Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7938–7942, September 1991
Neurobiology

Light-dependent channels from excised patches of *Limulus* ventral photoreceptors are opened by cGMP

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Communicated by John E. Dowling, May 28, 1991

ABSTRACT **The identity of the second messenger that directly activates the light-dependent conductance in invertebrate photoreceptors remains unclear; the available evidence provides some support for cGMP and Ca^{2+}. To resolve this issue we have applied these second messengers to membrane patches excised from the light-sensitive lobe of *Limulus* ventral photoreceptors. Our results show that these patches contain channels that can be opened by cGMP, but not by Ca^{2+}. These cGMP-activated channels closely resemble the channels activated by light in cell-attached patches. This evidence suggests that cGMP is the messenger that opens the light-dependent channel in invertebrate photoreceptors.**

In photoreceptors, large numbers (10^2–10^3) of membrane channels are affected by the absorption of a single photon (1, 2). This amplification is produced by a second messenger cascade (3). In vertebrate rods and cones (4–8) the final step of this cascade involves the direct control of ion channels by cGMP. Efforts to establish which second messenger controls the light-dependent channels in invertebrate photoreceptors have given conflicting results (9). Injection of inositol trisphosphate (IP_3) (10, 11) or Ca^{2+} (12) into *Limulus* ventral photoreceptors leads to the activation of the light-dependent conductance. This evidence, together with biochemical evidence that the inositol phospholipid pathway (11, 13–15) is stimulated by light, suggests that the Ca^{2+} released by IP_3 is the messenger that turns on the light-dependent conductance. However, injection of cGMP into *Limulus* also leads to activation of the light-dependent conductance and supports a role for this cyclic nucleotide (16). Because of the complexity of transduction processes and the possibility that second messenger systems may interact in living cells, it is not possible to determine from such experiments which second messenger interacts directly with the light-dependent channels. To resolve this issue, we have applied these second messengers to membrane patches excised from *Limulus* photoreceptors. Our results indicate that light-dependent channels are opened by cGMP and not by Ca^{2+}.

METHODS

To apply the patch clamp technique to *Limulus* ventral photoreceptors, it is first necessary to remove the glial cells and connective tissue that surround the photoreceptor cell that is to be studied. This was done mechanically by using a suction pipette while observing the cell through a ×100 compound microscope, as described (17). Gigaohm seals were made on the light-sensitive microvillar membrane of the photoreceptor (18) by using 1- to 2-MΩ electrodes filled with artificial seawater (ASW) and coated with Sylgard (Dow). After obtaining the seals, patches were excised in the inside-out configuration into an "internal" solution (described below) resembling the ionic composition of the cytoplasm. As described in *Results*, it was important to prevent exposure of the excised patch to ASW. In our initial work, we achieved this by exposing the region of the cell-attached patch to internal solution delivered from a separate pipette. We found, however, that this procedure was cumbersome and that obtaining stable excised patches was rare. We then switched to a different strategy that gave a higher yield of stable excised patches. In this procedure we perfused the entire bath with internal solution before making the seals (depolarizing the membrane voltage to near zero). The patch could therefore be excised directly into the bath without exposure to ASW. To apply different solutions to the excised patch, the pipette tip was placed near the mouth of one of a series of parallel glass capillaries through which different solutions

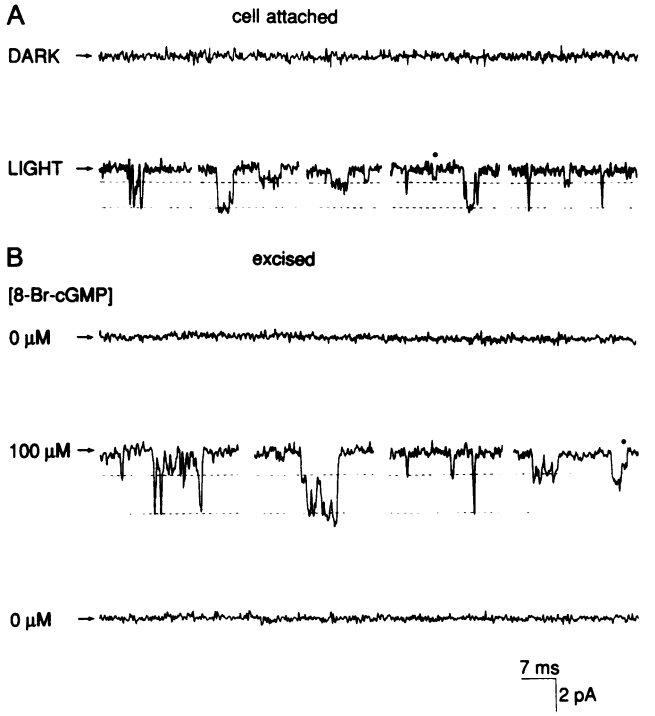

FIG. 1. Activation of channels by light and 8-Br-cGMP. (*A*) Cell-attached current in the dark (top trace) and in the light (bottom trace). Arrows indicate the baselines, and dashed lines indicate the large and small conductance states of the light-activated channel. Membrane voltage during light ≈ −30 mV. (*B*) Excised patch current in cGMP-free internal solution (top trace), in internal solution containing 100 μM 8-Br-cGMP (middle trace), and after return to control solution (bottom trace). Arrows indicate the baselines, and dashed lines indicate the two major conductance levels. The membrane potential was −70 mV; note the difference in the driving force from that in *A*. In *A* and *B*, the dots mark yet smaller conductance events.

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Abbreviations: IP_3, inositol trisphosphate; ASW, artificial seawater.

were driven by gravity. With this set of "optimal" procedures, the overall success rate was still very low; we estimate that for each useful excised patch obtained, experiments were attempted on about a dozen animals.

ASW composition was 425 mM NaCl, 10 mM KCl, 22 mM $MgCl_2$, 26 mM $MgSO_4$, 10 mM $CaCl_2$, and 10 mM Hepes at pH 7.8. The control internal solution contained 300 mM KCl, 2 mM $MgCl_2$, 5 mM Hepes (pH 7.0), 2.42 mM $CaCl_2$, and 5 mM bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA; Molecular Probes) to give a $-\log[Ca^{2+}]$ (pCa) of 7 (in a few experiments the pCa was set to 8, but it produced no obvious difference). To this solution we added cGMP or 8-Br-cGMP (Calbiochem) to the various final concentrations. Solutions containing high Ca^{2+} (pCa 5.0 and 3.0) were made with no added BAPTA. All experiments were done at room temperature.

The data were digitally stored in a video recorder. Before input into the computer for analysis, the data were filtered with a Bessel filter set at a cutoff frequency of 20 kHz. Analysis was done using PCLAMP 5.5 software for single-channel analysis. This program performed digital Gaussian filtering. For the current traces shown, the overall cutoff frequency of the system was 1.3 kHz. For determination of amplitude and open-time distributions, a 3-kHz cutoff frequency was used. For construction of amplitude and open-time histograms, events were detected by using an interactive program (FETCHAN in PCLAMP 5.5.1). An event was included in amplitude histograms only if its duration was long enough to allow the digital Gaussian filter to settle (200 μs). Closings shorter that 0.3 ms are due to flicker (18) and were ignored in the determination of open-time distributions. For this reason, the open-time distribution should technically be considered the distribution of burst times. The open time was calculated as the time between two 50% threshold crossings from an event that was open long enough for the amplitude to be accurately determined. For construction of current–voltage plots, amplitude histograms were made of the raw current data. These histograms were then fit with Gaussian distributions by using a standard Levenberg–Marquardt algorithm as implemented in PCLAMP 5.5.1. The peaks of the Gaussian curves at each patch potential were used to construct the current–voltage plots. To measure the probability of being open as a function of voltage or cyclic nucleotide concentration, the following procedure was used. A histogram of the raw digitized current data was constructed, and the baseline noise was fit with a Gaussian, the width of which was determined from data obtained without application of cyclic nucleotide. The points not accounted for by the baseline were integrated and divided by the charge that would have passed through a single continuously open 40-pS channel. This computed quantity is an estimate of the probability of being open multiplied by the number of channels in the patch (unknown in this case). More elaborate ways of computing the probability of being open in patches with multiple channels having multiple conductance states were not feasible because of limited data.

RESULTS

Experiments were begun by obtaining seals on the light-sensitive lobe of the photoreceptor (17). Before excising the patch, we briefly tested for the presence of light-dependent and voltage-dependent channels (1, 19). The appearance of light-activated channels in cell-attached patches is shown in Fig. 1*A*. The figure shows both 40- and 15-pS events, which previous work (18) has shown to be due to different conduc-

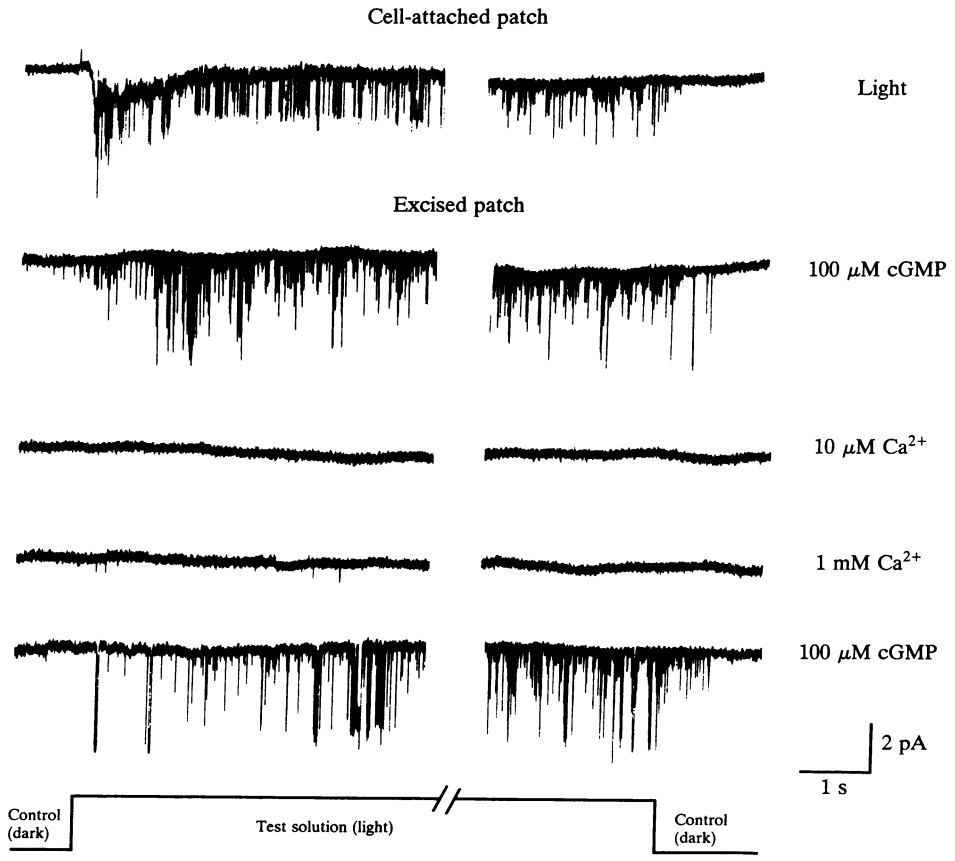

FIG. 2. Application of Ca^{2+} and cGMP to excised patch. The top trace shows the response to light in a cell-attached patch (membrane potential ≈ -30 mV). All other traces are from the same patch after excision (membrane potential = -70 mV). The bar below the traces indicates exposure to test solution or light, as indicated. Note the difference in the driving force under cell-attached and excised conditions.

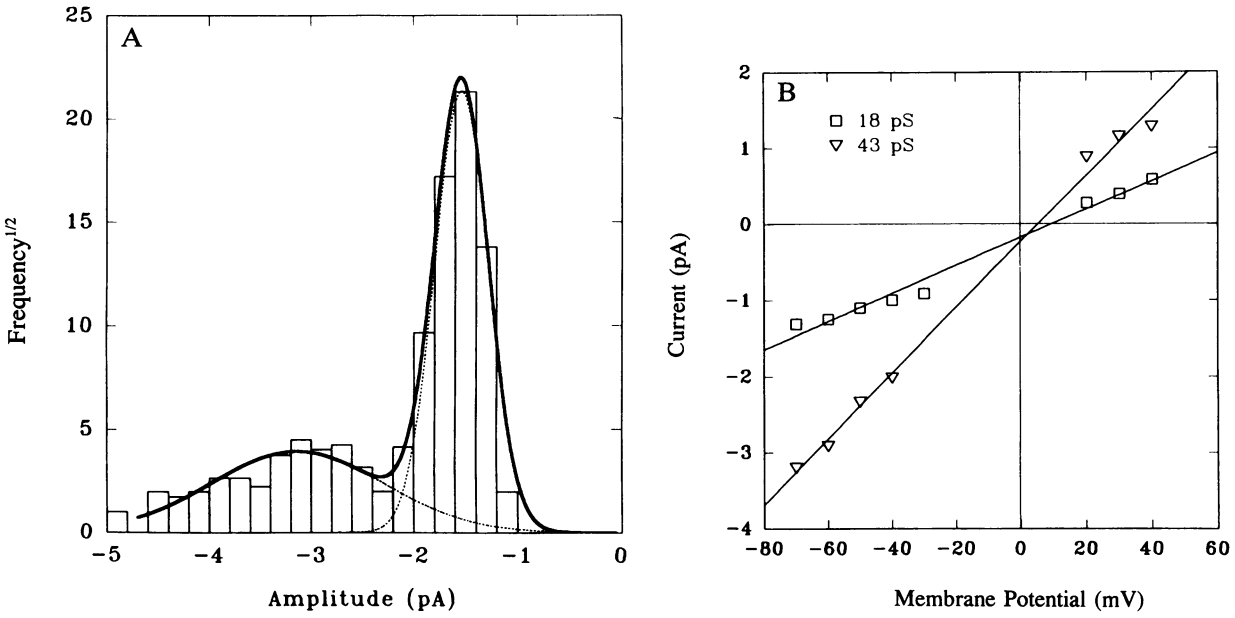

FIG. 3. Conductances of events evoked by 100 μM 8-Br-cGMP in an excised patch. (*A*) Amplitude histogram of events long enough for current to settle (see *Methods*). The membrane voltage was −60 mV. The ordinate is the square root (25) of the number of observations per 0.2-pA bin. The data are fit with the sum of Gaussian distributions (bold line) [level 1: weight 209, −1.5 ± 0.18 pA; level 2: weight 23, −3.2 ± 0.6 pA (mean ± SD)]. (*B*) Current–voltage relation for the 8-Br-cGMP-dependent channel. Plotted currents were obtained from the peaks of the raw current histograms. Solid lines are slopes fit by using linear regression.

tance states of the same channel. To check for voltage-dependent channels, we applied depolarizing steps in the dark (typically up to 100 mV). Depolarization in the dark usually evoked no channel activity. We proceeded with an experiment only if the patch contained solely light-dependent channels.

Membrane patches were excised in an inside-out configuration (20) into internal solution. Under these conditions, patches were silent (Fig. 1*B*, top trace). We found that it was essential to excise the patches directly into internal solution because even brief exposure of excised patches to normal extracellular solution (ASW) often altered the channel behavior in an irreversible way. These "damaged" patches exhibited spontaneous openings when subsequently examined in control internal solution (21). Patches were most vulnerable to damage at the moment of excision; patches were not damaged if excised into internal solution and subsequently exposed to ASW or high Ca^{2+} internal solution. This suggests that damage is caused by an enzyme (e.g., Ca^{2+}-dependent proteases) that can diffuse away from excised patches.

In some rare cases, excised patches were silent in the dark, but responded to light (22), indicating that these patches were metabolically active. Such patches were not used for the experiments described below since metabolic activity makes patches unsuitable for determining which second messenger interacts *directly* with the channel.

To determine whether cGMP can directly activate channels in excised patches, we applied cGMP or the slowly hydrolyzable cGMP analog 8-Br-cGMP (23) to the cytoplasmic face of the excised patch. The analog was used in most experiments because of the possibility that phosphodiesterase activity is associated with the patch, as is the case with vertebrate rod photoreceptors (24). Application of cyclic nucleotide evoked channel activity (Fig. 1*B*; 100 μM 8-Br-cGMP) that disappeared when cyclic nucleotide was re-

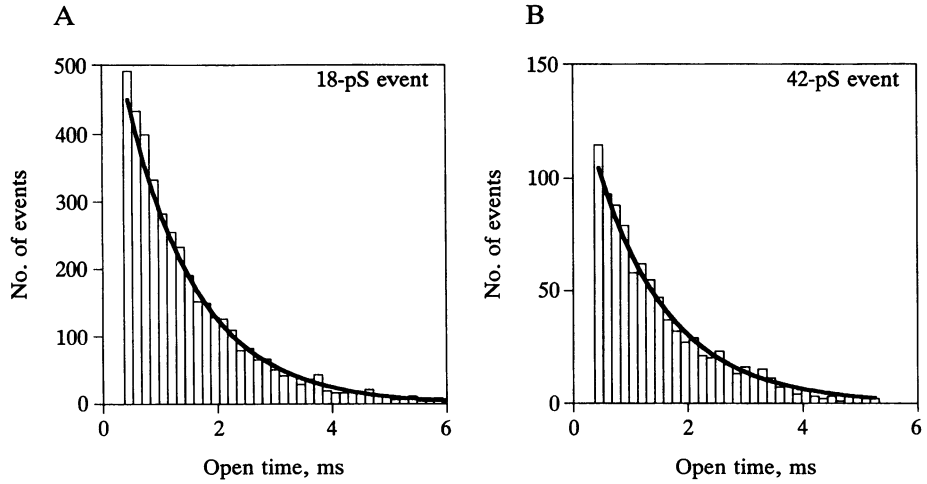

FIG. 4. Distribution of open times for small (*A*) and large (*B*) events during application of 30 μM 8-Br-cGMP to excised patch (membrane potential = −60 mV). Solid lines are exponentials fit by using the Levenberg–Marquardt algorithm (τ = 1.21 ms for the small and 1.25 ms for the large event).

moved. Fig. 2 shows a similar experiment using cGMP. Here it can be seen that the activation occurred <1 s after the application of cGMP, that activity was maintained for the duration of the application, and that the activity reversed rapidly when cGMP was removed. The most stable excised patch we studied was maintained for >30 min and remained responsive to cGMP over this entire period, strongly suggesting that the responsiveness does not depend on metabolism within the patch. We observed channel activation by either 8-Br-cGMP or cGMP in 16 patches, whereas 21 patches that were silent upon excision did not respond to cyclic nucleotide. This unresponsiveness was probably due to the formation of vesicles that would prevent access of cGMP to the binding site on the channel (20). Procedures used in other preparations to break such vesicles (exposure to air or mechanical force) usually led to the loss of the seal in the *Limulus* preparation.

The amplitude distribution (Fig. 3*A*) of channel openings induced by 8-Br-cGMP shows both large and small events. The current–voltage curves (Fig. 3*B*) for both large and small events were linear in the range of −70 mV to +40 mV. The conductance, as determined from the slope of these curves, was 18 and 43 pS. In the five experiments in which sufficient data were collected to determine the conductances, the values were 18 ± 3 pS and 42 ± 4 pS (mean ± SD, n = 5 patches). These values are similar to the conductances of the small and large subconductance states of the light-activated channel measured (18) in cell-attached patches (14.9 ± 3.1 pS and 39.7 ± 3.8 pS, respectively; mean ± SD). The reversal potential for cGMP-activated channels was 6 ± 4 mV for the small conductance and 4 ± 5 mV for the large conductance state (mean ± SD, n = 5 patches), in the same range (≈10 mV) as that of the light-dependent channel (18). We occasionally observed still smaller cGMP-activated events (Fig. 1*B*, dot) having a conductance of about 9 pS and a reversal potential near zero. This conductance could sometimes be detected in cell-attached patches during light (Fig. 1*A*, dot) but was not studied in detail.

Fig. 4 shows the open-time distribution for large and small events activated by 8-Br-cGMP at a membrane potential of −60 mV. These distributions are well fit by a single exponential with a time constant of 1.21 ms for the small events and 1.25 ms for the large events. This can be compared to values for the light-activated channel observed in cell-attached patches of 2.38 ± 0.73 ms for the large conductance state and 1.34 ± 0.49 ms for the small conductance state (18). In most patches the data available were more limited, so the open times of large and small events were lumped. The average time constant was 1.16 ± 0.52 ms (mean ± SD, n = 5). Fig. 5*A* shows channel openings at both positive and negative voltages. It can easily be seen that the probability of being open is much higher at positive voltage. This is demonstrated more quantitatively in Fig. 5*B*, in which a measure of the probability of being open (see *Methods*) is plotted as a function of voltage. This probability is nearly independent of voltage at negative voltages and increases dramatically at positive voltages, a property similar to that of the light-activated channel in cell-attached patches (1, 18, 19).

We obtained very limited information about the concentration dependence of the cGMP-activated channel. In one experiment using cGMP and one using 8-Br-cGMP we found that 10 μM cyclic nucleotide produced clearly noticeable channel activity. A 3-fold increase in concentration to 30 μM produced a dramatic increase in activity; a further 3-fold increase to 100 μM produced a less dramatic further increase, indicating an approach to saturation. These data suggest that the $k_{1/2}$ of the channel is in the range of 10–30 μM.

Cell-attached patches typically contain a few light-activated channels but rarely contain a single channel (18). In excised patches the maximum current activated by cGMP was typically several times larger than the single-channel current, indicating that patches contained several cGMP-activated channels; in one case a patch contained only a single cGMP-activated channel. Thus the number of cGMP-activated channels per patch appears to be roughly comparable to the number of light-activated channels per patch.

To test whether Ca^{2+} can directly activate channels in excised patches, we applied Ca^{2+} in the range of 10 μM to 1 mM. In no case did Ca^{2+} lead to channel activation (n = 28) in the absence of cGMP. This is illustrated in Fig. 2, which shows a patch that responded to cGMP, but failed to respond to 10 μM or 1 mM Ca^{2+}. The patch retained responsiveness to cGMP, indicating that the failure to respond to Ca^{2+} was not due to a change in the accessibility of the channel. The lower concentrations of Ca^{2+} (10 and 100 nM) in our control solutions also failed to activate channels. The only effect of

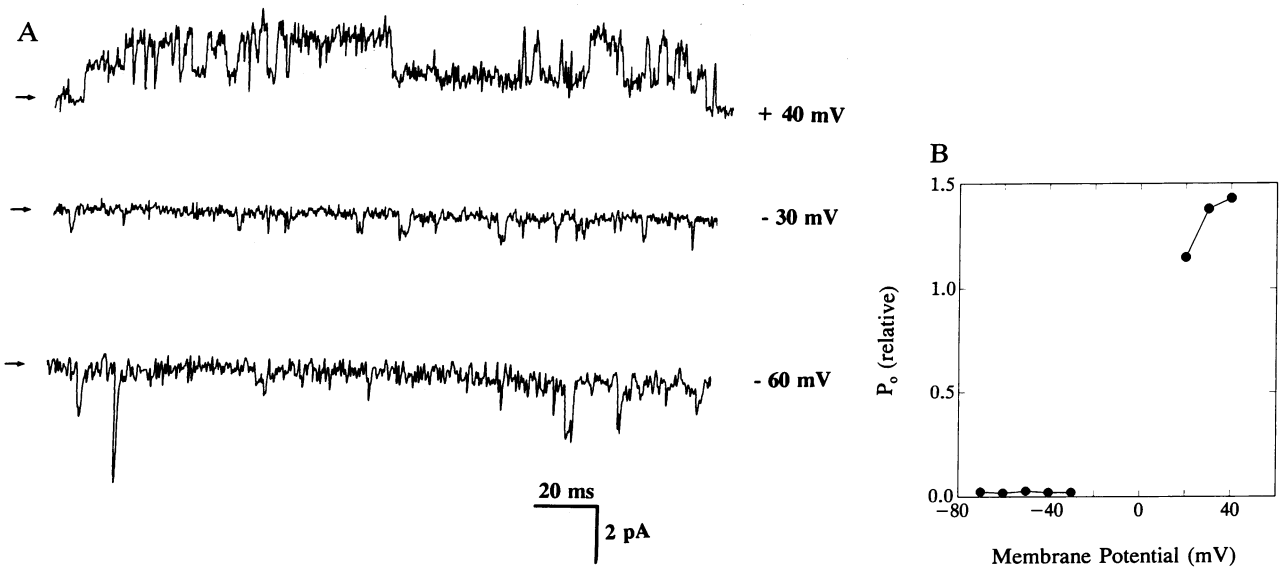

FIG. 5. Voltage dependence of gating of the cGMP-activated channel in excised patches. (*A*) Current traces showing channel events activated by 8-Br-cGMP at various membrane voltages, which are shown beside each trace. (*B*) A measure of the probability of the channel being in the open state is plotted as a function of membrane voltage. The measure used is described in *Methods*. The 8-Br-cGMP concentration was 100 μM.

Ca^{2+} we observed was on damaged patches (see above); application of 1–10 μM Ca^{2+} to such patches *decreased* channel activity (21).

DISCUSSION

Our results show that membrane patches excised from the light-sensitive lobe of *Limulus* ventral photoreceptors contain a channel that can be activated by application of cGMP or 8-Br-cGMP (Figs. 1 and 2). This activation does not appear to depend on metabolism since patches remained responsive to cGMP for as long as 30 min. Furthermore, channels in these patches could be activated by light before excision but were not light activated after excision. Since the activation of the channels in excised patches occurred in the absence of ATP or GTP, the activation is unlikely to be mediated by phosphorylation. Our results thus suggest that the channels were *directly* activated by cGMP.

The cGMP-activated channels that we observed in excised patches closely resemble the channels activated by light in cell-attached patches. Both have similar conductances, reversal potentials, and mean open times (see *Results*). Perhaps most telling is the voltage dependence of the probability of being open. For both the light-dependent (1, 17, 18) and cGMP-dependent channels, the probability of being open is relatively independent of voltage at negative potentials but increases dramatically at positive voltages (Fig. 5). We thus conclude that the light-dependent channel can be activated by cGMP.

Recent results using bilayer reconstitution methods indicate that squid photoreceptors also contain a cGMP-activated channel (26), but the similarity of this channel to the squid light-dependent channel remains to be determined. The *Limulus* cGMP-activated channel has similarities to that found in vertebrate photoreceptors: both have lifetimes in the millisecond range (5, 27), have subconductance states (6, 27), and show a voltage dependence of gating that leads to an increase in the probability of being open at positive voltages (27, 28). There is one dramatic difference between the *Limulus* and vertebrate channels: the invertebrate channel has a conductance in the picosiemen range in the presence of physiological levels of divalent ions, whereas the apparent conductance of vertebrate channels is in the femtosiemen range due to the blocking action of divalent ions (5, 6).

We found no indication of channels in excised patches that could be activated by Ca^{2+}. This argues strongly against models in which the Ca^{2+} liberated by IP_3 production is directly responsible for channel activation during light. Previous work had already ruled out the possibility that IP_3 directly activated plasma membrane channels since the effect of IP_3 injection can be blocked by intracellular injection of calcium buffer (29). The possibility that Ca^{2+} can bring about channel activation through the production of cGMP is an interesting possibility that is by no means ruled out by our experiments. A question of obvious interest is whether Ca^{2+} can affect the channels that are turned on by cGMP. Preliminary evidence suggests that Ca^{2+} acts to inhibit such activity (21).

The demonstration that *Limulus* light-dependent channels are activated by cGMP suggests that light opens channels through the production of cGMP or a related nucleotide and raises important questions about intermediate steps in transduction that must now be answered. cGMP-metabolizing enzymes exist in invertebrate photoreceptors (30, 31), but there is not yet any compelling evidence for their control by light. cGMP metabolism might be controlled by a product of the light-activated phospholipase C pathway or might be under the control of an altogether separate pathway.

We thank Gordon Fain for comments on the manuscript. This work was supported by National Institutes of Health Grant EY 01496 to J.E.L., and National Science Foundation Grants BNS-8812455 (to E.C.J.), and INT-8610625 (to J.B.).

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