Diffusion Coefficient of the Cyclic GMP Analog 8-(Fluoresceinyl)Thioguanosine 3′,5′ Cyclic Monophosphate in the Salamander Rod Outer Segment

Y. Koutalos,* R. L. Brown,‡ J. W. Karpen,‡ and K.-W. Yau*§¶

Departments of *Neuroscience and §Ophthalmology and the ¶Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and ‡Department of Physiology, University of Colorado School of Medicine, Denver, Colorado 80262 USA

ABSTRACT Cyclic GMP (cGMP) is the intracellular messenger mediating phototransduction in retinal rods, with its longitudinal diffusion in the rod outer segment (ROS) likely to be a factor in determining light sensitivity. From the kinetics of cGMP-activated currents in the truncated ROS of the salamander (*Ambystoma tigrinum*), the cGMP diffusion coefficient was previously estimated to be $\sim 60 \times 10^{-8}$ cm^2 s^{-1}. On the other hand, fluorescence measurements in intact salamander ROS using 8-(fluoresceinyl)thioguanosine 3′,5′-cyclic monophosphate (Fl-cGMP) led to a diffusion coefficient for this compound of 1×10^{-8} cm^2 s^{-1}; after corrections for differences in size and in binding to cellular components between cGMP and Fl-cGMP, this gave an upper limit of 11×10^{-8} cm^2 s^{-1} for the cGMP diffusion coefficient. To properly compare the two sets of measurements, we have examined the diffusion of Fl-cGMP in the truncated ROS. From the kinetics of Fl-cGMP-activated currents, we have obtained a diffusion coefficient of 3×10^{-8} cm^2 s^{-1} for this analog; the cGMP diffusion coefficient measured from the same truncated ROSs was $\sim 80 \times 10^{-8}$ cm^2 s^{-1}. Thus, a factor of 27 appears appropriate for correcting differences in size and intracellular binding between cGMP and Fl-cGMP. Application of this correction factor to the Fl-cGMP diffusion coefficient measurements by Olson and Pugh (1993) gives a cGMP diffusion coefficient of $\sim 30 \times 10^{-8}$ cm^2 s^{-1}, in reasonable agreement with the value measured from the truncated ROS.

INTRODUCTION

The rod and cone photoreceptors of the retina utilize cyclic GMP (cGMP) as the intracellular transmitter for transducing light into an electrical signal. The better understood rods are responsible for vision at low light intensities, and they can detect individual photons. Phototransduction takes place in the rod outer segment (ROS), which is cylindrical in shape and contains a large number (~1000) of membranous disks stacked on top of each other (see, for example, Rodieck, 1973). The light-sensitive pigment rhodopsin is a membrane protein residing in the disk membranes. In the dark, cGMP directly binds to cation channels located on the plasma membrane, keeping them open and maintaining an inward current. Absorption of light by rhodopsin initiates a reaction cascade that leads to the activation of a cGMP-phosphodiesterase. This results in a decrease in the cGMP concentration, causing the closure of the cGMP-gated channels and therefore a membrane hyperpolarization, which constitutes the electrical response to light. The recovery of the cell from light involves the decays of the active intermediates in the cascade, with the subsequent restoration of the cGMP concentration accomplished through synthesis by the guanylate cyclase (for recent reviews, see Lagnado and Baylor, 1992; Detwiler and Gray-Keller, 1992; Pugh and Lamb, 1993; Koutalos and Yau, 1993; Yarfitz and Hurley, 1994; Yau, 1994).

The stimulation of the cGMP cascade by a photon is a local event, confined to a single disk surface. At the same time, however, the electrical excitation spreads over at least 30–50 disks, as judged from the closure of 3–5% of the open channels by a single photon (Baylor et al., 1979). The longitudinal diffusion coefficient of cGMP should therefore be a factor in determining the spread of the light response, and hence the sensitivity of the cell to light. This diffusion coefficient of cGMP in the ROS is expected to be lower than in aqueous solution because of the baffling effect of the disks and possibly the viscosity of the cytoplasm.

Two recent measurements of the cGMP diffusion coefficient have given substantially different values. Fluorescence measurements by Olson and Pugh (1993) in intact salamander ROS with 8-(fluoresceinyl)thioguanosine 3′,5′-cyclic monophosphate (Fl-cGMP) have given a diffusion coefficient for this compound of 1×10^{-8} cm^2 s^{-1}. After corrections for differences in size and binding to cellular components, this value led to an upper limit of 11×10^{-8} cm^2 s^{-1} for the cGMP diffusion coefficient. On the other hand, an analysis of the kinetics of the cGMP-activated currents in a truncated salamander ROS (Koutalos et al., 1995b) has produced a directly measured diffusion coefficient of $\sim 60 \times 10^{-8}$ cm^2 s^{-1} for cGMP, more than fivefold higher. To compare the measurements from the two preparations, we have taken advantage of the ability of Fl-cGMP to open the cGMP-gated channels (Caretta et al., 1985), and

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Address reprint requests to Dr. Y. Koutalos, University of Colorado School of Medicine, Department of Physiology, Box C-240, 4200 East Ninth Avenue, Denver, CO 80262. Tel: 303-270-4418; Fax: 303-270-8110; E-mail: koutalos@essex.hsc.colorado.edu.

The present address of Dr. Brown is R. S. Dow Neurological Sciences Institute, Portland, OR 97209.

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examined its diffusion in the truncated salamander ROS in much the same way as with cGMP.

MATERIALS AND METHODS

Tiger salamanders (*Ambystoma tigrinum*) were from Charles D. Sullivan (Nashville, TN). The methods for obtaining isolated rod photoreceptors, making suction pipettes, and recording from a truncated ROS with a suction pipette were as described previously (Koutalos et al., 1995b). In all experiments, the suction pipette contained normal Ringer's solution (in mM: 110 NaCl, 2.5 KCl, 1.6 $MgCl_2$, 1 $CaCl_2$, 5 tetramethylammonium hydroxide-HEPES, 5 glucose, pH 7.55); for dialysis, we used a pseudointracellular solution (in mM: 12.5 NaCl, 100 potassium gluconate, 1.6 $MgCl_2$, 5 tetramethylammonium hydroxide-HEPES, 5 glucose, pH 7.55) containing 0.5 mM 3-isobutyl-1-methylxanthine and different concentrations of Fl-cGMP or cGMP. The concentration of 3-isobutyl-1-methylxanthine is enough to inhibit the basal phosphodiesterase activity in the truncated ROS (Koutalos et al., 1995b). With this solution arrangement, the membrane current was inward, carried by Na^+ ions from the pipette solution. Changes of bath solution were effected by a system of pneumatically controlled valves and were complete within about 300 ms (Nakatani and Yau, 1988).

cGMP was from Sigma. Fl-cGMP was synthesized by a modification of the method of Caretta et al. (1985). 8-thio-cGMP (75 mg) was first prepared and partially purified as described previously (Brown et al., 1993). It was then dissolved in 30 ml of methanol, and 200 mg of 5-iodoacetamidofluorescein (Molecular Probes) and 150 mg of sodium methoxide were added. The mixture was allowed to react at room temperature for several hours, and formation of Fl-cGMP was found to be complete by silica thin-layer chromatography (TLC) developed with 1-butanol/acetic acid/water (5:3:2). The mixture was concentrated to 6 ml in a Speed-Vac, and the product was purified by preparative silica TLC (band visualized by UV shadowing at the plate edges). The silica was scraped from the plate and extracted three times in methanol, and the product was dried in a Speed-Vac. The Fl-cGMP was further purified by reversed-phase high-performance liquid chromatography on a Vydac semipreparative C_{18} column using a 0–45% gradient of methanol in water (5 mM ammonium acetate, pH 5.0 with acetic acid, throughout). The yield was 65 mg. Analytical reversed-phase high-performance liquid chromatography and silica TLC indicated no detectable UV-absorbing or fluorescent impurities. The absorption spectrum was identical to that reported previously (Caretta et al., 1985). The concentration of the analog in the experimental solutions was determined by absorbance, assuming an extinction coefficient of 72,200 M^{-1} cm^{-1} at 492 nm in 0.1 M KOH.

The experiments were carried out in the dark and at room temperature. Electrical records were low-pass filtered at 10–30 Hz. In Figs. 1 and 3, inward current is plotted as negative.

Data analysis

The one-dimensional diffusion of a substance undergoing binding to immobile sites is described by

$$\frac{\partial C(x,t)}{\partial t} = D_0 \cdot \frac{\partial^2 C(x,t)}{\partial x^2} - \frac{\partial S(x,t)}{\partial t} \quad (1)$$

where $C(x, t)$ and $S(x, t)$ are the concentration of free and immobilized substance at distance x and time t, respectively, and D_0 is the diffusion coefficient. Assuming rapid equilibrium of the binding reaction compared with diffusion and a proportionality of the local concentration of immobilized substance to the concentration of substance free to diffuse, Eq. 1 can be rewritten as the equation describing free diffusion:

$$\frac{\partial C(x,t)}{\partial t} = D \cdot \frac{\partial^2 C(x,t)}{\partial x^2} \quad (2)$$

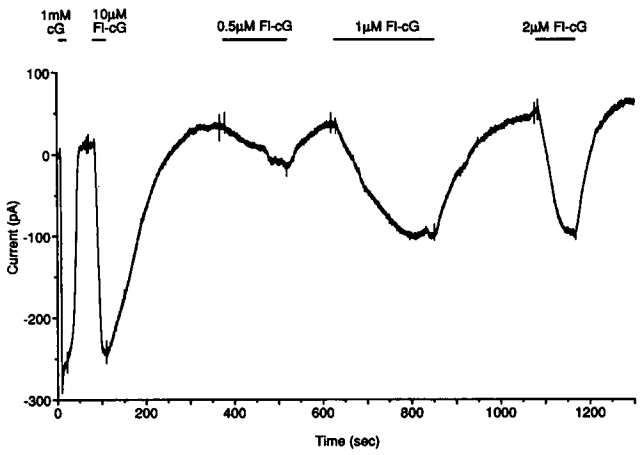

FIGURE 1 Membrane currents elicited from a truncated salamander rod outer segment by 1 mM cGMP and different concentrations of Fl-cGMP. The pipette contained normal Ringer's solution, and a potassium gluconate solution was used for intracellular dialysis. The length L of the truncated outer segment was 27 μm. Bandwidth, DC-20 Hz.

The apparent diffusion coefficient, D, is related to the diffusion coefficient in the absence of binding by

$$D = \frac{D_0}{B} \quad (3)$$

where B is the ratio of total to free substance (Crank, 1975, pp. 326–327, equations 14.1–14.3).

The diffusion coefficient of Fl-cGMP can be obtained by applying the solution of Eq. 2 to the kinetics of the Fl-cGMP-activated currents, in the same way as previously described for cGMP (Koutalos et al., 1995b). For comparison, measurements were also carried out with cGMP in the same truncated ROS. In experiments where 1 mM cGMP and 10 or 20 μM Fl-cGMP were used, the concentration of nucleotide inside the outer segment was not necessarily allowed to reach equilibrium with that in the bath; in these cases, the diffusion coefficient was measured from the current decay after the bath nucleotide concentration was rapidly removed. This current decay is exponential (Koutalos et al., 1995c), with a rate constant of nr, where n is the Hill coefficient for channel activation by the nucleotide, and

$$r = \frac{\pi^2}{4}\frac{D}{L^2} \quad (4)$$

L being the length of the truncated ROS. Because n is approximately the same for cGMP and Fl-cGMP (see Results), the ratio of the current decay rates is just the ratio of the diffusion coefficients. This analysis assumes that the off rate of cGMP or Fl-cGMP from the liganded channel is fast compared to diffusion in the outer segment. The dissociation of cGMP from the channel occurs on a time scale of milliseconds (Karpen et al., 1988). Fl-cGMP binds to the channel apparently more tightly (by, say, 40-fold; see below), but should still dissociate from the channel rapidly relative to diffusion through the outer segment.

RESULTS

Fig. 1 shows membrane currents activated by Fl-cGMP and cGMP from a truncated salamander ROS. The length, L, of the ROS inside the pipette was 27 μm. The steady-state currents, J, are plotted against the Fl-cGMP concentration in Fig. 2 A, after normalization against the saturated current,

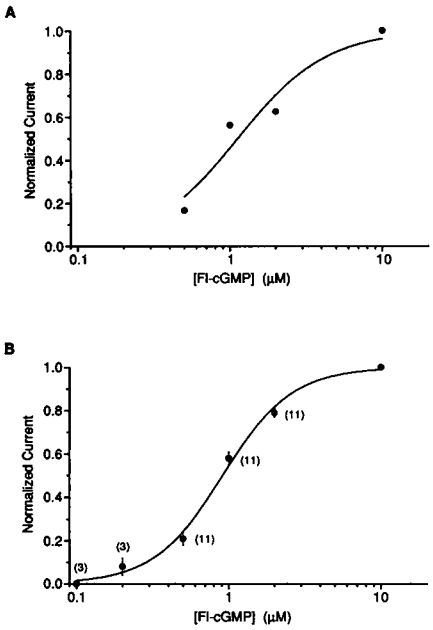

FIGURE 2 Dependence of steady-state current on Fl-cGMP concentration. (*A*) Data from Fig. 1, normalized by the current activated by 10 μM Fl-cGMP. The smooth curve was drawn according to the Hill equation, with $K_{1/2}$ = 1.1 μM and n = 1.5. (*B*) Averaged results from 11 rods. Error bars indicate standard errors. The numbers in parentheses indicate the number of determinations for each point. The smooth curve is a least-squares fit according to the Hill equation, giving $K_{1/2}$ = 0.91 μM and n = 1.9.

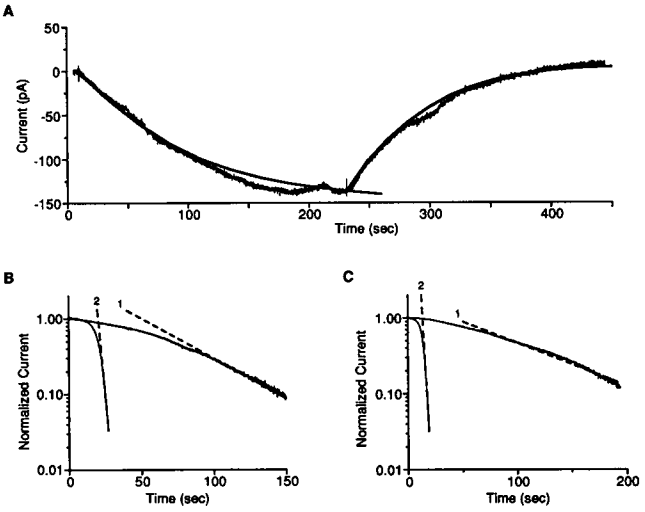

FIGURE 3 (*A*) Estimation of the Fl-cGMP diffusion coefficient from fitting calculated templates to the kinetics of the Fl-cGMP-activated current. Experimental trace shows the current elicited by 1 μM Fl-cGMP in Fig. 1. The calculated diffusion coefficients were 2.9 and 4.2 $\times$ 10^{-8} cm^2 s^{-1} for the rising and falling phase, respectively. (*B*) Estimation of the Fl-cGMP and cGMP diffusion coefficients from the relaxations of the currents elicited by 10 μM Fl-cGMP (*trace 1*) and 1 mM cGMP (*trace 2*). Experimental records are also from Fig. 1. The currents, plotted on a logarithmic scale, have been normalized with respect to their respective initial values and synchronized at the time of switching the bath nucleotide concentration to zero. The slopes of the current decays were 0.024 s^{-1} for Fl-cGMP and 0.50 s^{-1} for cGMP, giving diffusion coefficients of 3.5 $\times$ 10^{-8} cm^2 s^{-1} and 72 $\times$ 10^{-8} cm^2 s^{-1}, respectively, with a ratio of 20. (*C*) Decays of currents from another outer segment, elicited by 10 μM Fl-cGMP (*trace 1*) and 1 mM cGMP (*trace 2*). L = 26 μm. Bandwidth, DC-20 Hz. The slopes of the current decays were 0.013 s^{-1} for Fl-cGMP and 0.63 s^{-1} for cGMP, giving diffusion coefficients of 1.9 $\times$ 10^{-8} cm^2 s^{-1} and 91 $\times$ 10^{-8} cm^2 s^{-1}, respectively, with a ratio of 48.

J_{max}, elicited by 10 μM Fl-cGMP. The curve is drawn according to the Hill equation

$$\frac{J}{J_{max}} = \frac{C^n}{C^n + K_{1/2}{}^n} \quad (5)$$

where C is the Fl-cGMP concentration. The curve fit gives a half-maximal activation constant, $K_{1/2}$, of 1.1 μM Fl-cGMP and a Hill coefficient, n, of 1.5. Fl-cGMP opens the channels with a much lower $K_{1/2}$ than cGMP; this reflects a higher affinity for the binding site or a higher open probability of the liganded channel, or both. Averaged results from 11 rods are shown in Fig. 2 *B*. The Hill-equation fit gave $K_{1/2}$ = 0.91 μM Fl-cGMP and n = 1.9, in good agreement with results from excised patches (Tanaka et al., 1989) and comparable to biochemical results from membranes of bovine rod outer segments (Caretta et al., 1985). For comparison, the $K_{1/2}$ for cGMP is 36 μM, and the cooperativity of 1.9 is similar (Koutalos et al., 1995a).

The diffusion coefficient for Fl-cGMP can be obtained from the current kinetics. For the cell of Fig. 1, templates were constructed for the rising and falling phases of the Fl-cGMP-activated currents based on the measured values of $K_{1/2}$, n, and J_{max}. These templates were then scaled on the time axis to fit the experimental records, with the scaling factors providing estimates for the diffusion coefficient (see Koutalos et al., 1995b). Fig. 3 *A* shows such an analysis for the current activated by 1 μM Fl-cGMP in Fig. 1; the derived diffusion coefficient was 2.9 $\times$ 10^{-8} and 4.2 $\times$ 10^{-8} cm^2 s^{-1}, respectively, for the rising and falling phase of the current. The scaled templates do not describe the kinetics of the Fl-cGMP-elicited currents nearly as well as those of the cGMP-elicited currents (Koutalos et al., 1995b), possibly because of the assumptions involved in simplifying Eq. 1 to Eq. 2 (see Materials and Methods). For example, some of the Fl-cGMP binding sites may be saturated as the nucleotide concentration inside the ROS is equilibrating with that of the bath, so that the proportionality between the immobilized and free concentrations does not hold. In such a case, we can still use Eq. 2, but the apparent diffusion coefficient for Fl-cGMP should be interpreted as an empirical parameter describing the overall kinetics of the current.

The diffusion coefficient can also be obtained from the decay of the current elicited by 10 μM Fl-cGMP upon removing the analog (Fig. 3 *B*, trace 1). Similarly, the diffusion coefficient of cGMP can be obtained in the same experiment by using 1 mM cGMP (Fig. 3 *B*, trace 2). It can be seen that the Fl-cGMP-activated current relaxed much more slowly than the cGMP-activated current. Thus, Fl-cGMP diffuses more slowly than cGMP. Because Fl-cGMP is only ~1.3 times larger than

cGMP in molecular size (see Olson and Pugh, 1993), most of the difference in diffusion coefficient presumably reflects the binding of Fl-cGMP to cellular components due to the fluorescein moiety. From the slopes of the semilog plots in Fig. 3 *B* and the length $L = 27$ μm for this outer segment, we obtain a diffusion coefficient of 3.5×10^{-8} cm^2 s^{-1} for Fl-cGMP and 72×10^{-8} cm^2 s^{-1} for cGMP, using Eq. 4. The ratio of the diffusion coefficients was 20. Fig. 3 *C* shows results from another ROS, giving a diffusion coefficient of 1.9×10^{-8} cm^2 s^{-1} for Fl-cGMP and 91×10^{-8} cm^2 s^{-1} for cGMP, with a ratio of 48. In both Fig. 3 *B* and 3 *C*, there is an indication that two exponentials are present in the decay of the current elicited by 10 μM Fl-cGMP, reflecting a more complex diffusion process. The straight-line fit provided an average estimate for the overall kinetics of the relaxation. Results from seven salamander rods gave average diffusion coefficient values of $3.3 \pm 0.8 \times 10^{-8}$ cm^2 s^{-1} for Fl-cGMP and $79 \pm 15 \times 10^{-8}$ cm^2 s^{-1} for cGMP, with a ratio of 27 ± 5.

DISCUSSION

The value for the Fl-cGMP diffusion coefficient measured in this study, 3×10^{-8} cm^2 s^{-1}, agrees moderately well with the 1×10^{-8} cm^2 s^{-1} measured in intact salamander ROS (Olson and Pugh, 1993). The slight difference may arise from a number of factors. One is the difference in experimental approach. Olson and Pugh (1993) used a patch pipette to infuse Fl-cGMP into an isolated ROS and estimated the diffusion coefficient from the time course of spread of fluorescence. The diffusion model and analysis they used were therefore substantially different from those employed in this work. Another factor is a possible difference in cytoplasmic viscosity between the truncated and the intact ROS. Collisions with cell solids can retard diffusion by as much as two- to threefold (Kushmerick and Podolsky, 1969; Caille and Hinke, 1974; Kao et al., 1993). Even though the membranous disks are unlikely to come off the truncated ROS, some soluble proteins might diffuse into the bath, thereby reducing the overall viscosity as compared to the intact ROS. We have not observed an increase in the measured cGMP diffusion coefficient with time, as might be expected from any significant loss of cytoplasmic solids, but some change happening in the first 30 s after truncation cannot be excluded. Finally, the truncation procedure we used may somehow disrupt the structural integrity of the ROS in such a way as to increase the diffusion coefficient. Previously, we argued that any such structural change is unlikely to be very important (Koutalos et al., 1995b), but again a small contribution is possible.

Based on their measured value of 1×10^{-8} cm^2 s^{-1} for the Fl-cGMP diffusion coefficient, Olson and Pugh (1993) obtained an upper limit of 11×10^{-8} cm^2 s^{-1} for the cGMP diffusion coefficient. This latter value was derived from two correction factors. One is an estimated binding ratio (the parameter B in Eq. 3) of 8.6 between total and free Fl-cGMP, and the other is a difference in size between Fl-cGMP and cGMP, calculated to be 1.3; thus, the overall multiplication factor for correction was ~11 (Olson and Pugh, 1993). In our experiments with truncated ROS, we have directly measured a ratio of 27 between the cGMP and Fl-cGMP diffusion coefficients. This ratio should be independent of any complications from changes in viscosity or structural integrity, because the measurements for both compounds were obtained in the same experiment. Instead, the ratio should reflect only differences in size and intracellular binding between the two nucleotides. Using this factor of 27 and Olson and Pugh's (1993) value of 1×10^{-8} cm^2 s^{-1} for the diffusion coefficient of Fl-cGMP, we arrive at $\sim 30 \times 10^{-8}$ cm^2 s^{-1} for the diffusion coefficient of cGMP. This is not far from our direct measurement of $60-80 \times 10^{-8}$ cm^2 s^{-1} from the truncated ROS (Koutalos et al., 1995b, and here).

It is worth examining more closely the relation between the cGMP longitudinal diffusion coefficient in the rod outer segment, D, and that in aqueous solution, D_{aq}. We can write (see equations 2, 9, and A13 in Olson and Pugh, 1993):

$$\frac{D}{D_{aq}} = \frac{f}{\eta B} \tag{6}$$

where f is a tortuosity factor quantifying the effect of baffling by the disks, η is the cytoplasmic viscosity relative to aqueous solution, and B is the effect of intracellular binding sites. In terms of the structural parameters of the ROS, the tortuosity factor f has previously been expressed as (Lamb et al., 1981; Olson and Pugh, 1993; Koutalos et al., 1995b)

$$f = \frac{F_A}{F_V} \tag{7}$$

where F_A and F_V are, respectively, the patent cross-sectional area and volume available for diffusion inside the ROS. Olson and Pugh (1993) employed Eq. 7, an F_A value of ~0.014 (measured from migrographs), and an F_V value of ~0.5 to arrive at a tortuosity factor of ~0.028. It was pointed out to us by Professor R. A. Cone, however, that a more rigorous analysis (see equations 7, 10, and 11 in Hardt, 1979) would give a tortuosity factor of

$$f = \frac{F_A}{F_V(1 - F_V + F_A)} \tag{8}$$

Substituting ~0.5 for F_V (Sidman, 1957; Blaurock and Wilkins, 1969; see also Korenbrot et al., 1973) and ~0.04 for F_A (from freeze-etching; see Rosenkranz, 1977, and discussion in Koutalos et al., 1995b) in Eq. 8, we obtain a tortuosity factor of 0.15, close to the ratio of 0.2 between the longitudinal and lateral diffusion coefficients of 6-carboxyfluorescein measured by Phillips and Cone (1985). Because the diffusion of cGMP does not appear to be influenced by binding in the 5–1000 μM concentration range (Koutalos et al., 1995b), we can set $B = 1$. Finally, the diffusion coefficient of cGMP in aqueous solution is expected to be ~4 ×

10^{-6} cm^2 s^{-1} (Bowen and Martin, 1964). Substituting these values in Eq. 6, and using a D value of 60×10^{-8} cm^2 s^{-1}, we obtain $\eta = 1$. With a D value of 30×10^{-8} cm^2 s^{-1} instead (see above), we get $\eta = 2$. These correlations are only approximate, because the calculations depend on the exact value of the tortuosity factor f. Nonetheless, a cytoplasmic viscosity value between 1 and 2 is close to the range expected from measurements in other cell types, as mentioned above (Kushmerick and Podolsky, 1969; Caille and Hinke, 1974; Kao et al., 1993).

In conclusion, a value of $30-60 \times 10^{-8}$ cm^2 s^{-1} for the cGMP diffusion coefficient in ROS is consistent with the measurements of both Olson and Pugh (1993) and Koutalos et al. (1995b). The original discrepancy of more than five-fold seems to have arisen from the estimates of Fl-cGMP binding and baffling by the disks.

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