a host of other processes, such as neurotransmitter release (Augustine and Eckert, 1984), activation of Ca-dependent Ca channels (Gorman and Hermann, 1979), and Ca extrusion mechanisms (Nasi and Tillotson, 1985). A total of 15 patches were tested with 220 mM Ba in the pipette. In eight of them, small light-induced fluctuations could be observed, as shown in Fig. 10. Again, the signal/noise ratio precluded determination of the size of unitary events. To minimize the possibility that the channels in the patch may be down-regulated by Ca entering through other channels in the vicinity of the patch

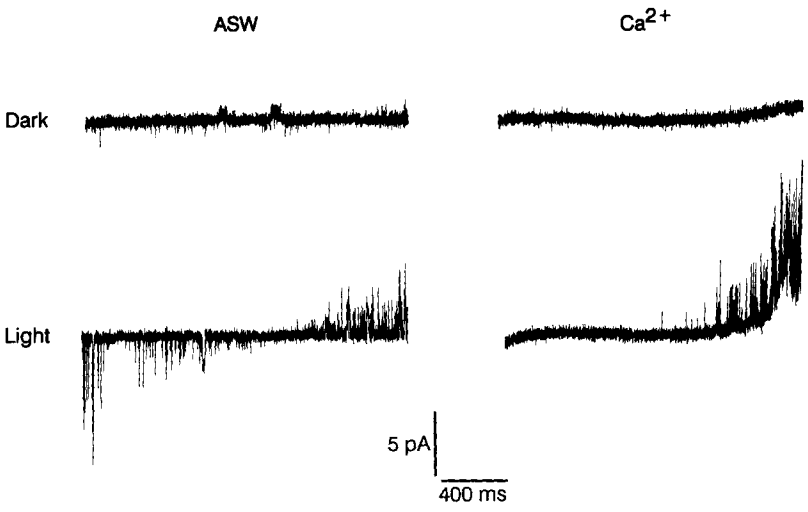

FIGURE 9. Lack of channel activity in high Ca is not due to patch vesiculation. The two traces on the left were recorded from a cell-attached patch with an electrode containing ASW. When a ramp of voltage was applied in the dark, gradually depolarizing the patch (V_p started at +60 mV and reached −50 mV at the end of the sweep), no channels were activated. A second ramp was delivered during steady illumination, resulting in clear single-channel currents, which were inward at negative voltages and eventually inverted direction. On the right, a similar procedure was used in a recording obtained with a patch pipette containing 100 mM $CaCl_2$, 10 mM HEPES/Tris. The patch was also silent in the dark. The ramp administered in the light, however, produced vigorous channel activity in the outward but not in the inward direction, indicating that the light-sensitive channels had not simply become uncoupled from the transduction machinery. (Light intensity 15×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$).

of membrane, we also replaced Ca in the ASW with Ba and used 220 mM Ba in the electrode solution; this precaution, however, also failed to reveal larger currents (n = 2). Finally, no light-induced channels could be seen when the pipette contained 220 mM Mg, as shown by the bottom trace displayed in the same figure. Taken together, the results described in the previous section indicate that light-dependent channels in molluscan photoreceptors are selectively permeable to Na over Ca, both according to the criterion of flux magnitude as well as reversal voltage.

Calcium and Voltage Dependence of the Photocurrent Time Course

Although only a small contribution of Ca influx to the inward photocurrent could be directly demonstrated, this factor may still play a key role regulating the decay of the light-sensitive conductance. To investigate such a possibility, the kinetics of the light response was measured in the presence of different divalent cations in the extracellular medium. The recordings shown in Fig. 11 were obtained with either 220 mM $CaCl_2$ or 220 mM $BaCl_2$, in the absence of Na and K (replaced by sucrose; intracellular Na and K were substituted with Tris), so that the light-activated inward current reflects the influx of either divalent cation. The peak amplitude of the photocurrent in the two conditions was similar, implying a comparable initial entry of either Ca or Ba, but the decay phase was significantly slower in the presence of barium (n = 3). In principle, these results could suggest that, even in a photoreceptor in which Ca permeation is minor, Ca influx may have a modulatory role, at least under conditions that favor the influx of divalent cations.

We next examined whether the accumulation of Ca that enters the cell during the light response is sufficient to account for the changes in kinetics at different membrane voltages. Fig. 12 *A* shows whole-cell current recordings of rhabdomeric cells from *Pecten* obtained under standard ionic conditions (ASW outside, K-based internal solution); light stimulation was applied while the membrane potential was clamped either above or below the reversal potential of the light response. The decay kinetics was significantly more rapid when the photocurrent was inwardly directed, an observation that qualitatively replicates the behavior of the photocurrent of *Drosophila* (Hardie, 1991; Ranganathan et al.,1991) and *Limulus* (Bacigalupo et al., 1986). A straightforward corollary of the proposition that the modulation by voltage of photoresponse kinetics reflects differences in Ca influx is that, by strongly buffering changes of cytosolic Ca, the differences in time course should be attenuated or eliminated. This can be accomplished by dialyzing the intracellular compartment with a high-affinity, rapid Ca buffer. Fig. 12 *B* shows the photocurrents elicited by bright flashes of fixed intensity delivered at different holding potentials, in a *Pecten* cell voltage clamped with a pipette containing 10 mM BAPTA. The buffer markedly slowed down the light response (compare traces in *A*; notice the different time scales) and reduced its sensitivity; to obtain light responses comparable to controls, the stimulus intensity had to be increased by >4 log units and its duration by a factor of 10. Even in the presence of intracellular BAPTA, it is evident that the inward photocurrent measured at a negative holding voltage (−60 mV) decayed significantly more rapidly than the outward photocurrent recorded at a holding potential of +80 mV. A similar effect was observed when calcium was added

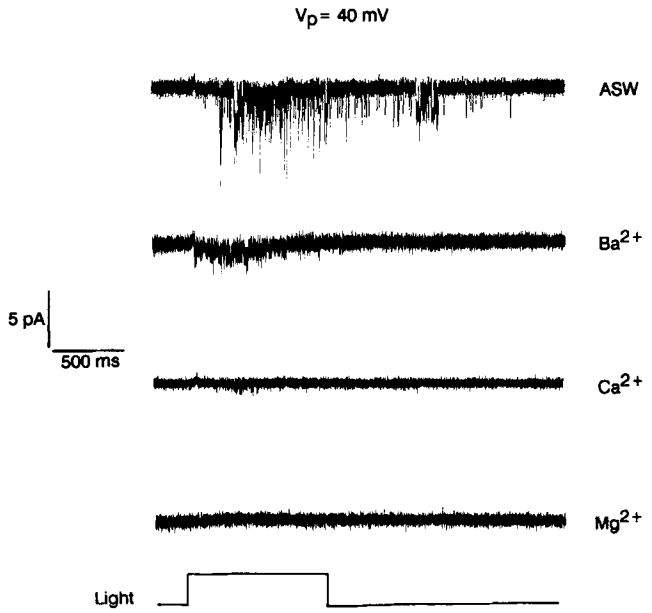

FIGURE 10. Effect of other extracellular divalent cations on light-dependent channel activity. Recordings were performed to compare calcium, barium, and magnesium, at a concentration of 220 mM. The top trace is a representative control, obtained with ASW in the pipette, showing light-activated channels responding to a 1-s light (26×10^{10} photons $\times$ $s^{-1} \times cm^{-2}$). None of the other divalents examined served as efficient carriers of light-dependent current, in spite of testing with higher stimulus intensity (4×10^{14} photons $\times$ $s^{-1} \times cm^{-2}$). Small-amplitude events barely above background noise were usually seen with barium (second trace); light-induced fluctuations were typically below the detection limit when Ca or Mg was used.

to the BAPTA-containing internal solution (free Ca^{2+} = 0.2 μM), to avoid possible exhaustion of intracellular Ca stores ($n = 2$). These results argue against the proposition that Ca influx is the main factor modulating the photocurrent decay phase at different voltages in these photoreceptors.

DISCUSSION

Permeation of ions other than Na through light-dependent channels of rhabdomeric photoreceptors was examined using a variety of experimental protocols designed to minimize the consequences detrimental to phototransduction that often accompany ionic manipulations involving Ca and Na. The results demonstrate that, as previously suggested by Brown and Mote (1974), potassium ions permeate through light-activated channels; this can account for the observation that, under physiological conditions, the reversal voltage of the photocurrent (≈+14 mV) is substantially more negative than the equilibrium potential for Na (≈+72 mV). Exact estimates of the permeability ratio of Na/K is rendered difficult by the concomitant permeability to other ions (see below). If such small additional contributions are disregarded, the apparent value of g_{Na}/g_K is ≈1.7.

At negative membrane potentials, removal of external Na (while maintaining divalent cation concentration constant) dramatically reduced the size of the photocurrent, which remained inward despite the fact that the driving force for potassium was outwardly directed. Chloride could not account for this phenomenon, on the grounds that (*a*) the residual light-induced current reversed at a more positive voltage than the equilibrium potential for Cl, and (*b*) when most $[Cl]_o$ was replaced with an impermeant anion, no shift on V_{rev} of the light response was observed. By exclusion, only Ca or Mg may be responsible for the observed inward photocurrent. Influx of Ca through the light-sensitive conductance could be directly demonstrated in the whole-cell recording configuration, using conditions that both ruled out inward currents carried by other ions and reduced competing outward currents. In the absence of Na and K, the amplitude of the photocurrent and its reversal potential depended on the concentration of extracellular calcium. However, the size of these responses was invariably minute, in spite of the precautions designed to avoid desensitization of the cell due to possible Ca loading of the cytosol. These results are consistent with the cell-attached recordings in patches of rhabdomeric membrane of *Pecten*, in which a solution containing solely a high concentration of $CaCl_2$ was used to fill the recording electrode. Under those conditions, it is unlikely that any significant perturbation of intracellular Ca could have occurred, yet photostimulation failed to induce resolvable unitary currents. By contrast, vigorous light-dependent channel activity could be routinely recorded when the patch electrode contained Na (with or without divalent cations). If the normal size of the light-dependent single-channel currents is scaled down by a factor corresponding to the reduction of the macroscopic photocurrent size observed after Na removal, one would in fact expect that the unitary currents would virtually disappear in the background current noise. In view of the convergent results obtained with different approaches, the most parsimonious conclusion is that Ca influx via the light-sensitive conductance is quantitatively small in molluscan photoreceptors. Considering the size of the currents normally measured in the presence of Na, an upper-bound estimate of the Ca contribution would be on the order of ≈3%.

A small Ca flux is not necessarily incompatible with the notion that a channel may favor calcium over other ions. For example, under physiological conditions, voltage-gated L-type channels of cardiac and skeletal muscle cells are highly Ca selective (for review see Tsien et al., 1987), as reflected by the direct relation between $[Ca]_o$ and the size and reversal potential of the current,

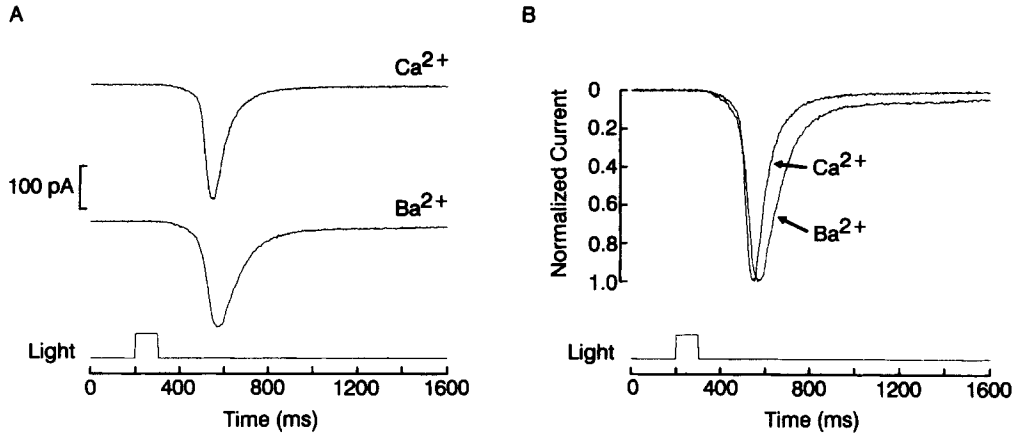

FIGURE 11. Comparison of the kinetics of photocurrents carried by Ca or by Ba. (*A*) A photoreceptor cell was dialyzed with an internal solution containing Tris (0-K, 0-Na), and voltage clamped at −50 mV. The top trace was in response to a test flash (63×10^{10} photons $\times$ s^{-1} $\times$ cm^{-2}) during superfusion with a 0-Na solution containing 220 mM $CaCl_2$, 10 HEPES/Tris, and 500 mM sucrose. After replacing Ca with Ba, a second, identical stimulus produced the response at the bottom. (*B*) Normalized responses to light carried by Ca and by Ba, underscoring the slower fall kinetics in the presence of barium.

and by the insensitivity to manipulations of other ions. However, in the absence of Ca, larger currents carried primarily by Na can be measured. This phenomenon was explained by the high affinity of the channel's selectivity filter for Ca ions, which would reduce transport rates while simultaneously excluding other ions. Removal of Ca eliminates this hindrance, so that other ions could be translocated at higher rates because of their weaker binding to intrapore sites. It is unlikely that a similar model could be satisfactorily applied to the light-sensitive channels of molluscan rhabdomeric photoreceptors, because $[Ca]_o$ is quite high (10 mM) under physiological conditions, and a high affinity of the channels for Ca ions would preclude large photocurrents carried by Na. This notion is also consistent with the measurements of V_{rev} after substituting both internal and external Na and K with poorly permeant species. It is difficult to gauge to what extent the reversal voltage of the residual response departs from E_{Ca}, because at the peak of the light response $[Ca]_i$ could increase by >100-fold (Ukhanov et al., 1995), largely because of internal release, so that E_{Ca} may be displaced by tens of millivolts. Nevertheless, it is clear that the *changes* in V_{rev} in response to manipulations of $[Ca]_o$ remained sub-Nernstian. Taken together, the present results underscore that, in *Lima* and *Pecten*, like in *Limulus*, the ionic selectivity of the light-dependent channels to Ca is substantially lower than to Na. By contrast, in *Drosophila*, photoreceptors' reversal potential measurements indicate that the light-dependent conductance may select Ca over Na by a factor as large as 40×. However, as shown by Hardie and Minke (1992), such selectivity for divalent cations is greatly attenuated in the *trp* null mutant, indicating that it concerns primarily a specific subpopulation of channels. The presence of multiple light-dependent ionic mechanisms seems relatively widespread across invertebrate species (e.g., Nasi, 1991*c*; Nasi and Gomez, 1992; Hardie and Minke (1992); Deckert et al., 1992). It is unlikely that, at least in mollusks, the secondary component that appears at high stimulus intensities may exhibit a higher Ca selectivity than the primary one, because its reversal potential is significantly more negative (Nasi, 1991*c*). The difficulties of examining this additional component in isolation preclude a more detailed analysis of its ion selectivity and conduction properties.

The effects of different divalent cations on kinetics of the light response in these cells (Fig. 11) indicate that Ca influx, in spite of its small contribution to the total photocurrent, may modulate light-dependent channels. Nevertheless, this interpretation is tempered by reports that barium can alter some metabolic function in rods, causing indirectly a prolongation of the light response (Menini et al., 1988). In addition, Ca influx alone is not sufficient to account for the effects of voltage on the time course of the light-induced current, because buffering internal Ca with BAPTA failed to eliminate the depolarization-induced slowing down of photocurrent decay (Fig. 12). The markedly reduced light sensitivity and the sluggish time course of the response during intracellular dialysis with BAPTA suggest that the chelator did in fact reach the sites where the transduction machinery is located. Although it is not possible to rule out that the increased buffering capacity may still be insufficient to counteract very large, rapid Ca changes near the sources, it seems more parsimonious to suggest that mechanisms other than Ca influx contribute to the modulation of the photocurrent

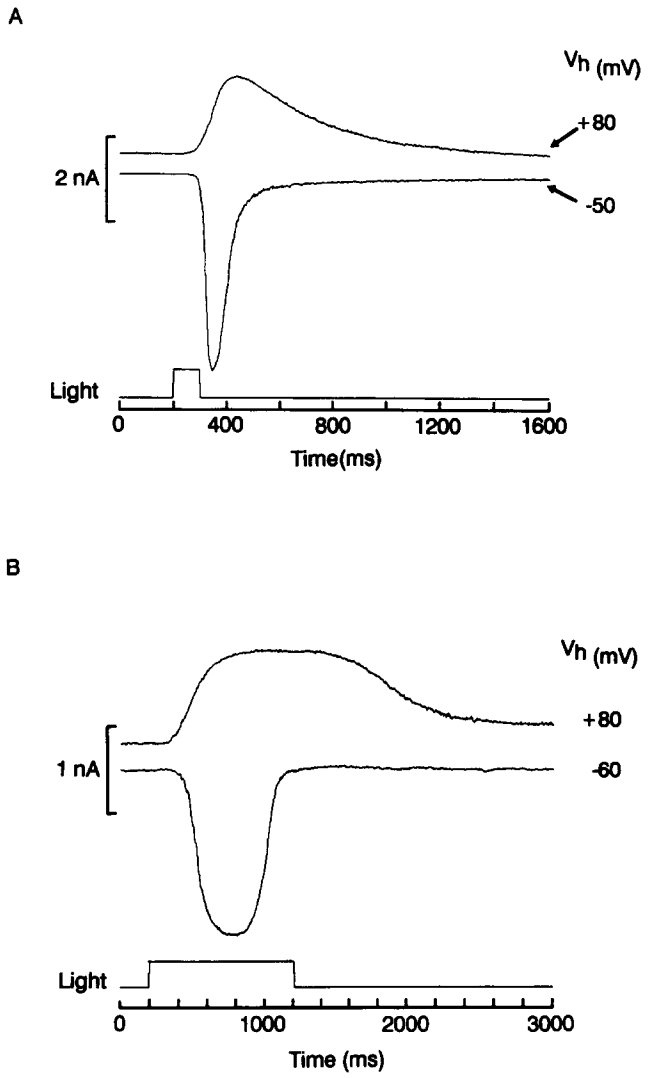

FIGURE 12. Effect of increased Ca buffering on photocurrent kinetics. (*A*) Differences in the falling phase of the light response when the membrane voltage is maintained either below or above the reversal potential. A *Pecten* photoreceptor was stimulated with a 100-ms flash (26×10^{12} photons $\times$ $s^{-1} \times cm^{-2}$), delivered at the indicated values of the holding potential. Depolarization resulted in a significantly slower decay of the photocurrent. (*B*) A similar experiment performed in a cell internally dialyzed with 10 mM BAPTA. The Ca buffer markedly reduced responsiveness, so that a much brighter light (5×10^{17} photons $\times$ $s^{-1} \times cm^{-2}$) had to be presented for 1 s to elicit a sizable photocurrent. The time course of the light response was considerably slower compared with control conditions (notice different time scales in *A* and *B*), but a striking difference persisted in the decay kinetics of the response at the two holding potentials.

by membrane voltage and by $[Ca]_o$: (*a*) depolarization is known to affect the gating of light-dependent channels in *Limulus* and *Pecten*, decreasing the closing rate and increasing the opening rate (Bacigalupo et al., 1987; Nasi and Gomez, 1992). These effects are likely to stem directly from the voltage changes, rather than secondarily from the decreased driving force on Ca, because exposure to divalent-free solutions per se failed to induce longer channel openings (Nasi and Gomez, 1992). (*b*) Reduced external calcium slows down the photoresponse via a mechanism that operates upstream of the gating of the light-sensitive conductance: Lisman (1976) and Martinez and Srebro (1976) observed that in *Limulus* Ca removal induced great variability in the latencies of the responses evoked by single photons. The time course of individual elementary waves, however, was similar to that observed in normal $[Ca]_o$, indicating that the mechanism responsible for shutting off the light-induced conductance was unaffected. Responses to brighter stimuli, composed of superimposed elementary waves, acquired a sluggish time course in Ca-free media as a result of the greater dispersion of the quantal events.

Some degree of permeability of light-dependent channels to divalent cations appears to be a general feature, necessary to account for the persistence of inward photocurrents in Na-free media that has been observed in a variety of rhabdomeric photoreceptors. The quantitatively disparate contribution of Ca influx to the photocurrent and its variable role as a modulatory factor may be related to the different mechanisms underlying the increase in cytosolic Ca that accompanies the photoresponse. In *Limulus*, the light-induced Ca transient survives superfusion with 0-Ca, implying that it is mostly due to internal release (Brown and Blinks, 1974). Because the peak concentration in the rhabdomeric lobe can reach tens of micromolar (Brown et al., 1977; Levy and Fein, 1985; Ukhanov et al., 1995), any additional Ca permeating through light-activated channels would not be expected to exert a major influence. In *Drosophila* and *Balanus*, by contrast, the Ca signal appears to be entirely dependent on external Ca (Peretz et al., 1994; Ranganathan et al., 1994; Brown and Blinks, 1974). The absence of a substantial internal release process in those cells may require Ca influx during the photoresponse for an effective modulatory role.

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