Biochimica et Biophysica Acta, 1022 (1990) 283–290
Elsevier

BBAMEM 74761

Spectrophotometric determination of photoreceptor cGMP-gated channel Mg^{2+}-fluxes using dichlorophosphonazo III

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(Received 3 August 1989)
(Revised manuscript received 16 November 1989)

Key words: Cyclic GMP; Magnesium ion; Dichlorophosphonazo III; Photoreceptor rod; Gated channel

We have characterised the spectroscopic properties of the metallochromic dye dichlorophosphonazo III and describe its use for the determination of changes of Mg^{2+} concentration in the micromolar range. Using a previously described reconstitution procedure, we incorporated the cGMP-gated channel from bovine rod photoreceptors into magnesium-containing liposomes and used the dye to monitor cGMP-activated Mg^{2+}-efflux. The K_m and cooperativity of the cGMP-dependence were identical regardless of whether Mg^{2+} or Ca^{2+} was the transported ion, however, the v_{max} for Ca^{2+} was more than 2-fold higher than that for Mg^{2+}. We thereby determined a channel selectivity (Ca^{2+} : Mg^{2+}) of 1.0 : 0.44 in the presence of symmetrical (30 mM) K^+. We also describe conditions where Mg^{2+} or Ca^{2+} effluxes can be selectively monitored in the presence of each other. This allowed the demonstration that magnesium ions can flow through the cGMP-gated channel even in the presence of an identically directed calcium gradient. Together these results indicate that magnesium ions may enter the photoreceptor rod outer segment cytosol through the cGMP-gated channel under dark conditions, thereby alluding to the existence of an as yet unknown Mg^{2+}-extrusion mechanism, distinct from the Na^+/Ca^{2+}-exchanger, in these cells.

Introduction

Magnesium is one of the most abundant biologically relevant cations. As well as being essential for numerous metabolic processes [1], it is also subject to several, as yet poorly characterized, transport processes [2]. In vertebrate photoreceptors magnesium is very abundant and its concentration is reduced by illumination [3]. It is an essential cofactor of, or interacts with, several enzymes [4–9,41], and is required to achieve the active form of nucleotide triphosphates. The 'light-dependent' channel of rod and cone photoreceptors has been shown to be appreciably permeable to this cation [10–12]. More recently it has been suggested that magnesium may be involved in an intracellular messenger system that mediates phototransduction [13].

Although numerous methods have been proposed for the determination of Mg^{2+} in biological systems [14–21], their usefulness for the study of Mg^{2+}-transport is relatively limited. Magnesium radioisotope studies are technically difficult due to the very short half life of ^{28}Mg, and the more popular spectroscopic methods [22] are either subject to interference or do not exhibit sufficient sensitivity. In this study we describe, for the first time, the use of the metallochromic dye dichlorophosphonazo III for the determination of Mg^{2+}-fluxes in the micromolar range. By employing a well characterized reconstitution system [23–25], we were able to select the intra- and extraliposomal ion concentrations most appropriate for optimal sensitivity and specificity. This allowed the investigation of several properties of the photoreceptor cGMP-gated channel's permeability to Mg^{2+} which were not accessible to electrophysiological methods.

Experimental

Materials. Dichlorophosphonazo III (DCP) was obtained from Fluka. Thin-layer chromatography using the conditions of Yoshikami and Hagins [26] revealed

Abbreviations: cGMP, guanosine 3′:5′-cyclic monophosphate; ROS, rod outer segment; DCP, dichlorophosphonazo III, bis-(4-chloro-2-phosphonobenzolazo)-2,7-chromotropic acid; arsenazo III, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthalenebis(azo)]dibenzenearsonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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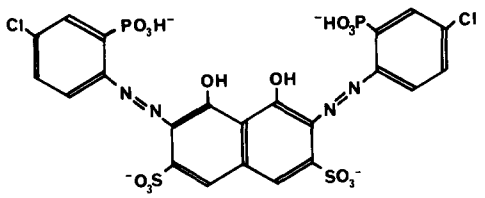


The structure of dichlorophosphonazo III.

two bands: DCP (R_F 0.49) representing more than 95% of total dye content, and a minor contaminant (R_F 0.59) representing the remaining dye content. DCP solutions were treated by passage through a Chelex 100 (Sigma) column and adjusted to 1 mM assuming a molar absorption coefficient (ϵ_{600}) of $4.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [26,27]. Arsenazo III (99% pure) and cGMP were from Sigma. All other chemicals and reagents were of analytical grade or better and obtained from the suppliers reported in previous publications [23–25,28].

Purification and reconstitution of the ROS cGMP-gated channel and Na^+/Ca^{2+} exchanger. The purification of the cGMP-gated channel and the Na^+/Ca^{2+}-exchanger from ROS membranes stripped of peripheral proteins

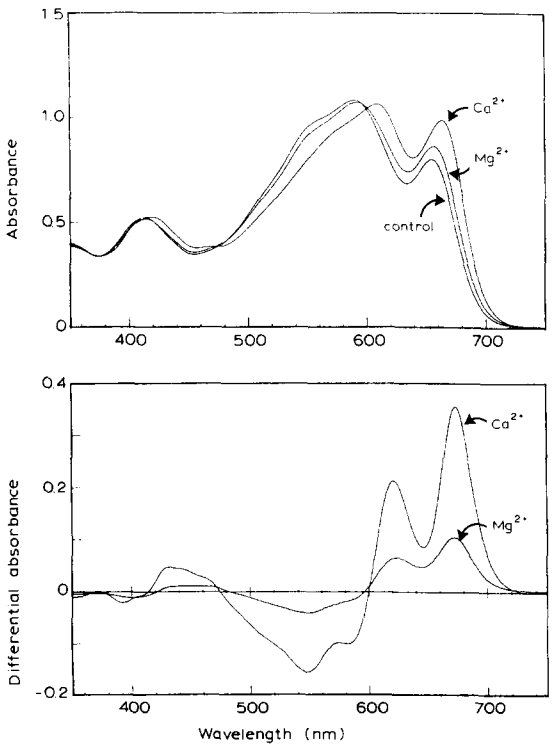


Fig. 1. Absorbance (above) and difference (below) spectra of dichlorophosphonazo III in the presence or absence of Ca^{2+} or Mg^{2+}. The spectra of the dye in the presence or absence of 10 μM Ca^{2+} or Mg^{2+} were obtained at room temperature using an Aminco DW-2000 spectrophotometer as described under Experimental. The sample cuvette contained 25 μM DCP, 0.1 M KCl and 10 mM Hepes-KOH (pH 7.4) and, where applicable, $MgCl_2$ or $CaCl_2$ in a total volume of 2 ml. The reference cuvette contained the same solution without the dye.

was as previously described [25,28]. Purified proteins were desalted and transferred into the appropriate buffers by gel filtration on PD-10 columns (Pharmacia). Reconstitution was carried out using a previously described detergent dialysis procedure [23] as follows: a concentrated phospholipid (asolectin) suspension was added to purified protein extract to give 10 mM CHAPS and 10 $mg \cdot ml^{-1}$ phospholipid (final concentrations) and the appropriate concentrations of buffer (10 mM Hepes-KOH or Hepes-arginine, both pH 7.4) and salts. This was followed by dialysis (48 h including three changes) against buffer containing the desired concentrations of KCl, $CaCl_2$ and/or $MgCl_2$. Transmembrane ionic gradients were established by a further dialysis against calcium- and magnesium-free buffer.

Spectrophotometric measurements. Dual-wavelength measurements and absolute and differential absorption spectra of dichlorophosphonazo III and arsenazo III were carried out on an Aminco DW-2000 spectrophotometer coupled to an IBM PS-II computer. All measurements were performed at room temperature with a 1 nm bandwidth and a 1 cm light path. Wavelength scans were performed at 2 $nm \cdot s^{-1}$. Dual-wavelength kinetic measurements were carried out using a stirring block attachment at a chopper setting of 250 Hz. The sample volume was 2 ml, and reagents (e.g., cGMP) were injected through an orifice in the lid of the cuvette holder. Efflux singles were calibrated by adding known concentrations of divalent cation to the cuvette. The divalent cation content of proteoliposomes was certified by adding ionophore (A23187) or detergent (0.3% w/v CHAPS) to liposome suspensions in the presence of metallochromic dye.

Results

Spectral properties of dichlorophosphonazo III

Fig. 1 shows the absorbance spectra and the corresponding difference spectra of DCP in the presence and in the absence of Ca^{2+} and Mg^{2+}. The addition of either of these divalent cations induces a large spectral change consisting of a decrease in absorbance with a λ_{min} at 548 nm and an increase in absorbance with values for λ_{max} at 610 and 672 nm, and isobestic points at 472 and 600 nm. Under all conditions investigated the dye exhibited a greater sensitivity for Ca^{2+} than it did for Mg^{2+} (see below).

In Fig. 2 titration curves of DCP with Ca^{2+} and Mg^{2+} are shown. The shape of the curves is suggestive of a complicated binding mechanism not analysable by simple kinetic procedures, e.g., double-reciprocal plots of absorbance change versus [Ca] or [Mg]. This is probably indicative of the formation of multiple DCP-divalent cation species, a phenomenon which has been extensively documented for another bis(arylazo) derivative of chromotropic acid, arsenazo III [29,30]. For this

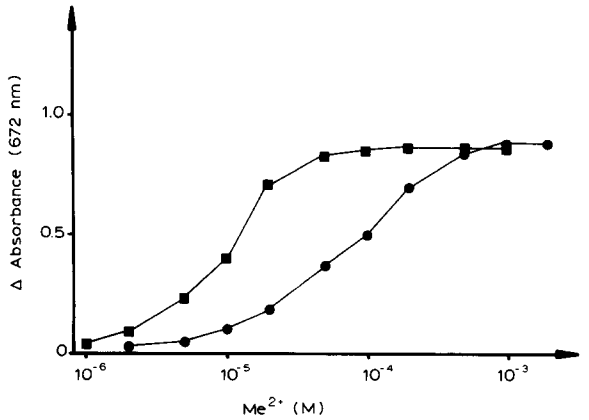


Fig. 2. Tritration of dichlorophosphonazo III with Ca^{2+} and Mg^{2+}. The change in absorbance at 672 nm is plotted as a function of the concentration of Ca^{2+} (■) or Mg^{2+} (●) in the cuvette. The sample cuvette contained 25 μM DCP, 0.1 M KCl and 10 mM Hepes-KOH (pH 7.4).

reason, in the experiments described below, instead of attempting to determine dissociation constants for a given set of conditions we have taken the concentration of divalent cation which gives the half-maximal absorbance change as a more direct and practical measure of DCP's affinity for Mg^{2+} or Ca^{2+}.

Fig. 3 shows the effect of pH on the affinity of DCP for Ca^{2+} and Mg^{2+}. Lowering the pH clearly decreases the affinity of the dye for magnesium, but has virtually no effect on the affinity for calcium. A similar effect of pH has also been extensively described for arsenazo III [31,32], and presumably reflects the competition of protons for the divalent cation binding site. Thus it is important to operate in a well buffered system when

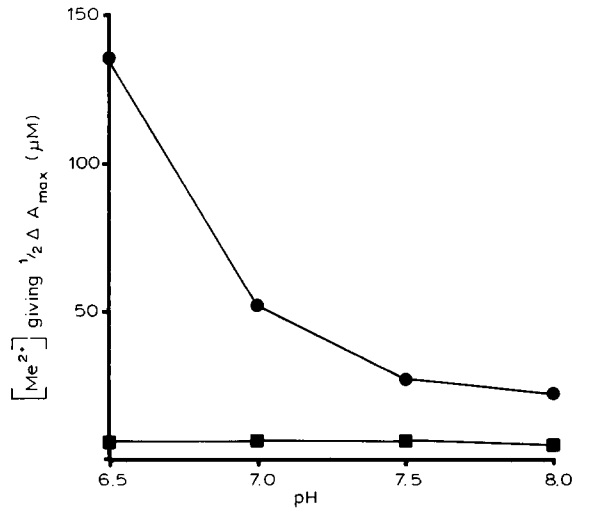


Fig. 3. The effects of pH on the interaction of dichlorophosphonazo III with Ca^{2+} (■) and Mg^{2+} (●). The concentration of divalent cation (Me^{2+}) giving the half-maximal absorbance change at 672 nm is plotted for different values of pH. The sample cuvette contained 25 μM DCP, 0.1 M KCl and 10 mM Hepes buffered to the indicated pH with KOH.

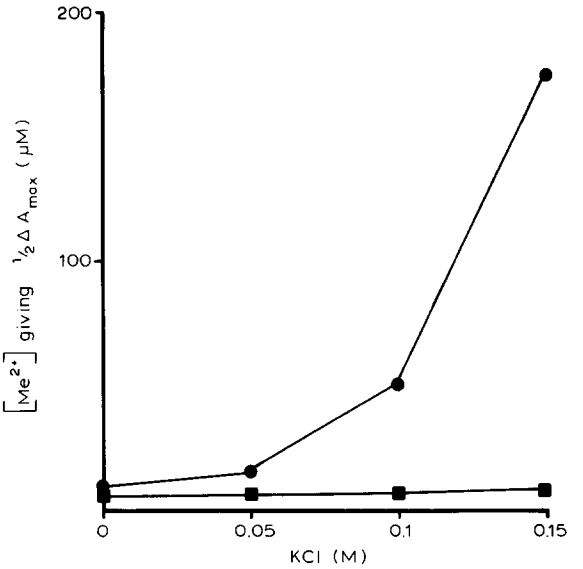


Fig. 4. The effects of ionic strength on the interaction of dichlorophosphonazo III with Ca^{2+} (■) and Mg^{2+} (●). The concentration of divalent cation (Me^{2+}) giving the half-maximal absorbance change at 672 nm is plotted for different concentrations of KCl. The sample cuvette contained 25 μM DCP, 10 mM Hepes-arginine (pH 7.4) and either 150 mM KCl, or 100 mM KCl and 100 mM sucrose, or 50 mM KCl and 200 mM sucrose, or 300 mM sucrose.

employing DCP for the estimation of micromolar changes of divalent cation (especially Mg^{2+}) concentration.

We also investigated the effects of ionic strength on the affinity of DCP for Ca^{2+} and Mg^{2+} (Fig. 4). With increasing ionic strength the dye displayed a decreased affinity for divalent cations. Again, this effect was far more pronounced for Mg^{2+} than for Ca^{2+}. This may be due to a combination of direct ionic strength effects and competition of K^+ ions for the divalent cation binding site, as has been reported for arsenazo III [32].

Determination of cGMP-gated Mg^{2+}-fluxes using DCP

For the analysis of changes in Mg^{2+}-concentration with time resolution, we performed spectroscopic measurements in the double-wavelength mode using the wavelength pair 672-600 nm. The isobestic point at 600 nm was selected as the reference wavelength (in preference to, say, wavelengths greater then 720 nm were the absorbance also does not change after magnesium addition) since at this wavelength the dye has an absorbance closer to that of the sample wavelength and therefore absorbance changes due to dilution effects (when adding reagents to the cuvette) are minimised. From the spectral properties of DCP described above, it is evident that certain conditions (pH ≥ 7.5, KCl concentration ≤ 50 mM) must be employed in order to measure micromolar changes of Mg^{2+} concentration with maximum sensitivity. The affinity of DCP for Ca^{2+} when measuring Mg^{2+} is not a problem since in a reconstituted system Ca^{2+}-free conditions can be ap-

plied simply by employing calcium-free buffers during the dialysis procedure.

Since high ionic strength would cancel out the possibility of measuring Mg^{2+}-concentration changes in the micromolar range, we determined the importance of the KCl concentration for the cGMP-gated channel. Potassium ions are included in the reconstitution system [23] not only for liposome stabilisation but also to serve as a counter-ion which enters the liposome after cGMP-activated Ca^{2+}- or Mg^{2+}-efflux to ensure electroneutrality [33]. Fig. 5 shows that liposomes reconstituted with the cGMP-gated channel exhibit a maximal efflux velocity at about 30 mM KCl. The inclusion of potassium ionophore (valinomycin) into the assay system did not enhance the rate of cGMP-activated cation efflux, thus K^+ presumably enters the intraliposomal space through the cGMP-gated channel itself, and does not require an alternative permeability to ensure electroneutrality. The results also indicate that in the absence of K^+ cGMP-gated fluxes can still be observed. Presumably proton-transport (to which asolectin liposomes are readily susceptible) is ensuring electroneutrality in such experiments. From these results we selected a KCl concentration of 30 mM (intra- and extraliposomal) when investigating the cGMP-gated Mg^{2+}-fluxes described below.

Fig. 6 shows spectroscopic Mg^2 + -efflux signals from liposomes reconstituted with the cGMP-gated channel after the addition of different concentrations of cGMP

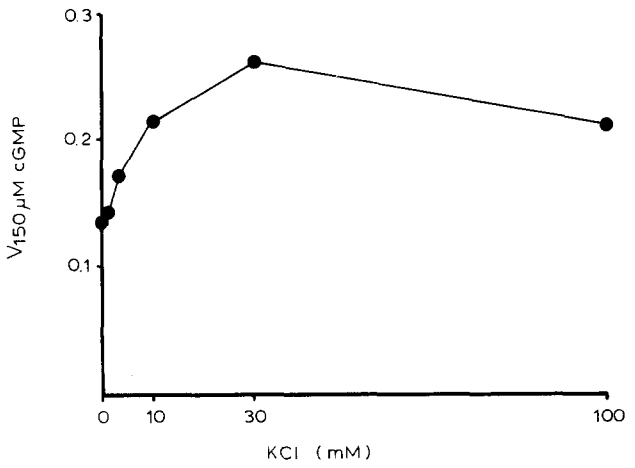


Fig. 5. The effects of KCl concentration on cGMP-gated channel Mg^{2+}-fluxes. cGMP-gated channel protein (1.5 $\mu g \cdot ml^{-1}$) was reconstituted in 10 mM Hepes-arginine (pH 7.4), 0.1 mM dithiothreitol and 10 mg asolectin per ml with different concentrations of KCl, and an intraliposomal $MgCl_2$ concentration of 5 mM. Mg^{2+}-efflux was initiated by adding 150 μM cGMP (i.e., a saturating concentration) to an aliquot (0.4 ml) of liposomes suspended (final volume 2 ml) in 25 μM DCP, 10 mM Hepes-arginine (pH 7.5) and the concentration of KCl equivalent to that present intraliposomally. Effluxes were monitored spectroscopically using the dual wavelength model (672–600 nm) and calibrated by adding known concentrations of $MgCl_2$ to the cuvette. The initial rates of efflux are expressed as nmol Mg^{2+} per s and were determined by tangent-fitting.

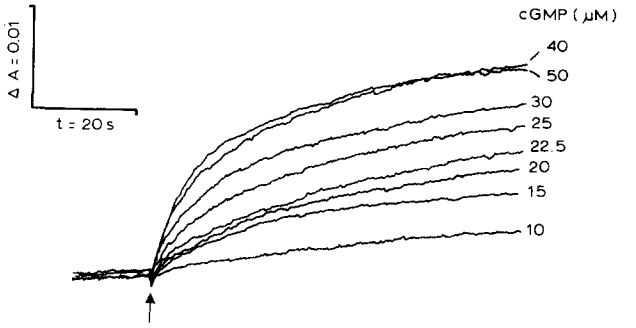


Fig. 6. Spectroscopic determination of cGMP-activated Mg^{2+} efflux from liposomes reconstituted with channel protein. The liposomes (6.4 μg channel protein per ml and 10 mg asolectin per mol contained 10 mM Hepes-KOH (pH 7.5), 30 mM KCl, 100 mM sucrose (all concentrations inside and outside) and 5 mM $MgCl_2$ (inside only). For spectroscopy, a 0.4 ml aliquot of liposomes was suspended (final volume 2 ml) in 10 mM Hepes-KOH (pH 7.5), 30 mM KCl, 100 mM sucrose and 25 μM DCP. cGMP was injected into the cuvette at the time-point indicated by the arrow, and Mg^{2+} efflux was monitored using the wavelength pair 672–600 nm.

to the cuvette. An intraliposomal Mg^{2+}-concentration of 5 mM was selected in order to obtain efflux signals of sufficient magnitude for kinetic analysis and because this concentration has been reported to be saturating with respect to magnesium's affinity for the channel protein [12]. A transmembrane magnesium-gradient (i.e., removal of extraliposomal magnesium) was established by a final dialysis against Mg^{2+}-free buffer. DCP was present extraliposomally at a concentration of 25 μM.

Kinetic analysis of the initial velocities of Mg^{2+}-efflux at different concentrations of cGMP is shown in Fig. 7(a–c). For comparison, we performed parallel experiments where either Ca^{2+} or Mg^{2+} was the transported ion (both present intraliposomally at a concentration of 5 mM and in the absence of the other divalent cation). From plots of normalised efflux rates versus cGMP concentration (Fig. 7a) and from Hill plots (Fig. 7b) it can be seen that Mg^{2+} and Ca^{2+}-effluxes are both activated by cGMP in a highly cooperative manner ($n = 3.4$ from Hill plots) and that the rates of efflux saturate at cGMP concentrations of 40–50 μM and exhibit a K_m of about 27 μM cGMP. The highly curved Lineweaver-Burk plots (Fig. 7c) also testify to cooperativity, and demonstrate that the v_{max} for Ca^{2+}-efflux is about double that for Mg^{2+}-efflux. Thus we conclude that the kinetics of cGMP-activation of the channel protein are identical whether Ca^{2+} or Mg^{2+} is the transported ion, however, the maximal rate of Ca^{2+}-efflux is about double that of Mg^{2+}-efflux. From these results we calculated that the Ca^{2+}/Mg^{2+} selectivity of the cGMP-gated channel is about 1.0 : 0.44 in the presence of symmetrical 30 mM KCl.

cGMP-gated Mg^{2+}-fluxes in the presence of Ca^{2+}

We were also interested to investigate whether Mg^{2+}-fluxes can occur in the presence of Ca^{2+}, since

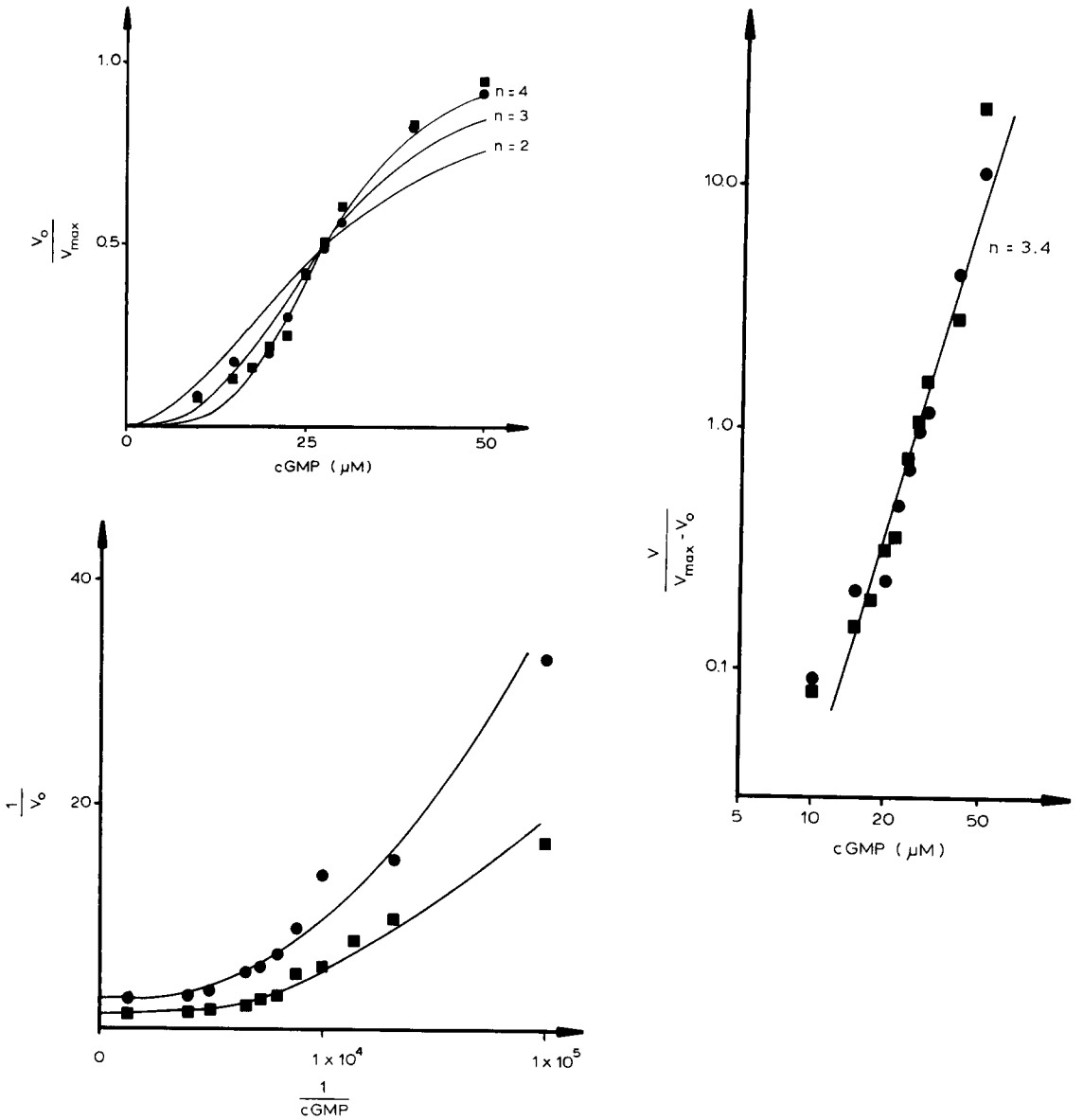


Fig. 7. Kinetic analysis of the cGMP-dependence of Mg^{2+} (●) and Ca^{2+} (■) efflux. The spectroscopic conditions were as described in the legend to Fig. 6. (a) Normalised velocities as a function of the concentration of cGMP injected into the cuvette. The continuous lines are calculated for a cooperative process using a K_m of 27 μM where $n = 2$, 3 or 4 as described in Ref. 33. (b) Hill plots for cGMP-activated Mg^{2+} and Ca^{2+} efflux. The slope of the straight line is $n = 3.4$. (c) Lineweaver-Burk plots (the v_{max} for Mg^{2+} is 0.37 nmol Mg^{2+}/s per μg channel protein reconstituted, and that of Ca^{2+} is 0.83 nmol Ca^{2+}/s per μg channel protein reconstituted).

both of these divalent cations are presumed to be present at relatively high (i.e., millimolar) concentrations in the extracellular space of vertebrate photoreceptors [10,11]. It was therefore necessary to establish conditions were Mg^{2+} (or Ca^{2+}) fluxes could be selectively monitored in the presence of simultaneously occurring Ca^{2+} (or Mg^{2+}) fluxes. In order to selectively monitor changes in magnesium concentration when changes in calcium concentration were simultaneously occurring, we added the Ca^{2+}-chelator EGTA (100 μM) to the extraliposomal suspension medium. Fig. 8 shows the changes in absorbance of DCP in the presence of EGTA when Mg^{2+} or Ca^{2+} are added to the cuvette. Magnesium induces a significant absorbance change whereas added calcium is rapidly and selectively buffered by EGTA, resulting in no absorbance change.

To selectively measure changes in Ca^{2+}-concentration in the presence of simultaneously occurring Mg^{2+}-fluxes, we employed a dye which possesses a much better selectivity for Ca^{2+} than DCP, arsenazo III. Fig. 9a shows the change in absorbance of arsenazo III in response to Ca^{2+} relative to the change in absorbance induced by the same concentration of Mg^{2+}, in the wavelength range 580 to 710 nm. It can be seen that the wavelength where this dye shows the best selectivity (about 15-fold better) for Ca^{2+} relative to Mg^{2+} is 665

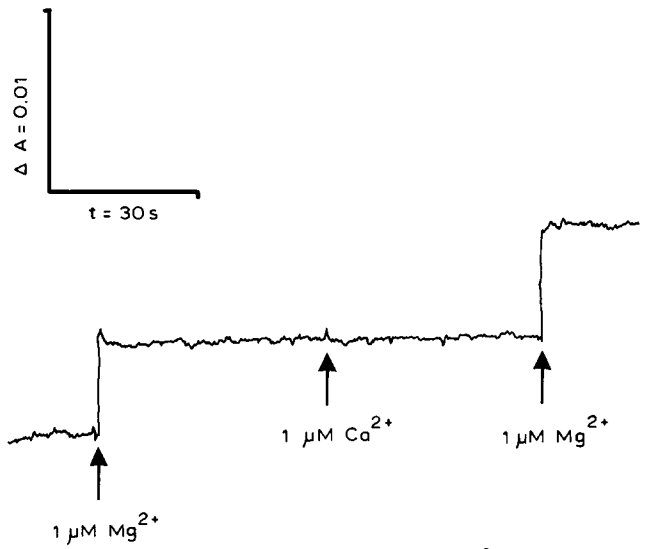


Fig. 8. Conditions for the determination of Mg^{2+} efflux in the presence of Ca^{2+} efflux. The cuvette contained 25 μM DCP, 10 mM Hepes-KOH, 100 mM KCl and 100 μM EGTA. At the time-points indicated either 1 μM $MgCl_2$ or 1 μM $CaCl_2$ was injected not the cuvette. Spectroscopy was performed in the dual-wavelength mode (672–600 nm).

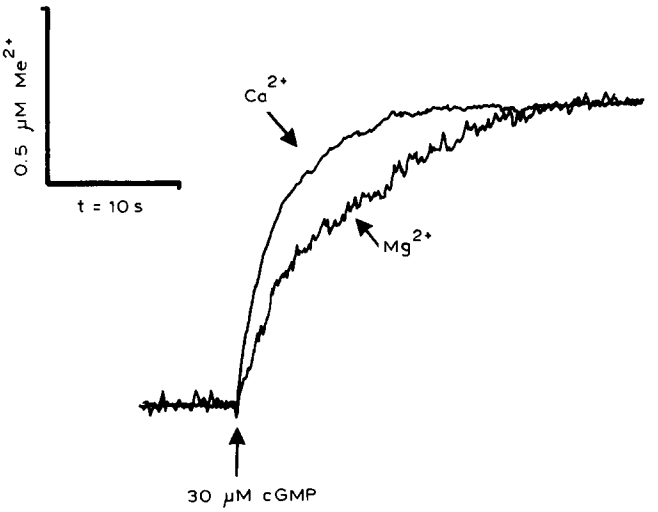
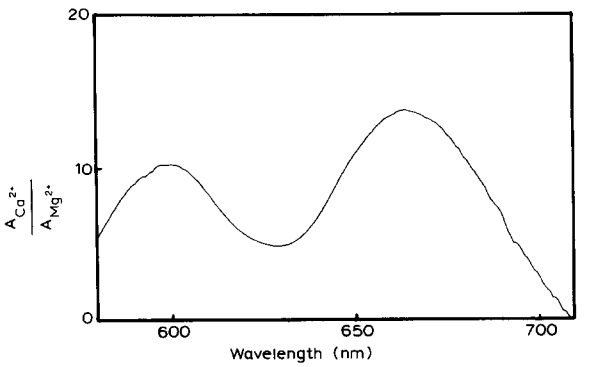


Fig. 10. Simultaneous Ca^{2+}- and Mg^{2+}-efflux from liposomes reconstituted with the cGMP-gated channel. Channel-containing liposomes were reconstituted to give internal $MgCl_2$ and $CaCl_2$ concentrations of 5 mM. cGMP-activated Mg^{2+} efflux was determined under the conditions described in the legend to Fig. 8, and cGMP-activated Ca^{2+} efflux was determined under the conditions described in Fig. 9. Signals have been normalized after calibration by the addition of known amounts of divalent cation.

nm. We therefore performed dual wavelength measurements using the wavelength pair 665-710 nm for Ca^{2+}-flux analysis. As can be seen in Fig. 9b, under these conditions addition of Ca^{2+} to the cuvette induces a significant change in absorbance, whereas Mg^{2+} induces such a minimal change in absorbance that it can be effectively ignored.

In Fig. 10, Mg^{2+}- and Ca^{2+}-efflux signals, activated by cGMP when both of these divalent cations are present intraliposomally at a concentration of 5 mM, are superimposed. It can be seen that when a transmembrane gradient for both of these divalent cations exists, cGMP induces their simultaneous efflux. As expected from the results presented in Fig. 7, the initial rate of Ca^{2+}-efflux is about twice that of the Mg^{2+}-efflux. These results would appear to indicate that, under the appropriate conditions (see Discussion), Mg^{2+}-ions would be capable of entering the photoreceptor outer segment via the light-dependent channels.

If Mg^{2+} does indeed enter the photoreceptor cytosol, there must exist some mechanism for its extrusion. We therefore examined the possibility that Mg^{2+} could be extruded by the by the same mechanism as Ca^{2+}, i.e.,



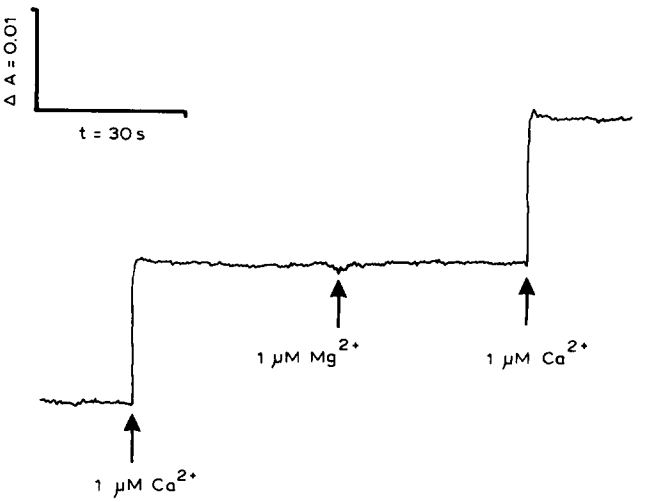


Fig. 9. Conditions for the determination of Ca^{2+} efflux in the presence of Mg^{2+} efflux. (a) The change in absorbance of arsenazo III after addition of 10 μM Ca^{2+} relative to the change in absorbance after the addition of 10 μM Mg^{2+}. (b) Dual-wavelength spectroscopy (665-710 nm). The cuvette contained 50 μM arsenazo III, 10 mM Hepes-KOH and 100 mM KCl. At the time-points indicated 1 μM $CaCl_2$ or 1 μM $MgCl_2$ was injected into the cuvette.

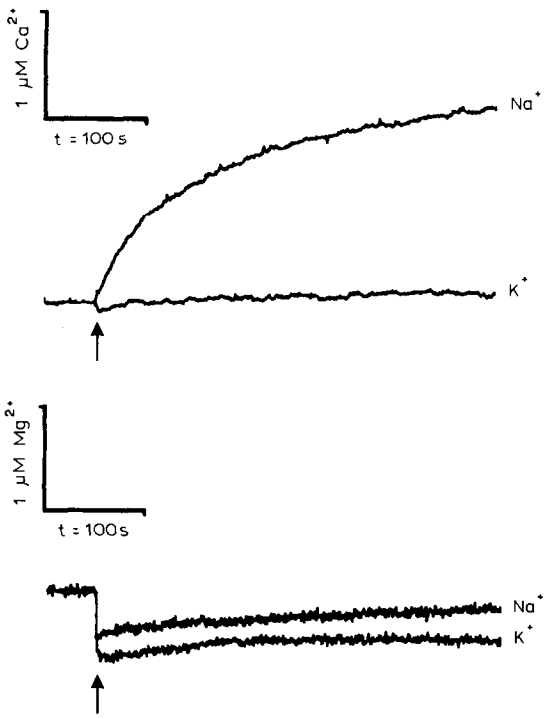


Fig. 11. Inability of the photoreceptor Na^+/Ca^{2+} exchanger to transport Mg^{2+}. Purified sodium-calcium exchanger was incorporated into asolectin liposomes containing either 5 mM $CaCl_2$ (above) or 5 mM $MgCl_2$ (below). Dual wavelength spectrophotometry was performed using arsenazo III for calcium efflux and DCP for magnesium efflux as described above. Addition of 50 mM NaCl to the cuvette at the time-points indicated by the arrows was found to release Ca^{2+}, but not Mg^{2+}. Addition of the same concentration of KCl to the cuvette did not induce Ca^{2+}, thereby demonstrating that the Ca^{2+} efflux was not due to a non-specific release mechanism caused by increased ionic strength.

via the sodium-calcium exchanger. Fig. 11 clearly demonstrates that this is not the case. When the purified sodium-calcium exchanger was reconstrituted into calcium-containing liposomes, Ca^{2+}-efflux could be specifically activated by adding Na^+ to the suspension medium (Fig. 11, above). Sodium was, however, unable to activate Mg^{2+}-efflux after incorporation of the exchanger into Mg^{2+}-containing liposomes (Fig. 11, below), in agreement with the results of Yau and Nakatani [34]. This indicates that if there does exist a magnesium-extrusion mechanism in vertebrate photoreceptors, it is distinct from the Na^+/Ca^{2+}-exchanger.

Discussion

DCP as a spectroscopic probe for Mg^{2+}

In this study, we have described the characterization and use of the dye dichlorophosphonazo III as a spectroscopic probe for changes in Mg^{2+}-concentration in the micromolar range. We have applied the method to the cGMP-gated channel of bovine rod photoreceptors after reconstitution into liposomes, a system where the channel protein has been amply demonstrated to exist in physiologically relevant condition. We were able to investigate several properties of the channel protein, e.g., the kinetics of cGMP-activation where Mg^{2+} is the transported ion at near-physiological concentration.

DCP was found to be more amenable to such studies than the more extensively used metallochromic dye arsenazo III due to its increased sensitivity for Mg^{2+} and lower toxicity. However, when selectively measuring Ca^{2+}-fluxes we preferred to use arsenazo III since it exhibits a far superior Ca^{2+} to Mg^{2+} specificity than DCP. We also found that the affinity of DCP for Mg^{2+} is particularly sensitive to pH and ionic strength, however, by employing a reconstitution procedure, we were able to impose the appropriate conditions and circumvent these problems. It is nevertheless evident that these spectral properties of DCP would severely limit its application for the measurement of changes in intracellular Mg^{2+}-concentration. The increasing availability of ion channel proteins in their purified and reconstituted forms may mean that the methods described here could be applied to other cation channels, such as the nicotinic acetylcholine receptor [35] known or suspected to conduct Mg^{2+}.

Mg^{2+}-permeability of the cGMP-gated channel

We have described for the first time the analysis of the kinetics of cGMP-activation of the light-dependent channel where Mg^{2+} is the transported ion. Our results indicate that the cGMP-dependence of Mg^{2+}-ion flux is identical to Ca^{2+}-ion flux, however, the maximal rate of Mg^{2+}-efflux was found to be about half that for Ca^{2+}. This presumably reflects the relative permeabilities of the channel pore for these divalent cations and agrees with results obtained with electrophysiological methods [10–13].

By reconstituting the cGMP-gated channel into liposomes containing identical concentrations of Ca^{2+} and Mg^{2+}, we were able to show that upon addition of cGMP the efflux of both divalent cations could occur simultaneously. The Ca^{2+}- and Mg^{2+}-effluxes presumably exhibit mutual inhibition [11], however, we were unable to investigate this quantitatively due to the technical difficulty of incorporating and maintaining a defined series of divalent cation concentrations into the liposomes during the relatively long dialysis procedure. We found that significant Mg^{2+}-efflux could occur long before the Ca^{2+}-efflux had reached completion. This would appear to contrast with results obtained from amphibian rod photoreceptors [12], where it has been reported that Mg^{2+} cannot flow through the light-dependent channel unless extracellular (i.e., in our system, intraliposomal) Ca^{2+} is reduced below 10 μM. One explanation for this could be that the cGMP-gated channel has been reported to exist in two distinct open states with respect to its permeability for divalent cations [42]. The results in Ref. 11 represent the first open

state; it is possible that the purified and reconstituted channel exists in the second state where Ca^{2+} and Mg^{2+} permeate more freely [42].

Magnesium status of the vertebrate photoreceptor cell in situ

Although vertebrate photoreceptors have been reported to have a high magnesium content [3], the free concentration of this cation in the cytosol has not been determined. Indeed much of the intracellular magnesium content is presumably bound to nucleotide triphosphates, since both ATP and GTP are known to exist in ROS at high concentrations [36]. It is reasonable to assume that the free Mg^{2+} may be of the order of 0.2–0.5 mM, the optimal concentration for cGMP-phosphodiesterase [7]. At this concentration, it may indeed be possible for this cation to modulate the cGMP-gated channel as has been implicated from electrophysiological studies [13].

The extracellular Mg^{2+}-concentration for vertebrate photoreceptors is generally assumed to be of the order of 1.6 mM. At this concentration, magnesium is presumably capable of entering the ROS cytosol, thereby contributing to the dark current. Our results confirm other reports [11] that Mg^{2+} can enter the photoreceptor cell even in the presence of millimolar concentrations of extracellular Ca^{2+}. The capacity for magnesium influx combined with the observation that rod photoreceptors loose magnesium upon illumination [3], suggests that there may exist a magnesium-extrusion mechanism, for example a Na^{+}/Mg^{2+} exchanger [37–40], in these cells. The further investigation and definition of the mechanism(s) of photoreceptor magnesium transport will become one of the most interesting goals of future research in the field of vertebrate phototransduction.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 169 Projekt C4), the Max-Planck Gesellschaft and a Leibniz-Programm Grant to Prof. Hartmut Michel.

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