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Excised patches of plasma membrane from vertebrate rod outer segments retain a functional phototransduction enzymatic cascade

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ABSTRACT **Ion channels in excised patches of plasma membrane are generally considered to be isolated from any intracellular regulation mechanisms. For example, in excised patches of vertebrate rod outer segment plasma membrane, the cGMP-activated cation channels have traditionally been studied in room light because the enzyme cascade linking photon absorption to channel closure was assumed to be inoperative. To investigate the possibility that, in fact, such excised patches retain a functional phototransduction enzymatic cascade, this same preparation was studied in darkness. Patches excised in the dark were found to retain the light sensitivity of their cGMP-induced conductance and the ability to synthesize cGMP. In the presence of guanosine 5'-[γ-thio]triphosphate (GTP[γS]), a nonhydrolyzable GTP analog, light suppresses the cGMP-induced conductance irreversibly. Furthermore, inhibitors of phosphodiesterase activity reduce light sensitivity, whereas activated phosphodiesterase or activated transducin does not directly affect the channels. These results (*i*) establish that excised patches from rod outer segment retain functional phototransduction enzymes, (*ii*) support the classical view that channel opening is modulated by phosphodiesterase-mediated cGMP hydrolysis, and, most surprisingly, (*iii*) demonstrate that diffusion in excised patches is so restricted that local enzymes can induce variations in the concentration of small molecules. The indication that excised patches are not as simple as usually surmised opens the possibility of using them to study other intracellular transduction mechanisms.**

In the traditional representation of excised patches, membrane proteins are considered to be isolated from any intracellular regulation mechanism involving either soluble enzymes or diffusible messengers. This isolation is presumed to arise from two phenomena: (*i*) soluble enzymes wash out from the patch when it is excised from the rest of the cell, and (*ii*) any intracellular messengers that might be synthesized by membrane enzymes diffuse instantaneously out into the "infinite" volume of the perfusion bath. In most cases, however, no attempt has been made to investigate the validity of these assumptions. The work reported here examines this problem in the specific case of excised patches of vertebrate rod outer segment (ROS) plasma membrane.

Vertebrate vision is initiated in photoreceptor outer segments by a process that links the absorption of photons to the closure of ion channels in the plasma membrane (1, 2). It is generally accepted that binding of intracellular cGMP opens these channels and that light closes them by decreasing cytoplasmic [cGMP]. The decrease in [cGMP] is due to a light-evoked increase in the activity of a cGMP-specific phosphodiesterase (PDE), triggered by the photoisomerization of rhodopsin (Rh) and the formation of an excited intermediate (Rh*). By catalyzing GTP/GDP exchange, Rh* activates transducin, a guanine nucleotide binding protein (G protein), which, in turn, stimulates PDE.

While extensive evidence supports this model, there are still questions about the signal that operates the channel. One problem is that light-induced changes in total [cGMP], based on chemical assays, are poorly correlated with the intensity dependence and time course of the changes in number of open channels measured electrophysiologically (3–5). Some find that subsecond flashes reduce [cGMP], yet the reduction is not graded with light intensity and channel reopening precedes [cGMP] recovery (4). Others show that dim light causes an increase in cGMP turnover (flux) in whole retinas with no change in total [cGMP] (5), and they propose that the flux of cGMP operates the light-regulated channels. Although the mechanism coupling flux to channel gating is not defined, it might involve PDE activity, which is directly linked to changes in flux. The possibility that PDE directly interacts with the channel is supported by a report that activated PDE, in the absence of cGMP, opens cGMP-gated channels in reconstituted lipid bilayers (6). A direct action of α subunits of transducin (T_α) has also been suggested. This was based on the finding that addition of guanosine 5'-[γ-thio]triphosphate (GTP[γS])-activated T_α (T_α^*-GTP[γS]) reduces the current induced in excised ROS membrane patches by 8-Br-substituted cGMP (8-Br-cGMP), a cGMP analog more resistant to hydrolysis by PDE (7). An alternative interpretation, however, is that excised patches contain PDE, which can be stimulated by T_α^*-GTP[γS], and cause sufficient hydrolysis of 8-Br-cGMP to reduce the current.

To investigate the presence of elements of the phototransduction cascade in excised membrane patches and their ability to mediate light-induced closure of the cGMP-activated channels, I obtained voltage-clamp recordings of tight-seal inside-out patches from lizard ROS in the dark. With this preparation, I demonstrate that ROS excised patches can retain, to a large extent, a functional phototransduction cascade, which, most surprisingly, is able to induce, in the limited volume of the patches, variations in the concentrations of small diffusible molecules such as cGMP. I also present additional evidence in support of the classical view that the opening of the cGMP-activated channels is modulated by PDE-mediated cGMP hydrolysis.

MATERIAL AND METHODS

Recordings were performed on excised plasma membrane patches of mechanically isolated ROSs from retinas of dark-adapted lizards (*Gekko gekko*). All experimental steps were performed at room temperature (22°C), either in total darkness or under infrared illumination, using image converters.

The animal was dark adapted for at least 3 hr before being sacrificed. The eyes were removed and hemisected, and the posterior half of each eyecup was cut in four pieces and

Abbreviations: Rh, rhodopsin; Rh*, rhodopsin photoproduct; G protein, guanine nucleotide binding protein; T_α, α subunit of transducin; GTP[γS], guanosine 5'-[γ-thio]triphosphate; T_α^*-GTP[γS], GTP[γS]-activated T_α; PDE, cGMP-specific phosphodiesterase; IBMX, 3-isobutyl 1-methylxanthine; ROS, rod outer segment.

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stored until use (<6 hr) in dark, cold, and oxygenated *Gekko* Ringer's (159 mM NaCl/3.3 mM KCl/2.8 mM Hepes/1.7 mM $MgSO_4$/1 mM $CaCl_2$, pH 7.4, with NaOH). The retina was isolated in Low divalent Ringer's (159 mM NaCl/3.3 mM KCl/2.8 mM Hepes, pH 7.4, with NaOH) and gently stretched to mechanically detach the ROSs. The patch pipettes were pulled from soft glass to a tip diameter of 1–2 μm. They were then filled with Low divalent Ringer's and connected to a List EPC-5 patch-clamp amplifier in the voltage-clamp mode (List Electronic, Darmstadt, F.R.G.). Gigaohm seals were obtained on the plasma membrane of a ROS and a patch was excised in the inside-out configuration by suddenly blowing away the ROS with the perfusion system. The patch geometry was examined under the microscope, after the experiment, in white light. The patches ranged from "flat patches," not visible under the microscope, to large "blebs," reaching up to 15 μm into the patch pipette. The total surface area was estimated by assuming the patches had a conical shape (a flask shape) and the resulting area ranged from 1 to 250 μm^2. The current was low-pass filtered at 1 kHz and PCM-recorded on videotape. It was then refiltered at 2.5 Hz and digitized at a 5-Hz sampling rate for the figures. Inward current (from extracellular side of the patch to cytoplasmic side) is negative and plotted downward. The solution bathing the cytoplasmic surface of the patch could be changed rapidly (≈150 ms) by moving a row of perfusion tubes laterally. The experiments were performed under an inverted fluorescence microscope (Nikon) modified to deliver infrared light (>780 nm) as a transmitted beam and diffuse unpolarized green light (540 nm) through the epifluorescence attachment. Visual observations were made with an infrared-sensitive video camera (RCA).

Nucleotides were added to a Low divalent Ringer's solution and pH was kept at 7.4 in all solutions. cGMP, 8-Br-cGMP, GTP, and 3-isobutyl 1-methylxanthine (IBMX) were from Sigma; GTP[γS] was from Boehringer Mannheim.

RESULTS

In agreement with experiments on excised patches in the light (8–10) and on truncated outer segments (11), I find that perfusion of cGMP on the cytoplasmic surface of a patch, in darkness, opens a conductance that increases with [cGMP]. This conductance exhibits a $K_{1/2}$ of 11.4 ± 2.1 and a Hill coefficient of 2.7 ± 0.3 for cGMP, does not desensitize, and displays little voltage sensitivity in the absence of divalent cations.

However, excised patches, like intact ROS (12), have a basal level of PDE activity in the dark. Addition of IBMX, a competitive PDE inhibitor, which by itself has no effect on patch conductance, either increases the size of the cGMP-induced current or is needed to record any current at all. Among 23 patches perfused with 20 μM cGMP, 6 patches (26%) required the additional presence of 1 mM IBMX to display any conductance in the dark. In another 11 patches (48%), the initial cGMP-induced conductance increased by a factor of 4.6 ± 1.2 upon addition of 1 mM IBMX. Only 6 patches (26%) were unaffected by IBMX. Similarly, among 60 patches perfused with 20 μM cGMP and 50 μM GTP, 13 patches (22%) required 1 mM IBMX to display any conductance, 39 patches (65%) saw their initial conductance increase by a factor of 15.5 ± 2.5 upon addition of 1 mM IBMX, and only 8 patches (13%) were unaffected. This result not only indicates that a dark PDE is present and active in excised patches but, more surprisingly, demonstrates that the enzyme is able to significantly reduce, in the microenvironment of the patches, the concentration of a steady perfusion of cGMP.

Excised patches can retain the principal components of the phototransduction cascade. In the presence of 50 μM GTP, light suppressed the conductance induced by 20 or 100 μM cGMP in 80 of 103 patches tested (78%) (Fig. 1). A family of superimposed light responses (Fig. 1*B*) shows that the fractional suppression of the current increases with flash intensity and the time to peak decreases, reaching a minimum of ≈1 s for the brightest flashes. Compared to intact outer segments (12), light sensitivity is reduced ≈25-fold after correction for total external surface area, recovery is slowed by an order of magnitude for dim flashes, and bright flashes evoke responses that do not recover fully. The problems with recovery arise probably from the loss of soluble components needed to inactivate Rh*, such as Rh kinase and/or arrestin (48-kDa protein). Again, these results not only imply that a functional phototransduction enzymatic cascade is present in the patches but, more surprisingly, establish that the light-activated PDE can locally affect [cGMP].

Guanylate cyclase is also present in excised patches of ROSs. Here again, the enzyme not only functionally supports cGMP synthesis from GTP in the perfusate but is able, in spite of a steady washout by the perfusion flow, to build up a large enough local [cGMP] to support phototransduction. In

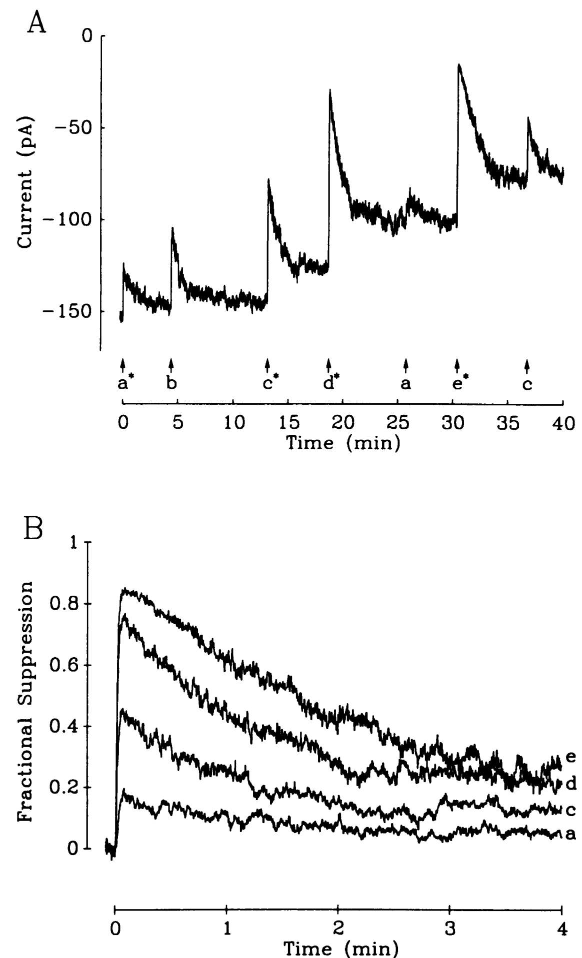

FIG. 1. Light-sensitive cGMP-induced current recorded from an inside-out membrane patch excised from a ROS. The recording pipette was filled with Low divalent Ringer's solution, and a Low divalent Ringer's solution containing 20 μM cGMP and 50 μM GTP was perfused on the patch. (*A*) Arrows indicate the timing of 20-ms flashes of light, which delivered the following: a, 684; b, 1510; c, 2986; d, 6984; e, 13,838 photons·μm^{-2}. Asterisks indicate the responses that are normalized to the current before flash and superimposed on a faster time scale in *B*. The remaining current at the peak of the response to the brightest flash is due to the leak resistance (3 GΩ). Holding potential was −10 mV (cytoplasmic minus extracellular).

the presence of 1 mM IBMX, 10 of 18 light-sensitive patches (56%) perfused with 5 mM MgGTP developed a light-suppressible conductance (Fig. 2). If Mg was omitted, none of the patches exhibited a current, suggesting that the Mg-GTP-induced current derived from the synthesis of cGMP by guanylate cyclase, which requires Mg for enzymatic activity (13). The light-evoked changes in the current induced by cGMP or by MgGTP were similar (Figs. 1 and 2), except response recovery was faster and more complete in MgGTP. The fact that 56% of patches pulled from random locations on the outer segments contain guanylate cyclase suggests that the enzyme is not restricted to a narrow longitudinal strip such as the axoneme, as was suggested by Fleischman *et al.* (14).

Light responses are mediated by G protein-coupled activation of PDE. The involvement of a G protein is supported by two observations. First, in agreement with results in truncated (11) or dialyzed (12) ROS, the cGMP-induced current is not light sensitive in the absence of GTP. Second, GTP[γS] blocks the recovery of the light responses, consistent with the persistent activation of G proteins. In Fig. 3, the cGMP-induced current is transiently reduced by a flash of light, applied in the presence of GTP, whereas it is fully and persistently suppressed by an identical flash in the presence of GTP[γS]. Exposure to IBMX restores the current, showing that its loss was due to increased PDE activity rather than a direct effect of T_α^*-GTP[γS] on the channel as proposed by Krapivinsky *et al.* (7).

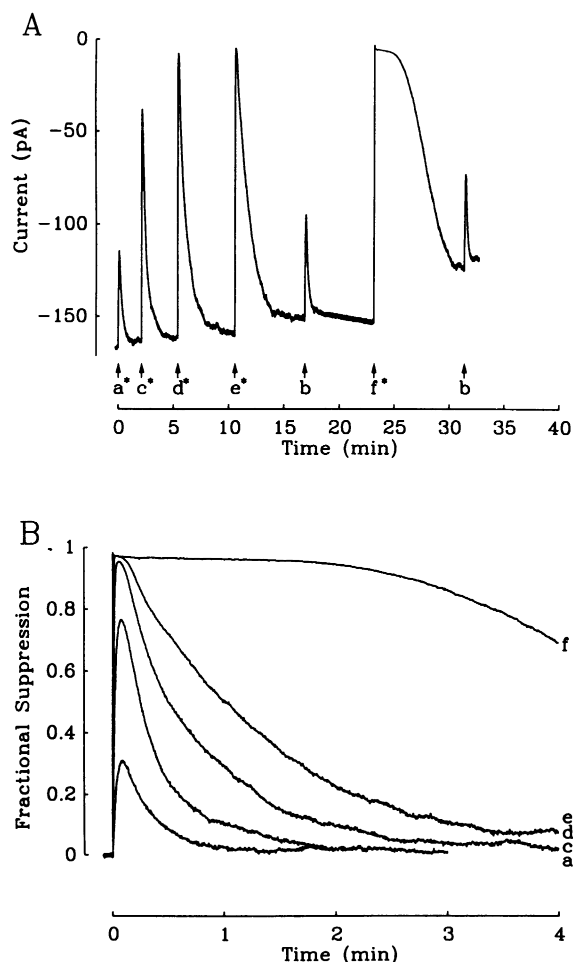

FIG. 2. Light-sensitive MgGTP-induced current recorded from an inside-out membrane patch excised from a ROS. The recording pipette was filled with Low divalent Ringer's solution, and a Low divalent Ringer's solution containing 5 mM GTP, 5 mM $MgSO_4$, and 1 mM IBMX was perfused on the patch. (*A*) Arrows indicate the timing of 20-ms flashes of light delivering the following: a, 70; b, 82; c, 291; d, 948; e, 2410; f, 98,942 photons·μm^{-2}. Asterisks indicate the responses that are normalized to the current before flash and superimposed on a faster time scale in *B*. The remaining current at the peak of the response to the brightest flashes is due to the leak resistance (1.4 GΩ). Holding potential was −10 mV.

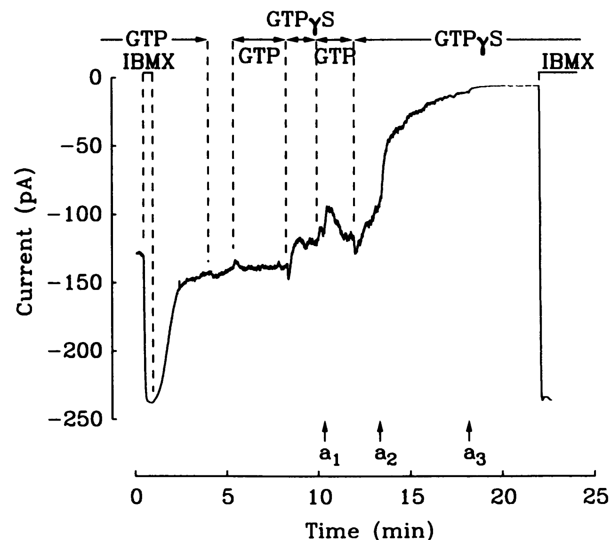

FIG. 3. Effects of GTP[γS] and IBMX on the light-sensitive cGMP-induced current in a ROS excised patch. The recording pipette was filled with Low divalent Ringer's solution, and a Low divalent Ringer's solution containing 100 μM cGMP and either 50 μM GTP, 50 μM GTP[γS], and/or 1 mM IBMX, as indicated at the top of the figure, was perfused on the patch. Arrows indicate the timing of 20-ms flashes of light, which delivered 40 photons·μm^{-2}. The initial addition of 1 mM IBMX increases the cGMP-induced current, demonstrating a basal PDE activity. Flash a_1 in cGMP and GTP elicits a small transient reduction in current but flash a_2 in cGMP and GTP[γS] suppresses the current fully, as shown by the absence of light-sensitive current when flash a_3 is applied. The remaining current at this point is entirely due to the leak resistance (2 GΩ). The final addition of 1 mM IBMX restores the current, demonstrating that its suppression was due to activation of PDE. Holding potential was −10 mV.

Experiments with 8-Br-cGMP confirm that T_α^* does not close channels directly. In excised patches in the dark, the current induced by 5 μM 8-Br-cGMP is similar in size to that evoked by a 20-fold higher concentration of cGMP, in agreement with other studies (15). However, the light sensitivity of the current in 5 μM 8-Br-cGMP and 50 μM GTP is reduced at least 1000-fold compared to that in 100 μM cGMP and 50 μM GTP. The fact that a hydrolysis-resistant cGMP analog dramatically decreases light sensitivity supports the notion that cGMP hydrolysis is required for the closure of the light-regulated channels.

In excised patches, activated PDE does not open the light-regulated channels in the absence of cGMP, contrary to results in reconstituted membranes (6). In patches that contain the elements needed for phototransduction, as shown by the light sensitivity of the cGMP-induced current, activation of PDE by a bright light, in the presence of GTP[γS] (no cGMP), does not open a conductance (Fig. 4). This result, observed in all of eight similar experiments, provides further evidence that neither activated PDE nor transducin directly gates channels.

DISCUSSION

Excised membrane patches are traditionally viewed as "simple" pieces of plasma membrane isolated from the internal machinery of the cell. It might be presumed that only

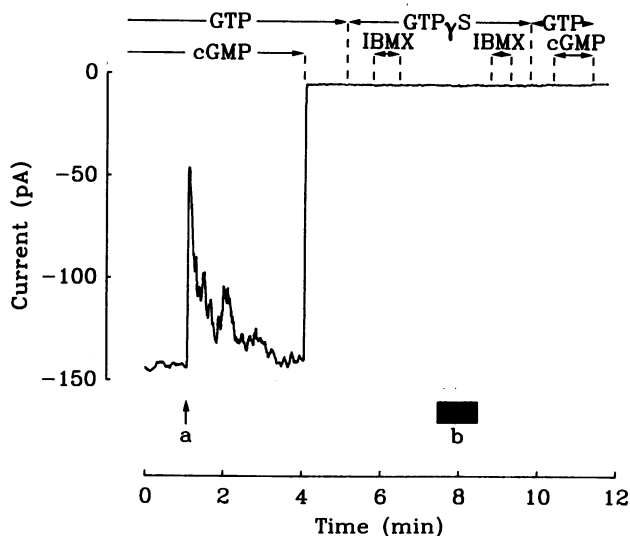

FIG. 4. Activated PDE has no effect on the conductance of a light-sensitive outer segment patch. The recording pipette was filled with Low divalent Ringer's solution, and a Low divalent Ringer's solution containing either 100 μM cGMP, 50 μM GTP, 50 μM GTP[γS], and/or 1 mM IBMX, as indicated at the top of the figure, was perfused on the patch. Arrow (a) indicates the timing of a 20-ms light flash, which delivered 590 photons$\cdot\mu m^{-2}$ and solid bar (b) indicates that of a 60-s step of light with a flux of 3.8×10^6 photons$\cdot\mu m^{-2}\cdot s^{-1}$. The patch contains the phototransduction machinery, as evidenced by the suppression of the cGMP-induced current by flash a, but no light-sensitive current is present in 50 μM GTP or 50 μM GTP[γS] with or without 1 mM IBMX. The remaining current at this point is entirely due to the leak resistance (3 GΩ). The activation of PDE by step b in GTP[γS] does not open a conductance even in the presence of 1 mM IBMX, yet the phototransduction cascade is fully activated as demonstrated by the complete suppression of the cGMP-induced current at the end. Holding potential was -10 mV.

membrane-associated proteins are retained and that these are not regulated anymore by mechanisms involving soluble enzymes or diffusible messengers. Two factors should aid this isolation: a washout of soluble enzymes and a rapid equilibration of the concentration of locally synthesized or degraded intracellular messengers with that of the "infinite" volume of the perfusion bath. Yet, Zimmerman *et al.* (16) have shown that excised patches exhibit a hindered ionic diffusion in the vicinity of their cytoplasmic surface. This was demonstrated by the observation of a slow relaxation of the membrane current when voltage pulses were applied to patches having a high ionic conductance and it was attributed to the presence of "intracellular material that adheres to the surface membrane." While the possibility of current-dependent variations in local ion concentrations was suggested, that of enzyme-dependent variations in local metabolite concentrations was not. The results reported here demonstrate the importance of such variations and provide further evidence of a restricted diffusion in the vicinity of the cytoplasmic surface.

If excised patches from photoreceptor outer segment plasma membrane were to retain a light-activatable PDE, it might affect the accuracy of measurements of the $K_{1/2}$ and Hill coefficient of the cGMP-gated channel for cGMP. The fact that such measurements, as reported in the literature, demonstrate a large and unexplained variability suggests that something is modulating the action of cGMP. Indeed, in cones, Haynes and Yau (17) have reported that the addition of IBMX has no influence on the cGMP-activated conductance of excised patches from outer segments in the light, but this has not been reported in the case of rods. While the large diameter of the pipettes used in the present work may increase the probability that active PDE molecules are retained and able to decrease the local cGMP concentration, the use of a standard way to suppress or diminish the possible activity of such enzymes might reduce the variability of the measurements of the ligand-binding properties of the channel. Possibilities include doing experiments in the dark and/or systematically using specific PDE inhibitors such as IBMX or, better but more difficult to use, May & Baker 22,948 (Zaprinast), which has a 25-fold better specificity than IBMX (18).

These observations and the finding that GTP appears to reduce or suppress the cGMP-induced conductance in many excised patches in the light (19) prompted me to examine the possibility that some functional part of the phototransduction machinery remains in excised patches and affects the microenvironment in their immediate vicinity. The results provide evidence that it is indeed the case. First, IBMX, a phosphodiesterase inhibitor, increases the dark current induced by cGMP, demonstrating that a dark PDE activity is present and significantly affects the effective cGMP concentration perceived by the channels. Second, light transiently suppresses the cGMP-induced current in a GTP-dependent manner, indicating the presence of Rh and the involvement of a G protein in the mechanism. The persistent suppression by light in the presence of GTP[γS] confirms this conclusion. Furthermore, light sensitivity decreases upon addition of IBMX, a PDE inhibitor, or replacement of cGMP by 8-Br-cGMP, a poorly hydrolyzable cGMP analog. These findings demonstrate that PDE-mediated cGMP hydrolysis is required for channel closure and they show that light-activated PDE can also considerably influence the effective cGMP concentration. Finally, perfusion of millimolar concentrations of GTP elicits a light-sensitive current in a Mg-dependent manner, suggesting that guanylate cyclase, a Mg-dependent enzyme using GTP to produce cGMP, is also retained in these patches. Again, these results show that an enzyme (guanylate cyclase in this case) can dramatically alter the concentration of metabolites in the vicinity of the patch.

In addition, the effects of PDE inhibitors contradict recent suggestions that channel closure can be mediated by the direct action of activated T_α on the channel (7). Also, no evidence was found that activated PDE could open the cGMP-activated channels of light-sensitive patches in the absence of cGMP, in disagreement with findings in reconstituted membranes (6).

These results demonstrate that the phototransduction cascade linking Rh to PDE is intact in excised patches and controls [cGMP] in the vicinity of the channels. This control takes place in spite of either the perfusion of a constant [cGMP] or the absence of any perfused cGMP if a substrate for its synthesis is provided. It indicates that diffusion into, as well as out of, the microenvironment at the vicinity of the cytoplasmic surface of patches is very restricted and that enzymes can either build up or reduce the local concentrations of metabolites in relative independence of the perfused solution.

Most patches extended up the pipette as a discernible membrane bleb, which could form a restricted space, but some light-sensitive patches (5–10%) appeared to be flat patches. While the absence of a visible bleb does not eliminate the possibility that a patch has a slight Ω shape, this result suggests that other diffusion barriers could be associated with ROS membranes. This is similar to the results of Zimmerman *et al.* for ionic diffusion (16) and supports the concept that hindered access may be due to cytoskeletal or diskal elements still attached to the membrane after excision. In intact rods, similar diffusional barriers might be present in the neighborhood of the surface membrane, where local changes in [cGMP] would then affect channel gating mark-

edly while influencing total [cGMP] negligibly. This may explain the difficulties in showing a correlation between light-induced changes in total [cGMP] and visual excitation.

In a more general perspective, since ROS excised patches may be obtained such as to create a microenvironment in the vicinity of their cytoplasmic surface where retained intracellular enzymes can alter the concentration of perfused metabolites and synthesize new products, this preparation might be tried in the study of other transduction mechanisms involving diffusible messengers such as cAMP or calcium. The unusual geometry of ROSs, where intracellular disks are tightly linked to the plasma membrane, could make them particularly amenable to this preparation but other cells do possess cytoplasmic elements tightly connected to the plasma membrane. Indeed, in myocytes, Weiss and Lamp (20) have reported that excised patches may retain part of the glycolytic enzymatic cascade.

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