Cyclic GMP-gated channels of bovine rod photoreceptors: affinity, density and stoichiometry of Ca^{2+}–calmodulin binding sites

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1. Ca^{2+}-loaded vesicles of bovine rod outer segment (ROS) membranes were used to examine the influence of Ca^{2+}–calmodulin (Ca^{2+}–CaM) on the activity of cGMP-gated channels.

2. In vesicles prepared from ROS membranes which were washed at zero free Ca^{2+}, Ca^{2+}–CaM reduced the Ca^{2+} flux to maximally 40%. The dose–response curve for activation of the cGMP-gated channel had a half-maximal value of $36 \cdot 8 \pm 2$ μM in the CaM-free state, and of $55 \cdot 6 \pm 5 \cdot 2$ μM in the Ca^{2+}–CaM-bound state. In both cases the Hill coefficients were $2 \cdot 2 \pm 0 \cdot 2$.

3. In vesicles prepared from ROS membranes which were washed at 100 μM Ca^{2+}, the dose–response curve was identical to the Ca^{2+}–CaM-bound state.

4. Titration of the Ca^{2+}–CaM-dependent decrease of the channel activity upon addition of 40 μM cGMP yielded half-maximal Ca^{2+}–CaM concentrations (EC_{50}^{CaM}) which were linearly correlated with the concentration of membrane vesicles. Extrapolation of EC_{50}^{CaM} to infinite dilution of vesicles yielded a Ca^{2+}–CaM affinity constant for the cGMP-gated channel of $1 \cdot 01 \pm 0 \cdot 20$ nM. Hill analysis of the Ca^{2+}–CaM titrations resulted in a Hill coefficient of $1 \cdot 36 \pm 0 \cdot 15$.

5. From the slope of the linear regression of EC_{50}^{CaM} plotted *vs.* the rhodopsin concentration, the molar ratio of rhodopsin to externally accessible Ca^{2+}–CaM binding sites of fused ROS membranes was determined to be 1439 ± 109. Therefore, there are about 720 molecules of rhodopsin per Ca^{2+}–CaM binding site present in ROS.

6. Based on these data, a density of 560 Ca^{2+}–CaM binding sites μm^{-2} is estimated for the plasma membrane of bovine ROS, suggesting that there are two Ca^{2+}–CaM binding sites per channel.

7. The Ca^{2+}–CaM effect did not become noticeable until the ROS membranes were hypotonically washed at free $[Ca^{2+}]$ below 100 nM, suggesting that an endogenous Ca^{2+}-binding protein was washed off in the absence of Ca^{2+}.

8. If the endogenous Ca^{2+}-binding protein of bovine ROS membranes was washed off at zero Ca^{2+} and then Ca^{2+}–CaM added, Ca^{2+}–CaM could only be washed off again at free $[Ca^{2+}]$ below 100 nM.

9. These findings strongly suggest that the endogenous Ca^{2+}-binding protein of the bovine cGMP-gated channel is CaM.

Light excitation and light adaptation are fundamental properties of photoreceptors. Two transmitter substances control these functions in vertebrates: cyclic guanosine-3′, 5′-monophosphate (cGMP) and Ca^{2+}. The target of the excitatory transmitter cGMP is the cGMP-gated channel which mediates an influx of Na^+ and Ca^{2+} into the outer segment of the photoreceptor. The intracellular cGMP concentration is controlled by light-dependent hydrolysis, which involves the chain reaction: light activation of

rhodopsin, activation of transducin (the retinal G-protein), and activation of cGMP phosphodiesterase. This biochemical pathway has been explored in considerable detail (for reviews see e.g. Hodgkin, 1988; Kaupp & Koch, 1992; Lagnado & Baylor, 1992; Yau, 1994).

Adaptation to background light appears to be mediated by Ca^{2+} because adaptation does not occur in photoreceptors where the $[Ca^{2+}]_i$ is experimentally kept constant (Matthews,

Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988). Several Ca^{2+}-dependent mechanisms have been reported: (1) in low $[Ca^{2+}]$, the synthesis of cGMP by the retinal guanylyl cyclase is strongly activated (Koch & Stryer, 1988); (2) Ca^{2+} enhances the light stimulation of the cGMP phospho-diesterase (Kawamura, 1993); (3) lowering of $[Ca^{2+}]_i$ reduces the catalytic activity of rhodopsin (Lagnado & Baylor, 1994); and (4) in the presence of calmodulin (CaM), Ca^{2+} decreases the affinity of the cGMP-gated channel for cGMP (Hsu & Molday, 1993, 1994).

The Ca^{2+}–CaM-dependent modulation of the rod cGMP-gated channel is mediated by its β-subunit (Chen, Illing, Molday, Hsu, Yau & Molday 1994; Körschen *et al.* 1995). The half-maximal reduction of the cGMP affinity by Ca^{2+}–CaM is in the nanomolar range (Hsu & Molday, 1994). Due to this high Ca^{2+}–CaM affinity, a thorough evaluation of a Ca^{2+}–CaM titration is not feasible without knowing the concentration of Ca^{2+}–CaM binding sites. Nothing is yet known about the number of Ca^{2+}–CaM binding sites per cGMP-gated channel, or whether the binding is co-operative. This study examines these issues and discusses the results in terms of the subunit stoichiometry of the cGMP-gated channel and the physiological relevance of CaM regulation of the activity of cGMP-gated channels. A short account of this work has been given to the Biophysical Society (Bauer, 1996).

METHODS

Preparation of bovine rod outer segments

Cattle eyes were obtained from a local slaughter house shortly after the animal had been killed. During transport, the eyes were kept on ice in a light-tight box. Photoreceptor rod outer segments (ROS) were prepared as described earlier (Bauer, 1988) with the modifications mentioned in Bauer & Drechsler (1992). Aliquots of purified ROS containing 5 mg of rhodopsin were shock-frozen in liquid nitrogen and stored at −80 °C until use. The time elapsed from obtaining the eyes to shock-freezing the purified ROS was at most 6 h.

Preparations of hypotonically washed ROS membranes

All membrane preparations described in this and the following paragraphs were carried out in dim red light. To wash ROS at nominally zero free Ca^{2+}, ROS (1–6 aliquots of purified ROS containing 5 mg rhodopsin per aliquot) were hypotonically lysed at 4 °C for 15 min in 40 ml of a solution containing: 0·5 mM Hepes, 0·5 mM Tris, 1 mM dithiothreitol (DTT), 5 µg ml⁻¹ leupeptin (L2884, Sigma), 5 µg ml⁻¹ aprotinin (A1153, Sigma), 2·15 µg ml⁻¹ E-64 (E3132, Sigma) and 1 mM EDTA (pH 7·8; wash buffer). They were then pelleted by centrifugation for 30 min at 48 000 *g* at 4 °C. The washing procedure was repeated, then the pellet was resuspended in 0·5 ml per aliquot of a K-Hepes solution (10 mM Hepes–KOH (pH 7·4), 100 mM KCl, 1 mM DTT, protease inhibitors as before), equilibrated for 15 min at 4 °C, shock-frozen in liquid nitrogen and stored at −20 °C overnight before being used for vesicle preparation (see below).

Hypotonic washes at 0·1 mM Ca^{2+} were carried out as described above, except that the wash buffer contained 0·1 mM Ca^{2+} instead of 1 mM EDTA.

Preparation of Ca^{2+}-loaded fused ROS membrane vesicles

The washed and frozen ROS membranes were thawed and K-Hepes was added to give a final volume of 2 ml per 5 mg of rhodopsin. This freeze–thaw procedure in K-Hepes yielded fused ROS membranes, i.e. fusion between disc membranes and plasma membranes occurred, thus leading to the distribution of cGMP-gated channels and Na^+–Ca^{2+}–K^+ exchangers over virtually the whole membrane area (Bauer & Drechsler, 1992). A small volume of 200 mM $CaCl_2$ was added to give a final Ca^{2+} concentration of 10 mM. The suspension was gently homogenized by passing it through a hypodermic needle (0·9 mm diameter, 38 mm long) several times. Finally, the suspension was extruded, using low pressure, through a 1 µm Nuclepore filter (diameter, 13 mm; Reichelt, Heidelberg, Germany) followed by extrusion through a 0·4 µm Nuclepore filter (diameter, 13 mm); the pressure was controlled such that about one drop of the suspension was forced through the filter in about 5 s. The resulting vesicles will be termed fused ROS membrane vesicles.

Measurement of Ca^{2+} efflux

To study Ca^{2+} efflux from fused ROS membrane vesicles, external Ca^{2+} was removed by ion exchange using Chelex-100 resin (BioRad; mesh 200–400) converted into the K^+ form. Two different protocols for Ca^{2+} removal were used depending on the purpose of the experiment.

In the first protocol, to obtain high Ca^{2+} releases from the vesicles, the external Ca^{2+} of an aliquot of the stock suspension was removed immediately before the experiment by passage of the suspension through K-Chelex-100 of 1 ml bed volume, using a Swinnex-13 filter holder (Millipore) with a 3 µm-pore size Nuclepore filter. Arsenazo III (40 µM) and $CaCl_2$ (20 µM, final concentrations) were then added, and two stirring cuvettes (OS119·004, Hellma, Müllheim, Germany) were both filled with 1 ml of suspension for the sample and reference for double-beam spectroscopy at 652 nm (Cary 1, Varian, Melbourne, Australia). The addition of 20 µM $CaCl_2$ served to convert all the CaM into the Ca^{2+}-complexed form. If the effect of CaM on the Ca^{2+} flux was to be studied, CaM was added in the sample cuvette. The cuvette holders of the spectrophotometer were maintained at a constant temperature of 24 °C, and the cuvettes were allowed to equilibrate for 10 min before the measurement was started. The rhodopsin concentration had to be determined for each experiment because the percentage of vesicle recovery after the Chelex procedure (*ca* 80%) was variable.
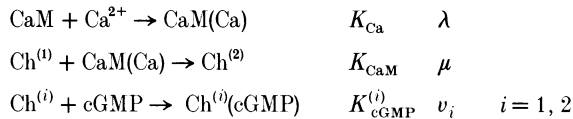
In the second protocol, to obtain more precise Ca^{2+} flux data as required for Ca^{2+}–CaM titration studies, the external Ca^{2+} was removed by passage of the whole stock suspension through a K-Chelex-100 column of 5 ml bed volume. The suspension was diluted with cold K-Hepes to give the final rhodopsin concentration (2–10 µM, depending on the experiment) and arsenazo III and $CaCl_2$ were added to final concentrations of 40 and 20 µM, respectively. The suspension was stored on ice until use. Since the Ca^{2+} leakage during the recording period (1 min) was minimal, no reference was used in these experiments. Trapped Ca^{2+} leaked out from these vesicles with a half-time of about 3 h. Therefore, the whole set of measurements for one titration was carried out within at most 2 h. As a control, Ca^{2+} fluxes upon adding a definite cGMP concentration (40 or 200 µM) were recorded at 30 min time intervals in each set of Ca^{2+} flux measurements. The Ca^{2+} leakage rate was determined from these controls as a single exponential of the Ca^{2+} fluxes *vs.* time. The Ca^{2+} fluxes of the titration measurements were then corrected using this leakage rate.

Initial Ca^{2+} fluxes were determined as the increase of the external Ca^{2+} concentration measured 3 s after initiating the release.

Quantitative evaluation of Ca^{2+}–CaM binding to cGMP-gated channels from cGMP-induced Ca^{2+} fluxes

In this section, the mathematical relations underlying the CaM-dependent reduction of the activity of cGMP-gated channels will be derived in order to evaluate the CaM titrations quantitatively.

Cyclic GMP-gated channels bind nanomolar concentrations of CaM in the Ca^{2+} complexed form (i.e. Ca^{2+}–CaM, which will be designated by CaM(Ca) in the following equations) thereby reducing the cGMP affinity of the channels. If $Ch^{(1)}$ and $Ch^{(2)}$ designate the CaM-free and Ca^{2+}–CaM-bound channel, respectively, this process is described by the following set of reactions:

$$CaM + Ca^{2+} \rightarrow CaM(Ca) \qquad K_{Ca} \qquad \lambda$$
$$Ch^{(1)} + CaM(Ca) \rightarrow Ch^{(2)} \qquad K_{CaM} \qquad \mu$$
$$Ch^{(i)} + cGMP \rightarrow Ch^{(i)}(cGMP) \qquad K_{cGMP}^{(i)} \quad \nu_i \qquad i = 1, 2$$

where the equilibrium constants and the Hill coefficients are noted on the right-hand side. If the Ca^{2+} concentration is high enough to saturate all Ca^{2+} binding sites of CaM the isotherm for CaM binding is given by:

$$\frac{[Ch^{(2)}]_{total}}{[Ch^{(1)}]_{total}} = \left(\frac{[CaM(Ca)]_{free}}{K_{CaM}}\right)^{\mu}, \qquad (1)$$

where the Hill coefficient (μ) may be greater than 1 if more than one Ca^{2+}–CaM binds per channel. The square brackets designate concentrations and the subscript 'total' indicates total concentrations, i.e. the sum of cGMP-free and cGMP-activated channels at a given cGMP concentration ($i = 1, 2$):

$$[Ch^{(i)}]_{total} = [Ch^{(i)}] + [Ch^{(i)}(cGMP)]. \qquad (2)$$

Ca^{2+}–CaM binding lowers the cGMP affinity of the channels, i.e.

$$[Ch^{(i)}(cGMP)] = [Ch^{(i)}]_{total}\left(1 + \left(\frac{K_{cGMP}^{(i)}}{[cGMP]_{free}}\right)^{\nu_i}\right)^{-1} \qquad (3)$$

($i = 1, 2$), with $K_{cGMP}^{(2)} > K_{cGMP}^{(1)}$. Because Ca^{2+} fluxes are monitored, only cGMP-activated channels are probed. With eqns (1) and (3), the ratio of Ca^{2+}–CaM-bound to CaM-free activated channels is given by:

$$\frac{[Ch^{(2)}(cGMP)]}{[Ch^{(1)}(cGMP)]} = \delta\left(\frac{[CaM(Ca)]_{free}}{K_{CaM}}\right)^{\mu}, \qquad (4)$$

where δ is the decrease of channel activity at a given cGMP concentration upon Ca^{2+}–CaM binding, i.e.

$$\delta = \left(1 + \left(\frac{K_{cGMP}^{(1)}}{[cGMP]_{total}}\right)^{\nu_1}\right) \Big/ \left(1 + \left(\frac{K_{cGMP}^{(2)}}{[cGMP]_{total}}\right)^{\nu_2}\right). \qquad (5a)$$

In this equation, the free cGMP concentration ($[cGMP]_{free}$) was replaced by the total cGMP concentration ($[cGMP]_{total}$) since $K_{cGMP}^{(1)}$ and $K_{cGMP}^{(2)}$ are more than three orders of magnitude greater than the concentration of cGMP binding sites. The strongest reduction of the channel activity (δ_{min}) is attained at very low cGMP concentrations:

$$\delta_{min} = (K_{cGMP}^{(1)})^{\nu_1}/(K_{cGMP}^{(2)})^{\nu_2}. \qquad (5b)$$

The free Ca^{2+}–CaM concentration is given by:

$$[CaM(Ca)]_{free} = [CaM(Ca)]_{total} - \gamma[B]_{total}, \qquad (6)$$

where $[B]_{total}$ is the total concentration of externally accessible Ca^{2+}–CaM binding sites, and γ denotes the fraction of occupied Ca^{2+}–CaM binding sites:

$$\gamma = [Ch^{(2)}]_{total}/([Ch^{(1)}]_{total} + [Ch^{(2)}]_{total}). \qquad (7)$$

In flux measurements, the fractional decrease of the Ca^{2+} flux (J), which is due to Ca^{2+}–CaM binding to the channel,

$$\alpha = (J_{max} - J)/(J_{max} - J_{min}), \qquad (8)$$

is primarily determined. Since the Ca^{2+} fluxes are proportional to the total concentration of activated channels (i.e. the sum of Ca^{2+}–CaM-bound and CaM-free activated channels) it can be readily shown, using eqn (3), that γ and α are equal, i.e.

$$(J_{max} - J)/(J_{max} - J_{min}) = [Ch^{(2)}]_{total}/([Ch^{(1)}]_{total} + [Ch^{(2)}]_{total}). \quad (9)$$

Therefore, the ratio of Ca^{2+}–CaM-bound to CaM-free activated channels is:

$$\frac{[Ch^{(2)}(cGMP)]}{[Ch^{(1)}(cGMP)]} = \delta\frac{\alpha}{1 - \alpha}, \qquad (10)$$

and eqn (4) transforms into:

$$\alpha/(1 - \alpha) = (K_{CaM})^{-\mu}([CaM(Ca)]_{total} - \alpha[B]_{total})^{\mu}. \qquad (11)$$

As one would expect intuitively, this equation no longer contains the cGMP concentration, since it could be reduced by the factor of δ. It should be noted, however, that the Ca^{2+}–CaM affinity of the closed and the cGMP-activated channel might be different. In this case, the above equations still hold for a given [cGMP] but K_{CaM} becomes a function of the [cGMP]. There is, however, no indication of such an effect from the experiments reported here (see Discussion).

For rod cGMP-gated channels, $[B]_{total}$ is normally equal to or greater than K_{CaM}. For this reason, both terms in the bracket of eqn (11) are similar in magnitude, and the total Ca^{2+}–CaM concentration for half-maximal binding (EC_{50}^{CaM}) and $[B]_{total}$ are linearly correlated:

$$EC_{50}^{CaM} = K_{CaM} + \tfrac{1}{2}[B]_{total}. \qquad (12)$$

This means that $[B]_{total}$ is high enough to exert a CaM buffering effect. $[B]_{total}$ is proportional to the rhodopsin concentration ($[Rh]_{total}$). If r represents the molar ratio of rhodopsin and Ca^{2+}–CaM binding sites ($r = [Rh]_{total}/[B]_{total}$) eqn (12) can be written as:

$$EC_{50}^{CaM} = K_{CaM} + (1/2r)[Rh]_{total}. \qquad (13)$$

It is obvious from eqn (13) that the values of EC_{50}^{CaM} and of $[Rh]_{total}$ are linearly correlated. A linear regression yields the dissociation constant for Ca^{2+}–CaM binding (K_{CaM}) as the intersect of the straight line with the ordinate. The concentration of binding sites ($[B]_{total}$) can be calculated from the slope of this linear regression. Plotting the Ca^{2+}–CaM titration data according to eqn (11) on a double-logarithmic scale yields the Hill coefficient (μ) for Ca^{2+}–CaM binding.

Dissociation of the endogenous Ca^{2+}-binding protein and of CaM from the cGMP-gated channel

To study the dissociation of the endogenous Ca^{2+}-binding protein from cGMP-gated channels at low free Ca^{2+} concentrations, the ROS were hypotonically washed, as follows. Equal amounts of purified ROS were hypotonically lysed, as described above, for 15 min at 4 °C in the presence of 0·1 mM Ca^{2+} and pelleted by centrifugation for 30 min at 48 000 *g* at 4 °C. Each pellet was then equilibrated for 30 min at 4 °C in 40 ml of one of the following hypotonic Ca^{2+} buffers containing (mM): 10 Hepes–KOH, 100 KCl, 1 DTT, protease inhibitors as before, 0·5 EGTA and 0·5 *N*-hydroxyethylethylene-diaminetriacetic acid (HEDTA), and one of the following total Ca^{2+} concentrations: 16·6 μM (5 nM), 32·3 μM

(10 nM), 61·2 μM (20 nM), 87·4 μM (30 nM), 133·1 μM (50 nM), and 220 μM (100 nM) $CaCl_2$ (pH 7·4). Given in parentheses are the free Ca^{2+} concentrations as calculated at 4 °C for an ionic strength of 120 mM, using the procedure of Schoenmakers *et al.* (Schoenmakers, Visser, Flik & Theuvenet, 1992) with the stability constants of Sillén & Martell (1964). After equilibration, the membranes were centrifuged for 30 min at 48000 g at 4 °C, resuspended in 0·5 ml K-Hepes per 5 mg rhodopsin, equilibrated for 15 min, shock-frozen in liquid nitrogen and stored at −20 °C.

To probe the dissociation of CaM from the cGMP-gated channels, the ROS membranes were washed twice in a virtually zero free Ca^{2+} solution, as described above. The pellet was then resuspended in 5 ml K-Hepes containing 1 mM Ca^{2+}, and the amount of rhodopsin determined spectrometrically (using a differential extinction coefficient between unbleached and bleached rhodopsin of 40000 cm^{-1} M^{-1} at 500 nm). A stoichiometric amount of CaM (i.e. 1 mol of CaM per 720 mol of rhodopsin; see Results) was added, and the suspension equilibrated for 15 min at 4 °C. Equal aliquots of about 0·5 ml were then centrifuged in Eppendorf tubes for 25 min at 18320 g at 4 °C. The pellets were equilibrated for 30 min at 4 °C in 40 ml of one of the above noted Ca^{2+} buffers, pelleted and shock-frozen, as described in the previous paragraph.

After thawing the pellets, vesicles were prepared with each pellet, as described above. The fraction of unoccupied Ca^{2+}–CaM binding sites of the cGMP-gated channels in the different membrane preparations was assayed using the Ca^{2+}–CaM-dependent reduction of the Ca^{2+} flux upon activation with 40 μM cGMP.

Quantitative evaluation of the dissociation of CaM and the endogenous Ca^{2+}-binding protein from the cGMP-gated channel

If the free Ca^{2+} concentration is buffered to low values, the coupled thermodynamic equilibria of (1) Ca^{2+} binding to CaM, (2) Ca^{2+}–CaM binding to the channel, and (3) cGMP binding to the channel have to be considered. In solution, the concentration of Ca^{2+}–CaM is given by the Hill equation:

$$[CaM(Ca)] = [CaM]_{total}\left(1 + \left(\frac{K_{Ca}}{[Ca]_{free}}\right)^{\lambda}\right)^{-1}. \qquad (14)$$

Only Ca^{2+}–CaM binds to the channel. In the presence of Ca^{2+}–CaM binding sites, eqn (14) still holds for the concentration of free Ca^{2+}–CaM, according to the principle of detailed balancing, provided that the total CaM concentration is replaced by the total concentration of unbound CaM. For CaM concentrations which are much higher than the concentration of Ca^{2+}–CaM binding sites, the unbound CaM concentration is approximately equal to $[CaM]_{total}$. If the $[Ca^{2+}]_{free}$ is buffered to a definite value, the fractional decrease of the Ca^{2+} flux (α) is given according to eqns (4) and (10) as follows:

$$\frac{\alpha}{1-\alpha} = \left(\frac{[CaM]_{total}}{K_{CaM}}\right)^{\mu} \Big/ \left(1 + \left(\frac{K_{Ca}}{[Ca]_{free}}\right)^{\lambda}\right)^{\mu}. \qquad (15)$$

The free Ca^{2+} concentration, where half of the Ca^{2+}–CaM binding sites are occupied (EC_{50}^{Ca}), is obtained from eqn (15),

$$EC_{50}^{Ca} = K_{Ca}\left(\frac{K_{CaM}}{[CaM]_{total} - K_{CaM}}\right)^{1/\lambda}, \qquad (16a)$$

which for $[CaM]_{total} \gg K_{CaM}$ becomes:

$$EC_{50}^{Ca} = K_{Ca}\left(\frac{K_{CaM}}{[CaM]_{total}}\right)^{1/\lambda}. \qquad (16b)$$

Therefore, the value of EC_{50}^{Ca} depends explicitly on $[CaM]_{total}$.

RESULTS

Ca^{2+}–CaM-induced shift of the cGMP affinity of cGMP-gated channels

In qualitative agreement with previous reports on cGMP-gated channels of human rods (Chen *et al.* 1994), frog rods (Gordon, Downing-Park & Zimmerman, 1995; Nakatani, Koutalos & Yau, 1995) and bovine rods (Hsu & Molday, 1993, 1994; Körschen *et al.* 1995), the activity of cGMP-gated channels in fused ROS membrane vesicles (see Methods) decreased markedly in the presence of Ca^{2+}–CaM. The addition of a saturating concentration of Ca^{2+}–CaM (600 nM) resulted in a shift of the cGMP concentrations for half-maximal channel activation from 36·8 ± 2 μM ($n = 5$) to 55·6 ± 5·2 μM ($n = 4$). Ca^{2+}–CaM binding did not change the Hill coefficients which were 2·15 ± 0·2 ($n = 5$) and 2·23 ± 0·27 ($n = 4$) in the absence and presence of Ca^{2+}–CaM, respectively. Using an average Hill coefficient of 2·2, a maximal reduction of the cGMP-induced Ca^{2+} flux of 40 % is obtained with the above K_{cGMP} values according to eqn (5b) for low cGMP concentrations.

The advantage in using fused ROS membrane vesicles is that cGMP stimulates an almost ten times greater Ca^{2+} signal in these vesicles than in non-fused ROS membrane vesicles (Bauer & Drechsler, 1992). This is because the fusion of disc and plasma membranes leads to the distribution of the cGMP-gated channels over a much larger membrane area; in native ROS, cGMP-gated channels are restricted exclusively to the plasma membrane (Bauer, 1988; Cook, Molday, Reid, Kaupp & Molday, 1989). The maximal difference in the cGMP-induced Ca^{2+} fluxes due to CaM occurred between 40 and 50 μM cGMP. Therefore, channel activation in this cGMP concentration range was most suited to study more closely the influence of CaM on the channel activity. At 40 μM, the reduction of the channel activity was found to be maximally 55 %, in good agreement with the value obtained from eqn (5a).

Ca^{2+} efflux from fused ROS vesicles at varying Ca^{2+}–CaM concentrations

The decrease of the Ca^{2+} efflux from fused ROS membrane vesicles with increasing Ca^{2+}–CaM concentrations was already evident in the nanomolar range. Figure 1 shows an example where the Ca^{2+}–CaM concentration for a half-maximal reduction of the Ca^{2+} efflux (EC_{50}^{CaM}) was 3 nM. This value is about 60 % greater than the value of 1·85 nM reported by Hsu & Molday (1994). In other experiments, values of EC_{50}^{CaM} near 2 nM were also determined. Actually, this value turned out not to be constant: in different sets of experiments, values of EC_{50}^{CaM} were obtained between 1 and ∼5 nM.

This variability can be understood by recalling that in these experiments the concentration of Ca^{2+}–CaM binding sites is probably in the nanomolar range which is the concentration range of the dissociation constant for Ca^{2+}–CaM binding. If the Ca^{2+}–CaM dissociation constant is similar or less than

Figure 1. Decrease of cGMP-induced Ca²⁺ efflux with increasing CaM concentrations

Ca^{2+} efflux induced from fused ROS membrane vesicles by adding 51 μM cGMP in the presence of the following CaM concentrations (nM): 0 (*a*), 0·75 (*b*), 1·45 (*c*), 4·46 (*d*), 8·85 (*e*), and 23·0 (*f*). The reduction of the Ca^{2+} flux measured in 23 nM CaM (*f*) is almost saturating because at 51 μM cGMP, according to eqn (5a) with the parameters given above ($K_{cGMP}^{(1)}= 36\cdot8$ μM, $K_{cGMP}^{(2)} = 55\cdot6$ μM, $\mu = 2\cdot2$) the maximal CaM-induced reduction of the Ca^{2+} flux is 67%. Ca^{2+} was removed with K-Chelex-100 immediately before the measurement. Rhodopsin, 2·8 μM; spectral sensitivity to Ca^{2+} of arsenazo III at 652 nm, 0·013 μM^{-1}.
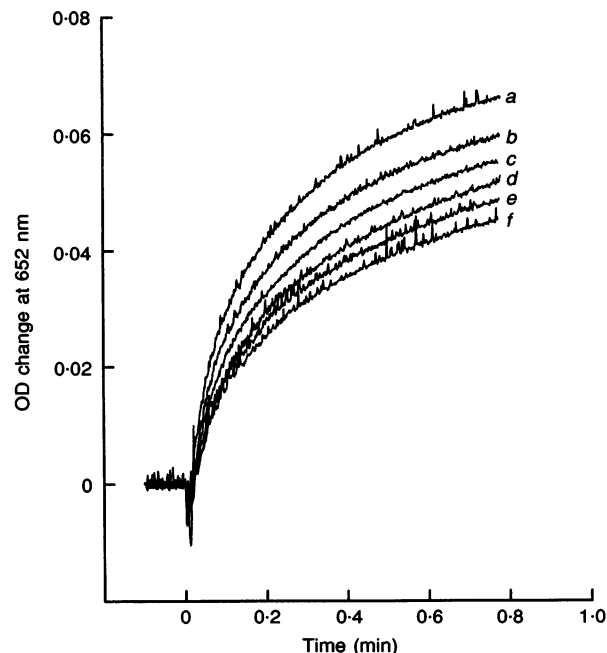
the concentration of binding sites, the latter is expected to enter the magnitude of the value of EC_{50}^{CaM} (see Methods, eqn (12)).

Figure 2 shows that the value of EC_{50}^{CaM} depends clearly on the concentration of membrane proteins as measured by the concentration of rhodopsin present: in the presence of 8·9 μM rhodopsin, the value of EC_{50}^{CaM} was 4·1 nM Ca^{2+}–CaM, whereas at 2·9 μM rhodopsin, half-maximal reduction of activity was already reached at only 2 nM Ca^{2+}–CaM. This difference cannot be due to different external Ca^{2+} concentrations since 20 μM Ca^{2+} was present in both titrations; that is sufficient Ca^{2+} to drive all CaM into the Ca^{2+}-complexed state. Also, protease inhibitors were present throughout these experiments to exclude the possibility that partial loss of Ca^{2+}–CaM binding sites occurred due to proteolysis.

Obviously, the observed EC_{50}^{CaM} depends on the concentration of Ca^{2+}–CaM binding sites. This means that the Ca^{2+}–CaM affinity constant cannot be determined without knowledge of the concentration of Ca^{2+}–CaM binding sites.

Determination of the affinity and the density of Ca^{2+}–CaM binding sites

If the Ca^{2+}–CaM binding constant is equal to or lower than the concentration of Ca^{2+}–CaM binding sites present in the titrated sample a linear relationship between the observed value of EC_{50}^{CaM} and the rhodopsin concentration is expected (Methods, eqn (13)). In fact, plotting the values of EC_{50}^{CaM} as determined in different experiments against the rhodopsin concentration yields a linear relationship (Fig. 3). The Ca^{2+}–CaM binding affinity of the cGMP-gated channel is obtained from Fig. 3 by linear extrapolation to zero rhodopsin concentration, $K_{CaM} = 1\cdot01 \pm 0\cdot2$ nM ($n = 22$).
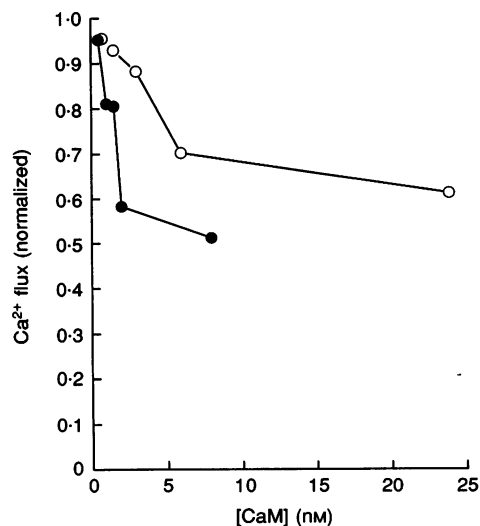
Figure 2. Buffering effect of cGMP-gated channels on Ca²⁺–CaM binding

The reduction of the channel activity with increasing CaM concentration was measured as the initial Ca^{2+} efflux from fused ROS membrane vesicles upon addition of 40 μM cGMP. The Ca^{2+} fluxes were normalized by dividing them by the maximal Ca^{2+} efflux which was measured in the absence of CaM. The rhodopsin concentrations were 8·9 (O) and 2·9 μM (●).
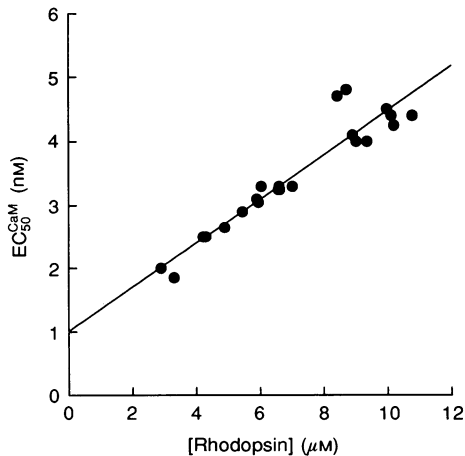
Figure 3. Dependence on the rhodopsin concentration of the half-maximal CaM blockage

Values of half-maximal CaM-dependent reduction of the cGMP-gated channel activity (EC_{50}^{CaM}) were determined for different concentrations of fused ROS membrane vesicles. Plotting these values against the rhodopsin concentrations present in the CaM titrations yields a straight line as expected from eqn (13). The linear regression line intersects the ordinate at $1\cdot01 \pm 0\cdot20$ nM and has a slope of $(3\cdot48 \pm 0\cdot26) \times 10^{-4}$. To titrate the CaM effect on the channel activity, the channels were activated with 40 μM cGMP. The external $[Ca^{2+}]$ was 25 μM. The protease inhibitors aprotinin (5 μg ml^{-1}), leupeptin (5 μg ml^{-1}) and E-64 (2$\cdot$15 μg ml^{-1}) were present throughout all procedures.

The dependence of the value of EC_{50}^{CaM} on the concentration of Ca^{2+}–CaM binding sites yields the molar ratio of rhodopsin to accessible Ca^{2+}–CaM binding sites in the vesicle preparation. Using eqn (13), the molar ratio of rhodopsin to externally accessible Ca^{2+}–CaM binding sites (r) was determined to be 1439 ± 109 ($n = 22$).

Based on this value, the density of Ca^{2+}–CaM binding sites in the plasma membrane of the rod photoreceptor can be estimated. The ROS membrane vesicles used in this study

consisted of fused disc and plasma membranes where the membrane proteins were distributed over the whole membrane area. It has been shown previously that inside-out and right-side-out orientation of the channel occur in these fused ROS membrane vesicles with about equal frequency (Bauer & Drechsler, 1992). Therefore, the cytoplasmic side of only half of the channels should be accessible on the external surface of vesicles, and there are approximately 720 rhodopsin molecules per Ca^{2+}–CaM
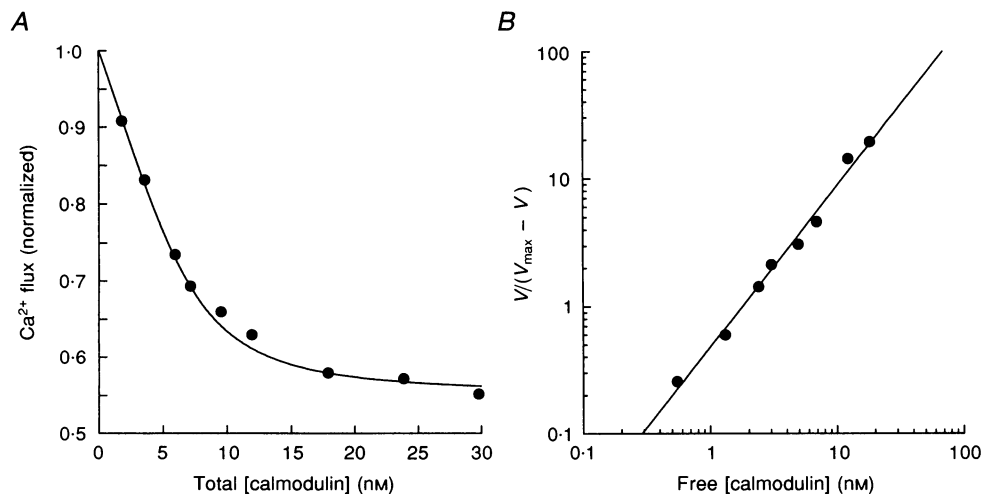

Figure 4. Hill analysis of the CaM-dependent reduction of channel activity

A, titration of the decrease of the channel activity with increasing [CaM]. To detect possible co-operative binding of Ca^{2+}–CaM to the channel, the transition to saturation of the CaM effect was more carefully traced in this experiment. The channel was activated with 40 μM cGMP. External Ca^{2+} was removed by passage of the fused ROS membrane vesicles through a K-Chelex-100 column. Ca^{2+} flux values were corrected for passive Ca^{2+} leakage as described in Methods. The same protease inhibitors as in Fig. 3 were present. Rhodopsin concentration, 8$\cdot$72 μM; spectral sensitivity to Ca^{2+} of arsenazo III at 652 nm, 0$\cdot$0073 μM^{-1}. The curve was plotted with the parameters obtained from the Hill plot shown in *B*. *B*, Hill plot of the CaM-dependent reduction of channel activity shown in *A*. The fraction of occupied Ca^{2+}–CaM binding sites (α) was determined as the ratio of the reduction of channel activity at a given [CaM] to the maximal CaM-dependent reduction of channel activity. Using the molar ratio of rhodopsin to accessible Ca^{2+}–CaM binding site of 1439 (as determined from the slope of the straight line in Fig. 3), the concentration of Ca^{2+}–CaM binding sites ($[B]_{total}$) was calculated to be 6$\cdot$06. The concentration of free Ca^{2+}–CaM was calculated as $[CaM(Ca)]_{total} - \alpha[B]_{total}$. The Hill plot yields an equilibrium constant of 1$\cdot$72 nM and the Hill coefficient of 1$\cdot$36.

binding site in ROS membranes. Since the density of rhodopsin molecules is about 25 000 molecules μm^{-2} (Daemen, 1973) this means that there are 35 Ca^{2+}–CaM binding sites μm^{-2} in fused ROS membranes. Recalling that the cGMP-gated channels are restricted exclusively to the plasma membrane of the ROS, i.e. to about one-sixteenth of the total membrane area for bovine ROS (Bauer, 1988), the density of Ca^{2+}–CaM binding sites in the native plasma membranes is 560 binding sites μm^{-2}.

The Ca^{2+}–CaM binding is co-operative

If the concentration of Ca^{2+}–CaM binding sites is greater than the dissociation constant for Ca^{2+}–CaM binding – as is the case in these experiments – most Ca^{2+}–CaM is bound at low total CaM concentrations irrespective of co-operativity. A measurable difference between a co-operative and a non-co-operative binding isotherm is only expected at near-saturating concentrations of Ca^{2+}–CaM where most of the Ca^{2+}–CaM binding sites are occupied. Therefore, this concentration range was carefully investigated. Furthermore, to determine the Hill coefficient, the ratio of occupied to non-occupied Ca^{2+}–CaM binding sites had to be plotted against the free Ca^{2+}–CaM concentration (Hill plot). In the nanomolar range, the free Ca^{2+}–CaM concentration is less than 50 % of the total Ca^{2+}–CaM concentration due to Ca^{2+}–CaM binding. However, since the total concentration of accessible Ca^{2+}–CaM binding sites can be determined from the rhodopsin concentration, the free Ca^{2+}–CaM concentration was calculated from eqn (6). Figure 4 shows such an experiment which yields a Hill coefficient (μ) of 1·36 for Ca^{2+}–CaM binding to the cGMP-gated channel. Averaging four similar experiments, a Hill coefficient (μ) of 1·36 ± 0·15 (S.D.) was obtained, which indicates a slightly positive co-operativity for Ca^{2+}–CaM binding to the channel.

Endogenous modulatory Ca^{2+}-binding protein associated with the channel

The Ca^{2+}–CaM-dependent decrease of the cGMP-gated channel activity was only observed with ROS membranes which were hypotonically washed at virtually zero Ca^{2+} (1 mM EDTA). If, however, the membranes were hypo-

tonically washed in the presence of 100 μM Ca^{2+}, no influence of Ca^{2+}–CaM on the cGMP-induced Ca^{2+} flux was found. Washing the membranes in 1 mM EDTA after a hypotonic wash in 100 μM Ca^{2+} restored the CaM-dependent reduction of the cGMP-induced Ca^{2+} flux. The cGMP concentration inducing half-maximal Ca^{2+} flux from vesicles of Ca^{2+}-washed ROS membranes was 51·4 μM, and the Hill coefficient was 2·35 (Fig. 5). Compared with vesicles of EDTA-washed ROS membranes (K_{cGMP} = 36·9 μM; Hill coefficient, 2·52), the dose–response curve of Ca^{2+}-washed vesicles was shifted to higher cGMP concentrations by 14·7 μM. These results strongly suggest that an endogenous Ca^{2+}-binding protein has been washed off the cGMP-gated channels at low Ca^{2+} concentrations.

The detachment of this protein from the bovine cGMP-gated channel has been studied with ROS membranes which were washed in various buffered free Ca^{2+} concentrations at rhodopsin concentrations of 4 μM. The membranes were then resuspended in 100 μM Ca^{2+}, freeze–thawed and extruded in 10 mM Ca^{2+} to obtain Ca^{2+}-containing vesicles (see Methods). The ratio of cGMP-induced Ca^{2+} fluxes with and without Ca^{2+}–CaM was taken as a direct measure of the fraction of Ca^{2+}–CaM binding sites which still contained the endogenous Ca^{2+}-binding protein after the wash procedure (Fig. 6, ●).

As expected, this ratio equals 1 for membranes washed in approximately micromolar free Ca^{2+}. This means that all Ca^{2+}–CaM binding sites are occupied in these membranes. A half-maximal Ca^{2+}–CaM-dependent reduction of the Ca^{2+} efflux is only observed if the ROS were washed in 20–30 nM free Ca^{2+}. This is a considerably lower free Ca^{2+} concentration than one would expect from eqns (15) and (16a) if the Ca^{2+} affinity (K_{Ca}) of 2·4 μM of free CaM (Huppertz *et al.* 1990) is used (Fig. 6, dashed line). The continuous line, which fits the experimental data reasonably well, was obtained with a value of K_{Ca} of 120 nM. This analysis suggests that the Ca^{2+} affinity of CaM increases 20-fold after the CaM binds to the channel.

The dissociation of Ca^{2+}–CaM from the cGMP-gated channels could be studied in a similar way, as follows. The

Figure 5. Dose–response curves of cGMP-induced Ca^{2+} fluxes in the presence and absence of the endogenous Ca^{2+}-binding protein

Initial Ca^{2+} fluxes from fused ROS membrane vesicles which were washed in 1 mM EDTA (O) and in 0·1 mM Ca^{2+} (●) before freeze–thawing. The data were normalized to the maximal cGMP-induced Ca^{2+} fluxes elicited with 205 μM cGMP. The curves were plotted with the equilibrium constants (K_{cGMP}) of 36·9 and 51·4 μM, and Hill coefficients of 2·52 and 2·35 for EDTA- and Ca^{2+}-washed ROS membranes, respectively.
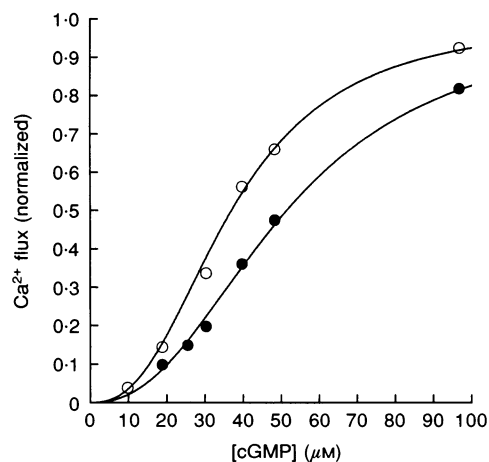
ROS membranes were washed in 1 mM EDTA. A stoichiometric amount of CaM was then added in the presence of 1 mM Ca^{2+}. The ROS membranes were then washed at various free Ca^{2+} concentrations, as before. With these membranes a half-maximal CaM effect was again only observed after a hypotonic wash in 20–30 nM free Ca^{2+} (Fig. 6, ○). There was no difference between endogenous Ca^{2+}-binding protein and CaM in the critical free Ca^{2+} concentration of detachment from the channel.

DISCUSSION

Ca^{2+}–CaM binding to the cGMP-gated channels of bovine rod photoreceptor membranes was found to result in a shift of the titration curve by about 18 μM to higher cGMP concentrations; this finding is in agreement with previously published data (Hsu & Molday, 1993). Although this moderate modification of the channel-gating sensitivity is unlikely to play a major part in the light adaptation of the rod photoreceptor (Lagnado & Baylor 1994; Nakatani et al. 1995), it is very interesting for several reasons: (1) the reduction by Ca^{2+}–CaM of the cGMP affinity of cGMP-gated channels is possibly a common feature of many (if not all) cGMP-gated channels; (2) in recombinant cGMP-gated channels of olfactory cells, the CaM effect is much more pronounced than in rod photoreceptor channels (Chen & Yau, 1994), suggesting that in these cells CaM regulation of the channel activity may play an important role; and (3) since the Ca^{2+}–CaM binding site of the rod cGMP-gated

channel is located on the β-subunit (Chen et al. 1994; Körschen et al. 1995), the CaM effect can also be used to study the subunit composition of the rod channel in greater detail.

The decrease of the channel activity by CaM was used to examine Ca^{2+}–CaM binding. The Ca^{2+}–CaM concentration for half-maximal reduction of the channel activtity (EC_{50}^{CaM}) is generally greater than the correct dissociation constant, (K_{CaM}) because the concentration of Ca^{2+}–CaM binding sites ($[B]_{total}$) is usually greater than K_{CaM}. Extrapolation to infinite dilution according to eqn (13) yielded the dissociation constant (K_{CaM}) of 1.01 ± 0.2 nM for Ca^{2+}–CaM binding to the rod cGMP-gated channel. This value of K_{CaM} agrees well with the value of 1.85 nM reported by Hsu & Molday (1994) although the two values were determined after different channel activations, namely 40 μM cGMP (this study) and 12 μM cGMP (Hsu & Molday, 1994). The somewhat smaller value reported here is probably due to the correction according to eqn (13) and not to a noticeable dependence of K_{CaM} on the cGMP concentration (see Methods). These values are, however, more than an order of magnitude smaller than the dissociation constant reported for Ca^{2+}–CaM binding to the recombinant olfactory cAMP/cGMP-gated channel (Chen & Yau, 1994).

Considering this low value of K_{CaM}, it appears unlikely that there are still other Ca^{2+}–CaM binding sites in these membranes because in that case the correct K_{CaM} would be even lower. In agreement with this conclusion, Hsu &
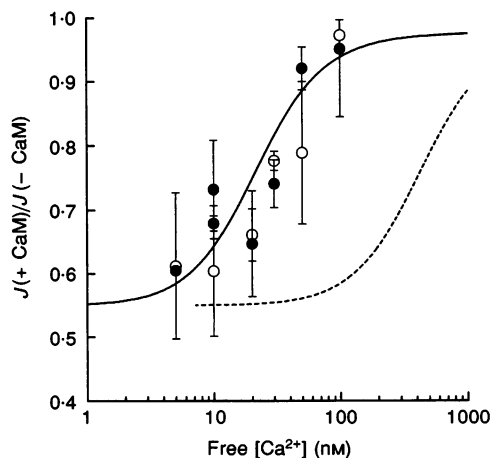

Figure 6. Detachment of the endogenous factor and of CaM from the ROS membranes by lowering the free Ca^{2+} concentration

Ratio of Ca^{2+} fluxes in the presence of 60 nM CaM, and the absence of CaM upon addition of 40 μM cGMP plotted *versus* the free Ca^{2+} concentration at which the ROS membranes were hypotonically washed before preparation of fused ROS membrane vesicles. Before exposure to the Ca^{2+} buffers, the ROS were either hypotonically washed in 0·1 mM Ca^{2+} (●) or in 1 mM EDTA followed by addition of a stoichiometric amount of CaM (○). The Ca^{2+} buffers contained 10 mM Hepes–KOH (pH 7·4), 100 mM KCl, 0·5 mM EGTA, 0·5 mM HEDTA, the protease inhibitors noted in Fig. 3, in addition to 16·6 μM (5 nM), 32·3 μM (10 nM), 61·2 μM (20 nM), 87·4 μM (30 nM), 133·1 μM (50 nM), and 220 μM (100 nM) $CaCl_2$; the free Ca^{2+} concentrations as calculated for an ionic strength of 120 mM at 4 °C are given in parentheses (for details see Methods). The mean rhodopsin concentration was 6·6 μM. The sigmoidal curves are plotted using eqn (15) with the parameters: $[CaM]_{total} = 9\cdot4$ nM; the Hill coefficients, $\lambda = 1\cdot3$, $\mu = 1\cdot3$; the equilibrium constants, $K_{CaM} = 1$ nM, and $K_{Ca} = 120$ nM (continuous line) and 2·4 μM (dashed line), respectively.

Molday (1994) reported only trace amounts of Ca^{2+}–CaM binding proteins besides the 240 kDa protein (β-subunit) in purified bovine plasma membranes, and no Ca^{2+}–CaM binding proteins in purified bovine disc membranes.

Owing to the high affinity of the rod cGMP-gated channel to Ca^{2+}–CaM, the molar ratio of rhodopsin to externally accessible Ca^{2+}–CaM binding sites (r) was determined to be 1439 ± 109 (see Results), which led to an estimated density of 560 Ca^{2+}–CaM binding sites μm^{-2} on the plasma membrane of the ROS. This value is twice as great as the density of cGMP-gated channels of 270 molecules μm^{-2} that has previously been estimated from Ca^{2+} flux experiments (Bauer & Drechsler, 1992), suggesting that there are actually two Ca^{2+}–CaM binding sites on each cGMP-gated channel in rod photoreceptors. This inference is further supported by the finding that the Ca^{2+}–CaM binding is slightly co-operative (Hill coefficient, $\mu = 1.36 \pm 0.15$; see Results). Assuming that there is only one Ca^{2+}–CaM binding site per β-subunit, this means that there are two β-subunits associated with each cGMP-gated channel of the rod photoreceptor.

Ca^{2+}–CaM modulated the channel activity only if the ROS membranes were hypotonically washed at zero Ca^{2+} but not if only washed in 0·1 mM Ca^{2+}. This means that there must be some Ca^{2+}-binding protein associated with the channel which can only be washed off at very low Ca^{2+} concentrations (see Fig. 6). Is CaM this endogenous factor?

All of the presently available data show that this is most probably the case. Hsu & Molday (1994) extracted a Ca^{2+}-binding protein from purified ROS plasma membranes which had the same molecular mass as bovine brain CaM. Moreover in the present study, it was found that CaM shifts the K_{cGMP} by about 15 μM to higher values. A similar shift is also observed for ROS membranes washed in 0·1 mM Ca^{2+} (Fig. 5), i.e. for membranes which still contain the endogenous factor. The dissociation from the cGMP-gated channel of the endogenous factor and of CaM occurs at the same free Ca^{2+} concentration (Fig. 6). Together, these results provide compelling evidence that the endogenous factor is most probably CaM.

This inference is further supported by the following consideration. As mentioned above, the CaM effect is completely absent with Ca^{2+}-washed ROS membranes. If, therefore, CaM is the endogenous factor, there must be at least as many CaM molecules in the cell as there are Ca^{2+}–CaM binding sites. In this study, it was estimated that there are 720 rhodopsin molecules per Ca^{2+}–CaM binding site. Kohnken *et al.* reported that the molar ratio of rhodopsin to CaM in bovine ROS is 700 (Kohnken, Chafouleas, Eadie, Means & McConnell, 1981). This means that there are identical numbers of CaM molecules and Ca^{2+}–CaM binding sites in the ROS. Recalling that the Ca^{2+}–CaM affinity of the cGMP-gated channel is very high, these data suggest that in bovine ROS, CaM is presumably permanently bound to the channel (see below).

The Ca^{2+}–CaM effect on the channel activity becomes noticeable already at nanomolar concentrations of free Ca^{2+}; a half-maximal effect (EC_{50}^{Ca}) has been observed at a $[Ca^{2+}]_{free}$ of about 70 nM (Hsu & Molday, 1993). On the other hand, CaM binds Ca^{2+} in the micromolar range. Under the experimental conditions of this study, a Ca^{2+} dissociation constant (K_{Ca}) of 2·4 μM has previously been determined for dissolved CaM (Huppertz, Weyand & Bauer, 1990), in agreement with an earlier report (Dedman, Potter, Jackson, Johnson & Means, 1977). The $[Ca^{2+}]_{free}$ at which half of the CaM is detached from the channel (EC_{50}^{Ca}) is given by eqns (16a and b). It should be stressed that, according to this equation, the observed value of EC_{50}^{Ca} is not constant but is rather strongly dependent on $[CaM]_{total}$. Since K_{CaM} for the rod cGMP-gated channel is 1 nM (see Results) the value of EC_{50}^{Ca} is – depending on the Hill coefficient (λ) – for e.g. 100 nM CaM, more than an order of magnitude smaller than K_{Ca} for Ca^{2+} binding to CaM. The value of λ for Ca^{2+} binding to CaM has been determined to be 1·3 (Crouch & Klee, 1980).

For the experiment shown in Fig. 6, the value of EC_{50}^{Ca} should be about 500 nM according to eqn (16b). However, the binding curve calculated with these parameters using eqn (15) is far off from the measured data (Fig. 6, dashed line). To obtain a reasonable fit, the Ca^{2+} affinity of the bound CaM had been taken to be twenty times higher than in solution (Fig. 6, continuous line). This evaluation suggests that the Ca^{2+} affinity of Ca^{2+}–CaM bound to the rod cGMP-gated channel is markedly increased, thereby shifting the value of EC_{50}^{Ca} to much lower values than predicted from eqn (16b) (Fig. 6, dashed line). Similar increases in the Ca^{2+} affinity of Ca^{2+}–CaM upon binding to its target have been found for other CaM binding proteins as well (Crivici & Ikura, 1995).

Ca^{2+}–CaM binding and unbinding has been suggested to constitute a modulatory mechanism for the activation of cGMP-gated channels (Hsu & Molday, 1993). Is this process likely to occur in the bovine rod photoreceptor? It has been reported here that (1) CaM detaches from the channel at a free Ca^{2+} concentration of about 20 nM (Fig. 6), and (2) that the dissociation of CaM from the channel depends strongly on the concentration of CaM present. In bovine ROS, the molar ratio of rhodopsin to CaM is 700 (Kohnken *et al.* 1981), the rhodopsin concentration in ROS is about 2·5 mM (Daemen, 1973), and therefore the total intracellular concentration of CaM is about 3·6 μM. Using this value for the $[CaM]_i$, a value for λ of 1·3 (Crouch & Klee, 1980) and a K_{Ca} of 2·4 μM (Huppertz *et al.* 1990), the EC_{50}^{Ca} is calculated from eqn (16b) to be 4·4 nM. This value constitutes an upper limit because the Ca^{2+} dissociation constant of bound Ca^{2+}–CaM is probably twenty times lower than that of free Ca^{2+}–CaM (see Results), and according to eqn (16b), EC_{50}^{Ca} should decrease by the same factor.

These calculated values of EC_{50}^{Ca} in the photoreceptor are most probably much smaller than the intracellular Ca^{2+}
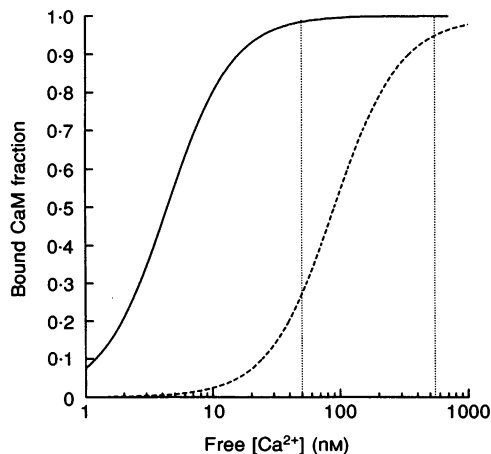
Figure 7. Theoretical Ca^{2+}–CaM binding isotherms

CaM binding curves according to eqn (15) are plotted for K_{CaM} values of 1 nM (continuous line) and 50 nM (dashed line) and a $[CaM]_i$ of 3·6 μM. The Ca^{2+} binding constant and Hill coefficient for free CaM of 2·4 μM and 1·3, respectively, were used. The Hill coefficient for Ca^{2+}–CaM binding to the bovine cGMP-gated channel of 1·3 (this paper) was taken. The dotted vertical lines indicate the concentration range from 50 to 550 nM of cytoplasmic free Ca^{2+} found in gecko photoreceptors (Gray-Keller & Detwiler, 1994).

concentration. For gecko photoreceptors, the cytoplasmic Ca^{2+} concentration has been determined to be 550 nM in the dark, dropping in the light at the most to 50 nM (Gray-Keller & Detwiler, 1994). These considerations suggest that under physiological conditions the intracellular CaM is permanently bound to cGMP-gated channels (Fig. 7, continuous line). In agreement with this inference, the ratio of rhodopsin to CaM binding sites reported here is equal to the molar ratio of rhodopsin to CaM in the ROS as reported by Kohnken *et al.* (1981). Therefore, dissociation of CaM from the channel is unlikely to play a modulatory role for bovine rod photoreceptors.

In two recent studies, convincing evidence has been reported for an endogenous modulatory Ca^{2+}-binding protein for cGMP-gated channels in frog rods as well (Gordon *et al.* 1995; Nakatani *et al.* 1995). In the report of Gordon *et al.* (1995), it has been suggested that this endogenous factor is not CaM since CaM, but not the endogenous factor, could be washed off at micromolar free Ca^{2+} concentrations. As pointed out above, there was no such difference observed between the endogenous factor and CaM in bovine rods; both CaM and the endogenous factor could only be detached from the channel in the nanomolar range of free Ca^{2+} (Fig. 6).

Nakatani *et al.* (1995) reported a value of 48 nM free Ca^{2+} for dissociation of the endogenous factor from truncated frog ROS. This figure was determined for the near-physiological situation. In frog rods, the ratio of rhodopsin to CaM is 800 (Nagao, Yamazaki & Bitensky, 1987), yielding an intracellular CaM concentration of 3·1 μM. If CaM is the endogenous modulatory factor of the cGMP-gated channel in rods, the binding affinity of CaM to the channel would be predicted from eqn (16b) to be about 20 nM. Therefore, if CaM is the endogenous factor of the frog cGMP-gated channel, the affinity of the channel to Ca^{2+}–CaM appears to be considerably lower than that of the bovine channel. Moreover, it should be noted that a 112 kDa protein is isolated by CaM affinity chromatography from frog rods as opposed to a 240 kDa protein isolated from bovine rods (Hsu & Molday, 1994). These results suggest

that there is a difference between species regarding the CaM mechanisms of rod photoreceptors.

The inference that CaM is unlikely to play a modulatory role in bovine rod photoreceptors was based on the finding that the affinity of the cGMP-gated channel for Ca^{2+}–CaM is extremely high. It should be noted, however, that often phosphorylation in or near CaM binding domains reduces CaM affinity (Crivici & Ikura, 1995). For the recombinant olfactory cAMP/cGMP-gated channel, K_{CaM} was reported to be 21 nM (Chen & Yau, 1994), thus shifting the binding curve for CaM in the more physiological range of intracellular free Ca^{2+}. In Fig. 7 it is shown that a K_{CaM} of 50 nM shifts the EC_{50}^{Ca} of the Ca^{2+}–CaM binding curve to 90 nM free Ca^{2+} (Fig. 7, dashed line), i.e. into the concentration range of cytoplasmic free Ca^{2+} found in several vertebrate photoreceptors (reviewed e.g. in Koch, 1994). Therefore, the Ca^{2+}–CaM affinity of the channel has a decisive functional importance which determines whether or not CaM modulates the activity of cGMP-gated channels under physiological conditions. Both the Ca^{2+}–CaM affinity of the cGMP-gated channel and the drop of cGMP affinity upon CaM binding appear to be the crucial parameters determining the physiological relevance of this modulatory mechanism.

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