Diacylglycerol Analogs Inhibit the Rod cGMP-Gated Channel by a Phosphorylation-Independent Mechanism

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ABSTRACT The electrical response to light in retinal rods is mediated by cyclic nucleotide-gated, nonselective cation channels in the outer segment plasma membrane. Although cGMP appears to be the primary light-regulated second messenger, cellular levels of other substances, including Ca^{2+} and phosphatidylinositol-4,5-bisphosphate, are also sensitive to the level of illumination. We now show that diacylglycerol (DAG) analogs reversibly suppress the cGMP-activated conductance in excised patches from frog rod outer segments. This suppression did not require nucleoside triphosphates, indicating that a phosphorylation reaction was not involved. DAG was more effective at low than at high [cGMP]: with 50 μM 8-Br-cGMP, the DAG analog 1,2-dioctanoyl-*sn*-glycerol (1,2-DiC8) reduced the current with an IC_{50} of ~22 μM (Hill coefficient, 0.8), whereas with 1.2 μM 8-Br-cGMP, only ~1 μM 1,2-DiC8 was required to halve the current. DAG reduced the apparent affinity of the channels for cGMP: 4 μM 1,2-DiC8 produced a threefold increase in the $K_{1/2}$ for channel activation by 8-Br-cGMP, as well as a threefold reduction in the maximum current, without changing the apparent stoichiometry or cooperativity of cGMP binding. Inhibition by 1,2-DiC8 was not relieved by supersaturating concentrations of 8-Br-cGMP, suggesting that DAG did not act by competitive inhibition of cGMP binding. Furthermore, DAG did not seem to significantly reduce single-channel conductance. A DAG analog similar to 1,2-DiC8—1,3-dioctanoyl-*sn*-glycerol (1,3-DiC8)—suppressed the current with the same potency as 1,2-DiC8, whereas an ethylene glycol of identical chain length (DiC8-EG) was much less effective. Our results suggest that DAG allosterically interferes with channel opening, and raise the question of whether DAG is involved in visual transduction.

INTRODUCTION

The light response in retinal rods is mediated by nonselective cation channels that are activated by the direct binding of cGMP (Fesenko et al., 1985). In the dark, an inward cation current ("dark current"), carried mainly by Na^+, flows through these channels, depolarizing the cell (reviewed in: Yau and Baylor, 1989; Lamb and Pugh, 1990; McNaughton, 1990; Yau, 1994). When excited by photon absorption, rhodopsin activates a G-protein (transducin), which in turn activates a phosphodiesterase. The phosphodiesterase hydrolyzes cGMP, and the resultant decrease in [cGMP] causes some of the channels to close. The decrease in Na^+ permeability hyperpolarizes the cell and leads to a reduction in neurotransmitter release from the synaptic terminal.

Additional control is added to this system through light-dependent changes in $[Ca^{2+}]$, which appear to regulate the production and hydrolysis of cGMP (recently reviewed in: Lagnado and Baylor, 1992; Detwiler and Gray-Keller, 1992) and may also modulate the cGMP-gated channels (Hsu and Molday, 1993; Gordon et al., 1995; Nakatani et al., 1995). Other regulatory mechanisms such as phosphorylation are also known to exist in the rod. For example, phosphorylation of rhodopsin by rhodopsin kinase is involved in shutting off rhodopsin activation of transducin (reviewed in: Hargrave and McDowell, 1992; Hofmann et al., 1992). Also, the cGMP sensitivity of the light-regulated ion channel can be reciprocally modulated by two phosphorylation sites (Gordon et al., 1992), although the physiological significance of this modulation remains to be determined.

Two other second messenger systems in the rod outer segment that are potentially sensitive to the level of illumination are the phospholipase A_2 system and the phospholipase C (PLC) system. Phospholipase A_2 is stimulated by light to liberate arachidonate from phosphatidylcholine by a mechanism that requires transducin (Jelsema, 1987), and arachidonate metabolism is greater in the light (Birkle and Bazan, 1989). Also, light has been found to stimulate PLC to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP_2) (Ghalayini and Anderson, 1984; Hayashi and Amakawa, 1985; Das et al., 1986; Van Rooijen and Bazan, 1986; Millar et al., 1988; Gehm and McConnell, 1990), probably to its usual metabolites, diacylglycerol (DAG) and inositol triphosphate (IP_3). Although intracellular injections of IP_3 have been found to hyperpolarize rods in a light-dependent manner (Waloga and Anderson, 1985), the reported effects were small, and the mechanism of action of IP_3 was unclear. One possible role for DAG in the rod PLC system is the stimulation of protein kinase C (PKC). For example, Binder et al. (1989) found that stimulating PKC with 1-oleoyl-2-

Received for publication 21 December 1994 and in final form 18 May 1995.

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0006-3495/95/08/409/09 $2.00

acetyl glycerol (OAG) significantly decreased the amplitude of the light response and of dark levels of cGMP, and Newton and Williams (1993) suggested a role for PKC in phosphorylation of rhodopsin.

We now show that two DAGs, 1,2-DiC8 and 1,3-DiC8, reversibly suppress the cGMP-activated conductance in excised patches of rod outer segment membranes. This suppression did not involve phosphorylation and appeared to reflect an allosteric inhibition of channel opening. Our findings also suggest that the use of these DAG analogs to stimulate PKC in cell systems in general may be complicated by direct actions of the analogs on ion channels. Preliminary accounts of this work have appeared in abstracts (Gordon and Zimmerman, 1993a,b).

MATERIALS AND METHODS

Preparation and solutions

Most experiments were performed on retinas from the leopard frog, *Rana pipiens*, but a few experiments using 1,2-DiC8 were performed on retinas from larval tiger salamanders (*Ambystoma tigrinum*) when healthy frog retinas were unavailable. The two types of preparations showed similar inhibition by 1,2-DiC8. The data in Fig. 5 are from a salamander; all other figures present frog data. Inside-out membrane patches (Hamill et al., 1981) were excised from mechanically isolated rod outer segments using previously described methods (Zimmerman and Baylor, 1992). The cell chamber had a 100-μl volume and was constructed with Plexiglas walls, a glass bottom, and a Teflon inlet tube. To immobilize the outer segments for approach by the patch pipette, the chamber bottom was precoated with poly-L-lysine (1 mg/ml or 2–4 μg/ml in water, rinsed with Ringer's solution before depositing cells). All manipulations were performed in normal room light. Retinae were stored in a modified Ringer's solution consisting of 111 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM D-glucose, 20 μM EDTA, and 3 mM HEPES (pH 7.6). In some macroscopic current studies, the above Ringer's solution was used in the pipette, and a pseudointracellular solution that contained the following was used in the bath: 10 mM NaCl, 120 mM KAsp, 648 μM $CaCl_2$ (300 nM free Ca^{2+}), 501 μM $MgCl_2$ (500 μM free Mg^{2+}), 1 mM EGTA (344 μM free), and 2 mM HEPES (pH 7.2). Free Ca^{2+} and Mg^{2+} concentrations were calculated using the method of Fabiato and Fabiato (1979). In other studies of macroscopic currents and in the single-channel experiments, the bath and pipette contained a low-divalent KCl solution (130 mM KCl, 2 mM HEPES, 20 μM EDTA). 8-Br-cGMP and the lipid compounds were applied to the intracellular surface of the membrane by addition to the bathing solution. Purities of 1,2-DiC8 and 1,3-DiC8 were tested with hydrogen nuclear magnetic resonance after the experiments.

1,2-DiC8, 1,3-DiC8, L-α-lysophosphatidyl-DL-glycerol (LG), and 8-Br-cGMP were obtained from Sigma Chemical Company (St. Louis, MO), and 1,2-dioctanoyl ethylene glycol (DiC8-EG), phosphatidic acid (PA), and OAG were obtained from Avanti Polar Lipids (Alabaster, AL). PA stock (2.5 mM) was prepared in the pseudointracellular solution. Stocks of 1,2-DiC8, 1,3-DiC8, DiC8-EG, and OAG (all 20 mM) and an 8 mM stock of LG were prepared in dimethyl sulfoxide (DMSO). A DMSO control solution consisting of either the intracellular or low-divalent solution plus 50 μM 8-Br-cGMP and the highest DMSO concentration used (1.25%, 0.75%, or 0.625%) was applied to each patch and did not affect the agonist-induced current or seal stability.

When the DiC8 solutions were applied from a fixed Teflon inlet tube attached to the chamber, rinsing with the DMSO control solution for several minutes was required to reverse the suppression, as though the DiC8 had adsorbed to the Teflon during its application and then leaked back out. Also, if the chamber and inlet tube were not rinsed well with the DMSO control solution between patches, suppression of the conductance of a new patch was observed in the DMSO control solution even before application of DiC8; this suppression could be prevented by proper rinsing of the inlet tube and chamber. A more efficient method used for most experiments was to have several inlet tubes on hand for each patch and to use a fresh (or prerinsed) one connected to a syringe to inject each solution into the chamber.

Electrophysiological recordings and analysis

Electrical recordings were performed at room temperature (18–22°C), as described previously (Zimmerman and Baylor, 1992). Patch clamp electrodes were made from borosilicate glass capillaries with or without an internal borosilicate filament for filling. For macroscopic current experiments, electrode orifices were ~1 μm in diameter, with resistances of 3.5–5.0 MΩ. When single-channel currents were studied, electrode orifices were ~0.2–0.5 μm in diameter, with resistances of 4–9 MΩ, and were coated with Sylgard 184 (Hamill et al., 1981) (Dow Corning Corporation, Midland, MI). Patch currents were acquired using an Axopatch 1C patch clamp amplifier (Axon Instruments, Foster City, CA) and either an Indec 11/73 computer (Indec Systems, Capitola, CA) or a Macintosh Quadra computer (Apple Computer, Cupertino, CA). Currents were filtered at 2 kHz (−3dB; 8-pole, low-pass Bessel filter; Frequency Devices, Haverhill, MA) and sampled at 10 kHz to prevent aliasing. Seal resistances ranged from 3–30 GΩ. Agonist-induced currents were taken as the difference between the steady-state currents in the presence and absence of 8-Br-cGMP. At saturating [8-Br-cGMP], currents were measured within 2 ms of switching the voltage, before significant ion depletion (Zimmerman et al., 1988). Maximum currents from these patches ranged from ~80–600 pA in the pseudophysiological solutions and reached more than 1 nA in the low-divalent solution, consistent with hundreds to thousands of channels per patch. No corrections were made for series resistance, but the resulting voltage errors were always less than 10%. The junction potential with Ringer's solution in the pipette and the pseudointracellular solution in the bath was measured to be −10.4 mV, and voltages were corrected accordingly. Single-channel currents were recorded with a constant applied voltage of +75 mV.

RESULTS

Suppression of currents by 1,2-DiC8

Application of 1,2-DiC8 (structure shown in Fig. 9 *A*) to the intracellular surface of excised patches from rod outer segments decreased the cGMP-activated current. The current traces of Fig. 1 are responses to the same saturating concentration of cGMP during voltage jumps from 0 to +90 mV. No 1,2-DiC8 was present during the recording of the largest current. Increasing concentrations of 1,2-DiC8 were then applied, yielding the smaller currents shown in the figure. When the highest concentration of 1,2-DiC8 was applied, the current response to saturating cGMP was indistinguishable from the "leak" current in the absence of cGMP, indicating that 1,2-DiC8 had suppressed all the cGMP-activated current. This suppression was reversible (see Fig. 3).

The suppression of current required many seconds after the introduction of DAG analogs into the bath. For the patch shown in Fig. 1 *B*, the suppression of current by 1,3-DiC8 (an analog with similar potency to 1,2-DiC8; see Fig. 9 *B*) was fit with one exponential with a time constant of 15 s. This was much slower than our perfusion time, which had a time constant of 0.4 s. The time required for suppression could reflect the time necessary for DiC8 to find its target in

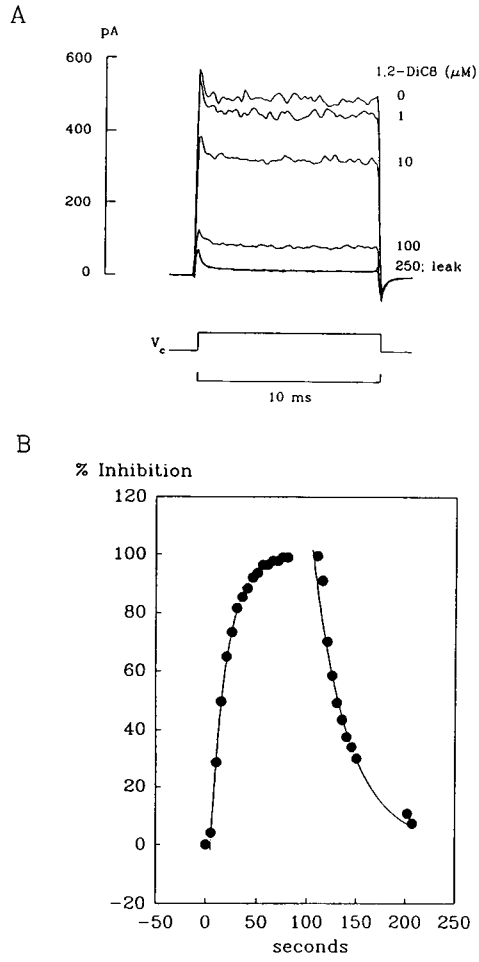

FIGURE 1 Suppression of the cGMP-activated current by DiC8. (*A*) The pipette contained Ringer's solution, and the bath contained the pseudointracellular solution with or without 50 μM 8-Br-cGMP and with the indicated concentration of 1,2-DiC8. The patch was held at 0 mV and jumped to +90 mV. The lowest two traces were essentially identical in magnitude, although one was recorded with 50 μM 8-Br-cGMP plus 250 μM 1,2-DiC8 and the other ("leak") with no 8-Br-cGMP or 1,2-DiC8. (*B*) Slow time course for inhibition and recovery. The bath and pipette both contained the low divalent KCl solution. During the onset of the response (*left*, beginning at time 0) the bath also contained 50 μM 8-Br-cGMP and 10 μM 1,3-DiC8. The smooth curves are single exponentials with time constants of 15 s (onset) and 36 s (recovery after removal of DiC8). The time constant for complete changeover of the chamber volume (100 μl) was 0.4 s.

or near the membrane (e.g., a site on the channel), or the time required for the concentration of DiC8 to reach equilibrium in the membrane. For each patch, washout of the suppression was approximately two to three times as slow as its onset. Thus, the recovery curve in Fig. 1 *B* was fit with an exponential with a time constant of 36 s. This slow reversal of the inhibition may reflect the rate of removal of DiC8 from the membrane. However, washout times were even slower than this when a Teflon chamber inlet tube was used (see Materials and Methods), suggesting that at least some of the washout time may reflect the time required to remove DiC8 from the chamber.

The dose-dependence of the suppression of current by 1,2-DiC8 could be quantified by fitting the Hill equation to the percentage current remaining at different concentrations of 1,2-DiC8. The data and a smooth curve representing the fit are shown in Fig. 2. This fit yielded an IC_{50} of 22 μM and a slope of 0.8 at saturating [8-Br-cGMP] (but see Fig. 3, regarding the much greater potency of DiC8 with lower [8-Br-cGMP]). Although 1,2-DiC8 is usually applied as an activator of PKC, the suppression we observed did not require a phosphorylation reaction, inasmuch as no ATP or GTP was present in either the bath or the pipette.

The primary effect of 1,2-DiC8 was on channel gating, with a decrease in apparent agonist affinity

The data of Fig. 3 illustrate that 1,2-DiC8 was more potent at low than at high [8-Br-cGMP]. The current families in Fig. 3 are responses to voltage pulses from 0 mV to between −100 and +100 mV in steps of 20 mV. A low dose of 1,2-DiC8 (1 μM) had little effect at saturating [8-Br-cGMP], but decreased the current at a low [8-Br-cGMP] to almost half of its control value. Thus, whereas the IC_{50} for channel inhibition by 1,2-DiC8 was 22 μM with saturating [8-Br-cGMP] (Fig. 2), only ~1–2 μM DiC8 was required to halve the current with 1.2 μM 8-Br-cGMP. It is this lower concentration of 8-Br-cGMP that is comparable to what is thought to be the free cGMP concentration (a few micromolar) in dark-adapted rods (Yau and Nakatani, 1985), with light absorption reducing the [cGMP] even further. These results suggest that DAG concentrations of 1 μM or less could have significant effects on the light-regulated, cGMP-

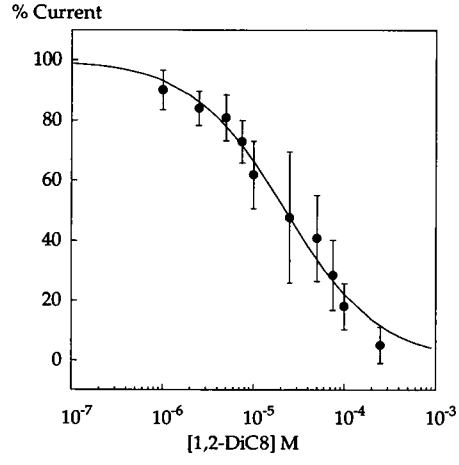

FIGURE 2 Dose-response relation for suppression of current by DiC8. Solutions were the same as in Fig. 1 *A*. Points are means ± SD (*bars*) of data from seven patches. The ordinate is 100 multiplied by the current (at +90 mV) in the presence of 1,2-DiC8, relative to the current in the absence of 1,2-DiC8. The smooth curve is a fit of the data with the Hill equation: % current = $100(1/1+([IC_{50}/[DiC8])^n)$, where IC_{50} = 22.3 μM and n = −0.8.

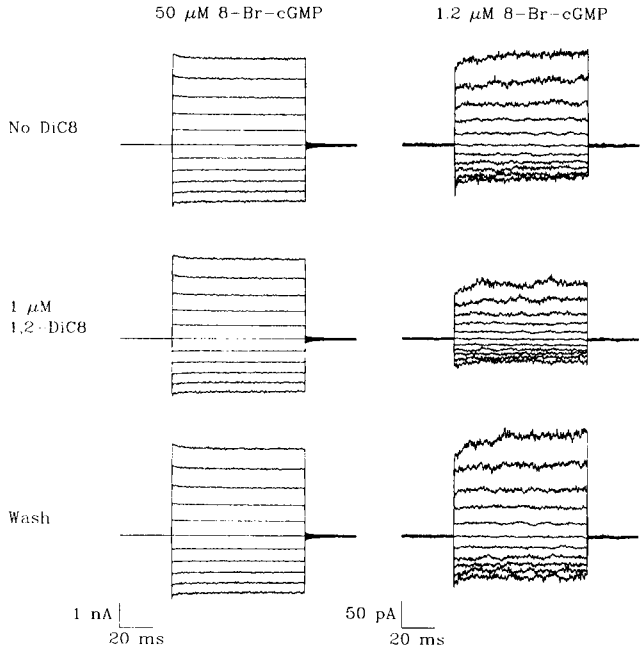

FIGURE 3 DiC8 reversibly suppressed the current more at low than at high [8-Br-cGMP]. The pipette contained the low-divalent KCl solution. The bath contained the low-divalent KCl solution with or without 50 μM (*left*) or 1.2 μM (*right*) 8-Br-cGMP and with (*center*) or without (*top*) 1 μM 1,2-DiC8. Bottom panels were obtained after complete rinsing of the 1,2-DiC8 from the chamber. Currents are responses to voltages from −100 to +100 mV in steps of 20 mV, from a holding potential of 0 mV. All data were obtained from the same patch.

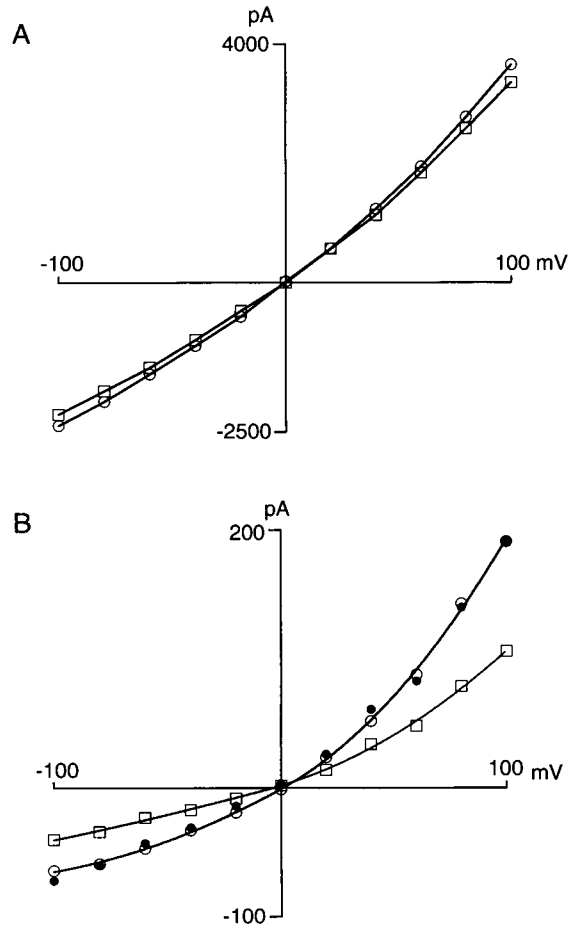

FIGURE 4 Steady-state current-voltage relations of the data shown in Fig. 3. Open circles are cGMP-activated currents recorded in the absence of DiC8. Open squares are currents in the presence of 1 μM 1,2-DiC8. (*A*) 50 μM 8-Br-cGMP. (*B*) 1.2 μM 8-Br-cGMP. Filled circles are currents measured after washout of DiC8.

gated current in vivo. A more quantitative comparison of the suppression at high and low [8-Br-cGMP] is presented in the current-voltage relations of Fig. 4, constructed from the data of Fig. 3. The lowest two panels of Fig. 3 demonstrate that the suppression of the current was reversible; after 1,2-DiC8 was washed out of the bath, the currents recovered to their original levels.

Dose-response curves for activation of the channels by 8-Br-cGMP, with and without 1,2-DiC8, show that the DAG analog decreased the apparent cGMP affinity of the channels. Fig. 5 illustrates that 4 μM 1,2-DiC8 increased the $K_{1/2}$ for channel activation by more than a factor of 3, from 1.6 to 5.2 μM, while not altering the Hill coefficient or the form of the relation. The maximum current was also reduced by a factor of 3, and as discussed below, this most likely reflects an inhibition of channel opening. The lower apparent affinity does not appear to result from competitive inhibition of cGMP binding by DiC8, inasmuch as it was not reversed by application of supersaturating (1 mM) 8-Br-cGMP (Fig. 6).

The reduction in the maximum current by DiC8 could represent either a decrease in the equilibrium constant for the allosteric conformational change producing channel opening (Gordon and Zagotta, 1995) or a decrease in single-channel conductance. To test for the latter mechanism, we examined single-channel currents with and without a DiC8 concentration that suppressed most of the cGMP-activated patch current. For the patch of Fig. 7 *A*, 100 μM 1,2-DiC8 suppressed nearly all of the current at saturating 8-Br-cGMP. At 8-Br-cGMP concentrations low enough to activate only one to a few channels at a time, both with (Fig. 7 *C*) and without (Fig. 7 *B*) 1,2-DiC8, single-channel currents demonstrated the rapid gating kinetics characteristic of cGMP-gated channels and especially striking in records from frog and toad rods (to compare toad with salamander rods, compare Matthews and Watanabe, 1987, with Haynes et al., 1986, and Zimmerman and Baylor, 1986). However, in the presence of DiC8, more 8-Br-cGMP was necessary to elicit channel activity than in its absence. There is slightly more channel activity in Fig. 7 *C* than in Fig. 7 *B* because it was difficult to predict in advance of the experiment exactly how much of an increase in [8-Br-cGMP] would be necessary to compensate for the dramatic decrease in channel activity caused by DiC8. In this case, a 20-fold increase in [8-Br-cGMP] (from 0.05 to 1.0 μM) was used to get channel activity, and this seems to have activated at least two or three channels at a time, instead of one. The diffi-

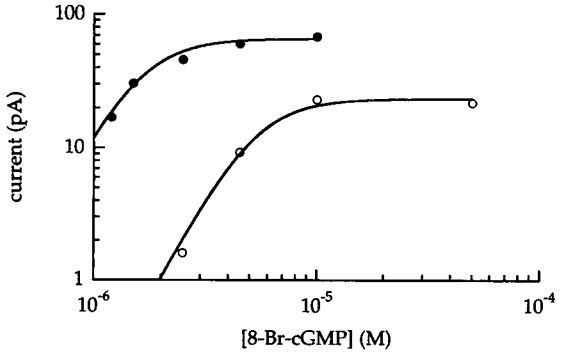

FIGURE 5 Shift in the cGMP dose-response relation by DiC8. The pipette contained the low-divalent KCl solution. The bath contained the low-divalent KCl solution with or without 8-Br-cGMP and with (○) or without (●) 4 μM 1,2-DiC8. All data were obtained from the same patch from a salamander rod. Steady-state cGMP-activated currents were measured at +100 mV. Smooth curves were drawn according to the Hill equation, $I/I_{max} = [\text{8-Br-cGMP}]^n/(K_{1/2}{}^n + [\text{8-Br-cGMP}]^n)$, with $n = 3.2$ for both curves. For the upper curve, $K_{1/2} = 1.6$ μM and $I_{max} = 65.6$ pA. For the lower curve, $K_{1/2} = 5.2$ μM and $I_{max} = 23.3$ pA.

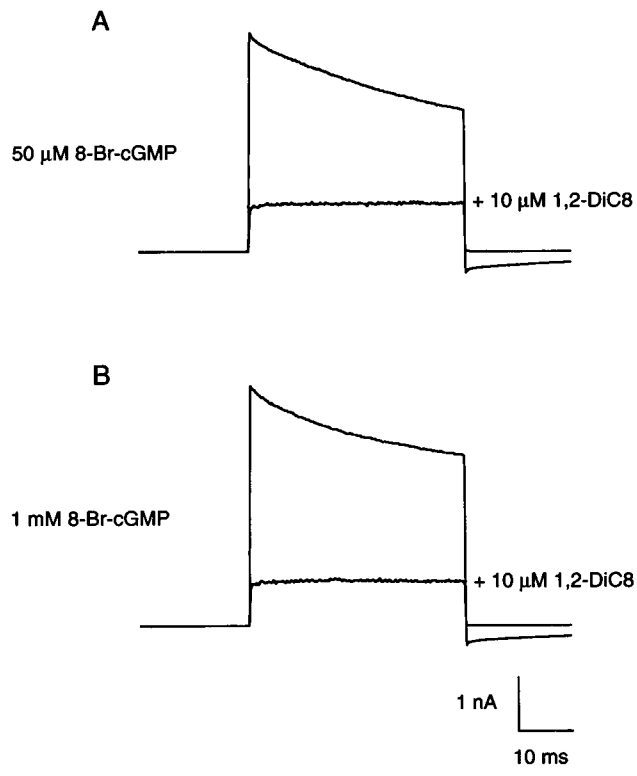

FIGURE 6 Evidence that DiC8 is not a competitive inhibitor of cGMP binding. Supersaturating 8-Br-cGMP did not relieve inhibition by DiC8. The pipette contained the low-divalent KCl solution. The bath contained the low-divalent KCl solution with 8-Br-cGMP and with or without 1,2-DiC8. All data were obtained from the same patch. Steady-state cGMP-activated currents. (*A*) Upper curve: 50 μM 8-Br-cGMP, no DiC8. Lower curve: 50 μM 8-Br-cGMP with 10 μM 1,2-DiC8. (*B*) Upper curve: 1 mM 8-Br-cGMP, no DiC8. Lower curve: 1 mM 8-Br-cGMP with 10 μM 1,2-DiC8.

culty in selecting the appropriate [8-Br-cGMP] arose from patch-to-patch variability in the $K_{1/2}$ for channel activation by cGMP and in the variable number of channels per patch (hundreds to thousands).

The rapid fluctuations of the single-channel currents with and without DiC8 precluded meaningful kinetic analysis of the records. In fact, channel openings were so brief that we were unable to obtain an accurate measure of single-channel conductance by direct inspection of the records. However, we were able to derive amplitude histograms from the raw records shown in Fig. 7, *B* and *C*. Fig. 8 shows these histograms along with gaussian fits. A gaussian component with a peak at 0.82 pA (11 pS) was present with and without 1,2-DiC8. The DiC8 histogram also contained a hint of a small peak at 2.48 pA (33 pS). Because the higher [8-Br-cGMP] used with 1,2-DiC8 elicited more channel activity than the [8-Br-cGMP] used in the absence of DiC8, the 33-pS peak may reflect the increased prevalence (at higher [8-Br-cGMP]) of the "large" conductance state of the channel relative to the "small" conductance state (Taylor and Baylor, 1995). In any case, the amplitude histograms suggest that a decrease in single-channel conductance was not the primary mechanism by which DiC8 reduced the macroscopic currents. We cannot rule out effects of DiC8 on ion permeation through the channels, but the dramatic reduction in macroscopic current does not seem to correspond to an equivalent reduction in the current through each channel. Thus, the clearest difference between the single-channel currents recorded under the two conditions (Fig. 7, *B* and *C*) was that more 8-Br-cGMP was required to open channels in the presence of 1,2-DiC8 than in its absence. This would be expected if 1,2-DiC8 inhibited channel opening or cGMP binding. Inasmuch as raising cGMP to supersaturating levels (Fig. 6) did not reverse inhibition by DiC8, the most likely mechanism of DiC8 action is an allosteric interference with channel opening after cGMP binding.

Specificity of 1,2-DiC8 effect

To examine the mechanism of the suppression further, we applied other lipids to the patches. These included 1,3-DiC8, DiC8-EG, PA, OAG, and LG. The eight-chain DAG analog, 1,3-DiC8 (structure shown in Fig. 9 *A*), suppressed the cGMP-activated current with similar potency to that demonstrated by 1,2-DiC8 (compare Figs. 2 and 9 *B*). Unlike 1,2-DAG analogs, 1,3-DAG analogs do not stimulate PKC (Ganong et al., 1986). However, these analogs are more similar than different with respect to other physiological activities: 1,2-DiC8 and 1,3-DiC8 are equally effective at inducing exocytosis of sperm acrosomes (Roldan and Harrison, 1992); 1,3-DiC8 is an inhibitor of 1,2-diacylglycerol kinase and also inactivates it with a rate constant only slightly greater than that for 1,2-DiC8 (Walsh et al., 1990); and the DAG analog 1,3-dioleoylglycerol activates 1,2-diacylglycerol kinase (Walsh and Bell, 1986). DiC8-EG, a compound of identical chain length and comparable mem-

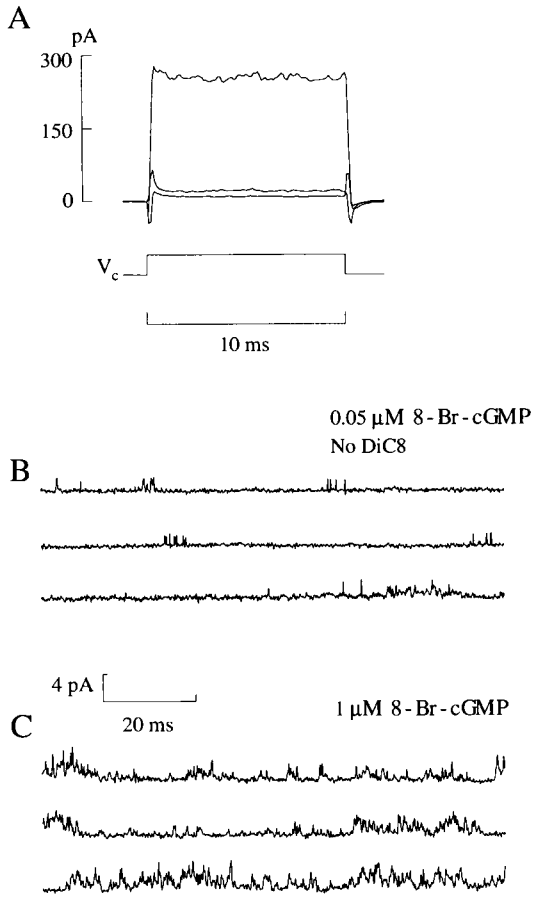

FIGURE 7 Macroscopic and single-channel currents recorded in the presence and absence of DiC8. The pipette contained the low-divalent KCl solution. The bath contained the low-divalent KCl solution with or without 8-Br-cGMP and with or without 100 μM 1,2-DiC8. All data were obtained from the same patch. (*A*) Suppression of the macroscopic current by 100 μM 1,2-DiC8. 50 μM 8-Br-cGMP was present in the bath for the top two traces, and the bottom trace shows the leak current obtained in the absence of 8-Br-cGMP. The voltage was held at 0 mV and jumped to 100 mV, as indicated. The difference current before 1,2-DiC8 treatment was 244.4 pA (top trace minus bottom trace) and in the presence of 1,2-DiC8 was reduced to 11.5 pA (middle trace minus bottom trace). (*B*) Single channel currents in response to 0.05 μM 8-Br-cGMP in the absence of 1,2-DiC8; holding potential +75 mV. (*C*) Single channel currents in response to 1.0 μM 8-Br-cGMP in the presence of 1,2-DiC8; holding potential +75 mV.

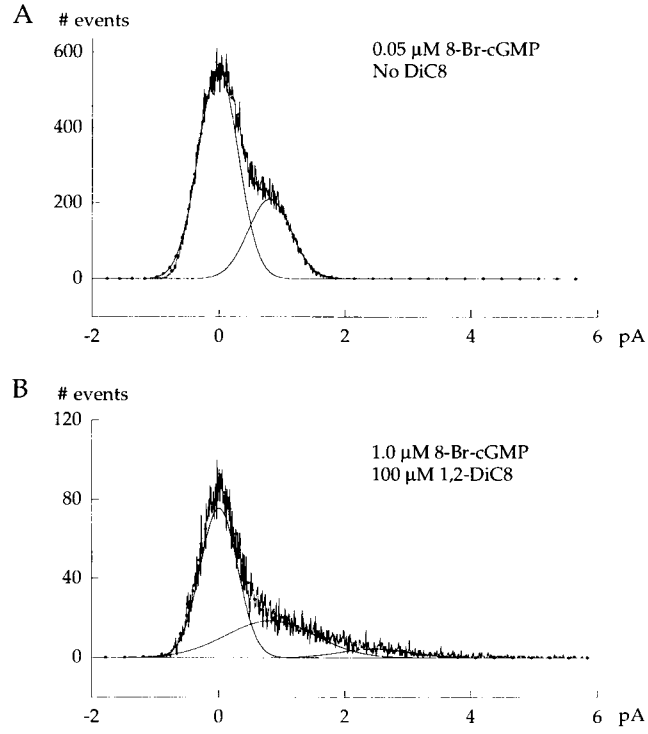

FIGURE 8 Amplitude histograms from records obtained in the presence and absence of DiC8. Same patch as in Fig. 7. (*A*) Solutions the same as in Fig. 7 *B*. The histogram was fit by the sum of two gaussians, with means of 0 and 0.82 pA. The dots are the sums of the two gaussians, and the smooth curves represent the individual gaussian components. (*B*) Solutions the same as in Fig. 7 *C*. The histogram was fit by the sum of three gaussians, with means of 0, 0.82, and 2.48 pA. This histogram was made from selected records that showed baseline at least one third of the time.

brane solubility but without known physiological activity, was much less potent than 1,2- or 1,3-DiC8 at reducing the cGMP-activated current. Thus, 100 μM DiC8-EG reduced the current by only 34.6% ± 5.5% (mean ± SD; Fig. 9 *B*). PA, OAG, and LG were also applied to the patch. As with other very hydrophobic substances (e.g., long-chain lipids and substances with detergent-like properties), OAG and LG disrupted rod outer segment seals quickly; however, seals lasted as long as a few minutes when exposed to PA, and one patch was stable long enough to obtain complete data. This patch showed no reduction in current by PA (Fig. 9 *B*). Because of the seal-disrupting behavior and low water solubility of long-chain lipids, we were unable to apply native DAG to the patches.

The inhibition of the cGMP-activated current was unlikely to be caused by changes in membrane fluidity. De Boeck and Zidovetzki (1992) found that 1,2-DiC8 induced only very slight perturbations in phosphatidylcholine bilayers at concentrations as high as 35 mol %; in contrast, similar concentrations of longer-chain DAGs (e.g., 1,2-*sn*-ditetradecanoylglycerol) induced the formation of a gel phase. Furthermore, in our experiments DiC8-EG had little effect on the cGMP-activated current even though it is a saturated lipid of the same length as 1,2- and 1,3-DiC8, and the long-chain unsaturated lipid PA had no effect. This suggests that alterations in membrane fluidity cannot account for our results.

DISCUSSION

Our results revealed a dramatic suppression of the cGMP-activated current by DAGs but a much smaller effect by a diacylethylene glycol of identical chain length. The suppression of current did not require nucleoside triphosphates, indicating that a phosphorylation reaction had not occurred. Furthermore, our results suggest that the suppression of current by 1,2- and 1,3-DiC8 did not result from changes in membrane fluidity. Suppression by 1,2-DiC8 was at least 10

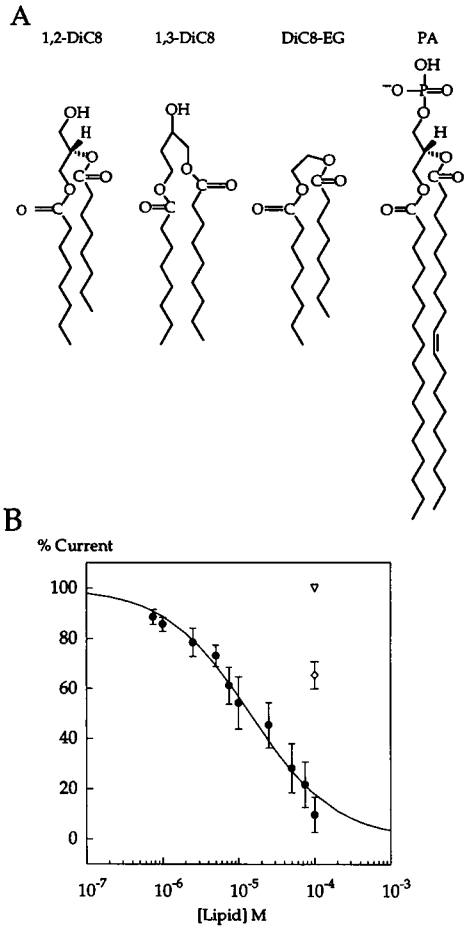

FIGURE 9 Suppression of the cGMP-activated current by other lipids. (*A*) Structures of the lipids. (*B*) Dose-response relation for 1,3-DiC8 (●) and data for 100 μM DiC8-EG (◇) and PA (△). Points for 1,3-DiC8 are means ± SD of data from eight patches, and the point for DiC8-EG is the average of four patches (±SD). Only one patch survived PA treatment long enough for complete data to be collected. The IC_{50} for 1,3-DiC8 was 14.1 μM, with $n = -0.8$. The pipette contained Ringer's solution, and the bath contained the pseudointracellular solution with 50 μM 8-Br-cGMP and the indicated concentration of lipid. Currents were measured at +90 mV.

times more effective at low than at saturating cGMP, with 1 μM DiC8 giving significant suppression in the expected physiological range of [cGMP]. Application of 4 μM 1,2-DiC8 gave a threefold increase in the $K_{1/2}$ for channel activation by 8-Br-cGMP. Supersaturating 8-Br-cGMP did not relieve the suppression by 1,2-DiC8, arguing against competitive inhibition of cGMP binding. Also, although more cGMP was required to open channels in the presence of DiC8, DiC8 did not seem to decrease the single-channel conductance. These findings suggest that DiC8 allosterically inhibited channel opening.

Lipids have been found to both inhibit and activate membrane proteins in other systems. They were found to stimulate the glutamate transporter in retinal glial cells (Barbour et al., 1989), to inhibit the Na^+/glucose transporter in renal epithelial cells (Le Grimellec et al., 1988), and to inhibit the Na^+/K^+/ATPase from brain (Ahmed and Thomas, 1971; Swann, 1984). However, in these cases the ability of lipids to affect the membrane transporters increased with increasing chain length and increasing number of double bonds, suggesting that the lipids acted by increasing membrane fluidity. This mechanism cannot explain our results or those of Bowlby and Levitan (1995), who found that DAG interacts directly with voltage-dependent K^+ channels, increasing their inactivation rate and decreasing peak current.

In vivo, DAG is produced primarily by the breakdown of PIP_2 by PLC (reviewed in Majerus et al., 1986; Majerus, 1992). The major consequences of DAG production in many systems are the release of intracellular calcium stores and the activation of PKC. The existence of light-stimulated PIP_2 turnover in rod outer segments remains controversial. Although some laboratories have found no evidence for such a system (Van Rooijen and Bazan, 1986; Gehm and McConnell, 1990), others have demonstrated a light-stimulated hydrolysis of PIP_2 (Ghalayini and Anderson, 1984; Hayashi and Amakawa, 1985; Das et al., 1986; Millar et al., 1988). Binder and colleagues (1989) found that the PKC activator OAG decreases the amplitude of the light response in frog rod outer segments, as well as the dark level of cGMP and the light-stimulated fall in [cGMP]. The outcome of the controversy over whether the PIP_2/PKC system is light-dependent is important in evaluating the potential role of DAG in modulating the cGMP-gated channels. However, even if the DAG concentration near the channels does not vary with light, the [cGMP] level near the channel does. Thus, if native DAG, like 1,2-DiC8, inhibits the channels more at low than at high [cGMP], then the decrease in [cGMP] in the light might lead to increased DAG-induced suppression of the channels even at a fixed level of cellular DAG.

How do the DAG concentrations used here compare with the concentration of DAG in vivo? The answer to this question is complicated by the fact that we applied the DAG analogs in aqueous solutions (containing DMSO) and were not able to measure their concentrations in the membrane patches. The concentration of native DAGs in frog rod outer segment membranes has been reported to be ~1–3 mol % of the total lipid, with a lipid/protein ratio of ~1:1 (by dry weight) (reviewed in Fliesler and Anderson, 1983). Neglecting membrane proteins and assuming that there are 5 million lipid molecules per square micrometer of bilayer (Alberts et al., 1983), that the thickness of the bilayer is 40 Å, and that native DAGs account for 1 mol % of the total lipids, one can calculate that there are ~50,000 molecules of native DAGs in 1 μm^2 of λ bilayer. The true number of native DAG molecules in the membrane would be somewhat less, given that membrane proteins are present as well. For comparison with the membrane density of native DAGs calculated above, a bath DiC8 concentration of 1 μM would give an average of 2.4 DiC8 molecules in a slab of solution of the same dimensions as that section of membrane (1 μm by 1 μm by 40 Å). Thus, although we do not know by how much the membrane concentrated the DiC8, the bath levels

of DiC8 required for inhibition do not seem unreasonably high.

It is not clear whether DAG analogs in rod outer segment membrane patches act directly on the channel or via some other cellular factor. Although PKC is the common target of DAGs, other targets exist in the cell as well. Neuronal calcium currents, for example, can be reduced by the PKC activator OAG (and to a much lesser extent by 1,2-DiC8 and phorbol esters) even in the presence of PKC inhibitors (Hockberger et al., 1989). No ATP or GTP was present in any of our solutions, indicating that DiC8 did not suppress the currents by initiating a phosphorylation reaction. However, the phosphorylation state of the channel or of other rod proteins may still be an important factor in the suppression of current by DiC8. Also, even though phosphorylation by PKC was not involved, PKC might mediate this effect by another mechanism. In other systems, PKC has been found to have phosphorylation-independent activities. For example, phorbol 12-myristate 13-acetate stimulation of phospholipase D in hamster fibroblast membranes required PKC but persisted in the absence of ATP (Conricode et al., 1992). Inasmuch as PKC activity has been observed in rod outer segments (Binder et al., 1989), such a PKC-mediated, phosphorylation-independent mechanism cannot be ruled out.

Our results indicate that DiC8 modulates gating of the cGMP-gated channel in the absence of a phosphorylation reaction. Regardless of the physiological role of DAG in modulating the cGMP-gated ion channel, elucidation of the molecular mechanism by which DiC8 acts will further our understanding of this channel and how it interacts with other components of the light response. Furthermore, potential phosphorylation-independent effects of DiC8 must be considered when using it to study other ion channels, especially in whole-cell preparations in which the ATP dependence is difficult to assess.

We thank Lisa Gentile for the nuclear magnetic resonance measurements, Drs. Diane Lipscombe, Lubert Stryer, and Bill Zagotta for comments on an earlier version of the manuscript, and Drs. Gene Anderson, Denis Baylor, Dena Gordon, Jeff Karpen, Justine Malinski, and Ted Wensel for helpful discussions.

This work was supported by a grant from the National Eye Institute (EY-07774) to Anita L. Zimmerman and by a graduate student fellowship from the National Science Foundation (NSF RCD-9054722) to Sharona E. Gordon.

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