Calmodulin controls the rod photoreceptor CNG channel through an unconventional binding site in the N-terminus of the β -subunit

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Calmodulin (CaM) controls the activity of the rod cGMP-gated ion channel by decreasing the apparent cGMP affinity. We have examined the mechanism of this modulation using electrophysiological and biochemical techniques. Heteromeric channels, consisting of α - and β -subunits, display a high CaM sensitivity ($EC_{50} \leq 5$ nM) similar to the native channel. Using surface plasmon resonance spectroscopy, we identified two unconventional CaM-binding sites (CaM1 and CaM2), one in each of the N- and the C-terminal regions of the β -subunit. Ca^{2+} co-operatively stimulates binding of CaM to these sites exactly within the range of $[Ca^{2+}]$ occurring during a light response. Deletion of the N-terminal CaM1 site results in channels that are no longer CaM-sensitive, whereas deletion of CaM2 has only minor effects. We discuss different models to explain the high-affinity binding of CaM.

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Introduction

Cyclic nucleotide-gated (CNG) channels form a family of ion channels that are gated open by cAMP and cGMP. In photoreceptors and olfactory neurons, these channels serve as final targets for cGMP- and cAMP-signalling pathways that are activated by light and odorants, respectively. A functionally significant feature of CNG channels is their Ca^{2+} permeability (Yau *et al.*, 1981; Capovilla *et al.*, 1983; Hodgkin *et al.*, 1984; Picones and Korenbrot, 1995; Leinders-Zufall *et al.*, 1997). At physiological extracellular Ca^{2+} concentrations, Ca^{2+} carries a substantial fraction of the total current passing through CNG channels (Hodgkin *et al.*, 1987; Nakatani and Yau, 1988; Perry and MacNaughton, 1991; Frings *et al.*, 1995). Ca^{2+} entry through CNG channels is crucially important for both excitation and adaptation of vertebrate photoreceptors and olfactory neurons as Ca^{2+} controls the activity of several signalling enzymes including the CNG channels

themselves (for reviews see Shepherd, 1994; Zufall *et al.*, 1994; Detwiler and Gray-Keller, 1996; Koutalos and Yau, 1996; Polans *et al.*, 1996). Ca^{2+} /calmodulin (CaM) attenuates the activity of rod and olfactory CNG channels by increasing their apparent $K_{1/2}$ for cGMP and cAMP (Hsu and Molday, 1993; Chen and Yau, 1994; for review see Molday, 1996). This modulation is believed to serve as one of several Ca^{2+} -mediated feedback mechanisms that terminate the electrical response and set the sensitivity of photoreceptor cells and olfactory neurons (for reviews see Finn *et al.*, 1996; Molday, 1996).

CaM lowers the ligand sensitivity of rod and olfactory CNG channels to different levels and by way of different subunits. CaM increases the $K_{1/2}$ of the olfactory CNG channel for cAMP up to 50-fold (Chen and Yau, 1994; Balasubramanian *et al.*, 1996) by binding to a short segment in the N-terminal region of the α -subunit (Liu *et al.*, 1994). The CaM target is involved in the interaction between the N- and C-terminal regions which controls gating of the olfactory channel. Binding of CaM interferes with this interaction and thereby decreases the apparent cAMP affinity (Varnum and Zagotta, 1997). Although Ca^{2+} -CaM regulation of the olfactory CNG channel has been well established *in vitro*, it is not the only mechanism that sets the channel's ligand sensitivity. Phosphorylation of a Ser residue near the CaM-binding site sensitizes the channel to cAMP and thereby extends the range of CaM modulation (Müller *et al.*, 1998). Furthermore, an unidentified factor has been reported to be responsible for a significant fraction of the sensitivity shift (Balasubramanian *et al.*, 1996). CaM regulation of the rod CNG channel and its significance as an adaptive mechanism has been questioned on various grounds (Gray-Keller and Detwiler, 1994; Nakatani *et al.*, 1995; for summary see Molday, 1996; Sagoo and Lagnado, 1996). The maximally 2-fold increase in $K_{1/2}$ of the native rod channel by CaM (Hsu and Molday, 1993; Gordon *et al.*, 1995; Bauer, 1996) seems small compared with the large changes that occur in the cell's sensitivity during light adaptation. CaM sensitivity is conferred to heteromeric rod channels by the β -subunit. The rod α -subunit itself does not bind CaM and a channel consisting of only α -subunits is not modulated by CaM (Hsu and Molday, 1993; Chen *et al.*, 1994; Körschen *et al.*, 1995). Although these results show that the rod channel is modulated *in vitro*, the mechanism of this modulation and the CaM-binding site(s) on the β -subunit is not known.

To address these questions, we employed surface plasmon resonance (SPR) spectroscopy to identify binding sites for CaM in the β -subunit of the rod CNG channel and to study quantitatively the interaction between CaM and these sites. In this paper we report the identification and characterization of two distinct binding sites, CaM1 and CaM2, that are located in the

Fig. 1. Location of putative CaM-binding sites within the β -subunit of the bovine rod CNG channel. **(A)** The β -subunit polypeptide consists of the GARP-part and the β' -part. Only structural features of the β' -part are shown. The putative CaM-binding sites (CaM1 and CaM2, black boxes), the six membrane spanning regions (S1–S6, grey boxes) and the cGMP-binding site (hatched box) are located within the β' -part. P1–P11 refer to peptides used for immobilization in SPR measurements. The black bar indicates a region in the C-terminal domain of the β -subunit that has been probed for CaM-binding using overlapping peptides P4–P11. Conserved positions of aromatic or long-chain aliphatic amino acids in CaM-binding motifs are shaded in grey. Numbers represent the position of amino acid residues according to Körschen *et al.* (1995). **(B)** Fusion protein constructs and mutant β' -subunits used for SPR spectroscopy and patch-clamp recording, respectively (see Material and methods); + indicates either binding of CaM to the various constructs or functional modulation in the electrophysiological experiments; – refers to no binding or no modulation.

N- and C-terminal domains of the β -subunit, respectively. Only the CaM1 site is necessary for channel modulation, whereas the function of the CaM2 site is unknown. These findings may have a general significance for CNG channel operation, because shorter splice variants of the rod β -subunit also exist in other types of CNG channels (Biel *et al.*, 1996; I.Weyand, personal communication; F.Sesti, W.Bönigk, J.Bradley, F.Müller, S.Frings and U.B.Kaupp, in preparation).

Results

Both N- and C-terminal regions bind CaM

We performed overlay experiments using biotinylated CaM (bCaM) to test N- and C-terminal fusion constructs for CaM binding. Both, N- and C-terminal regions were able to bind bCaM (construct FPβNT and FPβCT1 in Figures 1B and 2A). Staining with bCaM was not due to the presence of the maltose binding protein (MBP). After proteolytic cleavage of fusion constructs labelling of the respective channel-derived fragment persisted, whereas the MBP-part was not detected by bCaM (data not shown). Labelling of FPβCT1 was significantly stronger than labelling of FPβNT (Figure 2A, compare lanes 1 and 3). Comparison of labelling intensities also suggested that bCaM-binding to both constructs is significantly weaker than binding to calcineurin (Figure 2B, lane 6; $K_D = 16$ – 24 nM; Hubbard and Klee, 1987; Takano *et al.*, 1994). The presence of EGTA abolished specific labelling with bCaM (data not shown). Using smaller constructs (Figure 1B), the CaM-binding site in the C-terminal region was localized to a segment adjacent to the cGMP-binding

Fig. 2. Calmodulin binds to N- and C-terminal regions.

(A) Comparison of CaM binding to N- and C-terminal constructs with an overlay assay using biotinylated CaM. Each lane contained ~ 0.4 μ g of FPβNT (lane 1); FPβNTΔCaM1 (lane 2) and FPβCT1 (lane 3). **(B)** Identification of a CaM-binding region in C-terminal constructs. Each lane contained ~ 0.3 μ g of FPβCT1 (lane 1); FPβCT2 (lane 2); FPβCT3 (lane 4); FPβCT4 (lane 5); and calcineurin (lane 6).

domain (Figure 2B, compare lanes 2, 3 and 4). As described below, we identified the CaM-binding sites, CaM1 in the N-terminus and CaM2 in the C-terminus, using SPR spectroscopy. When these sites were deleted in the constructs FPβNTΔCaM1 and FPβCT3ΔCaM2, labelling with bCaM was abolished almost entirely (Figure 2A, lane 2 and Figure 2B, lane 5). In conclusion, bCaM specifically recognizes a site in both the N- and C-terminal regions. The binding site in the C-terminal region has a higher affinity than that in the N-terminal region. Weak labelling of the deletion construct FPβCT3ΔCaM2 might indicate the presence of another CaM binding site with

Fig. 3. Quantitative analysis of CaM binding to C-terminal targets. (A) Sensorgrams recorded with peptide P10 and CaM. The immobilization density was ~ 0.4 – 1 ng peptide/ mm^2 . During recording, the running buffer contained 3 mM Ca^{2+} (open bars) and 3 mM Ca^{2+}/X mM CaM (filled bar). Formation of the complex between CaM and the corresponding peptide was monitored as a change in resonance units ΔRU . The CaM concentrations in the running buffer were: 0 , 30 , 60 , 150 , 300 , 450 , 600 and 900 nM. During flushing of the flow cell after each recording with EGTA/ 0 Ca^{2+} (regeneration, hatched bar), sensorgrams exhibited a transient negative signal and then returned to the baseline indicating that all bound CaM dissociated from the peptide. Right panel: $\text{EC}_{50} = 537$ nM. (B) Sensorgrams recorded with FP β CT3 and CaM. The CaM concentrations in the running buffer were: 0 , 30 , 60 , 150 , 300 , 450 , 600 and 1200 nM. All other conditions as in (A). Right panel: Determination of the K_D value for CaM-binding from a Scatchard plot as described in Materials and methods. $K_D = 245$ nM.

lower affinity or that the CaM2 sequence does not represent the entire CaM binding site.

Identification of a CaM-binding site in the C-terminal region

Using SPR spectroscopy, we quantitatively studied CaM-binding to FP β CT3 and eight overlapping synthetic peptides that cover the entire sequence of the construct (P4–P11; Figure 1A and Materials and methods for sequences). Peptides were immobilized on the dextran matrix of sensor chips by means of an additional Cys residue at the N-terminus. Experiments were conducted at saturating $[\text{Ca}^{2+}]$ to ensure optimal interaction of CaM with immobilized peptides. Sensorgrams consisted of four parts: a pre-running phase without CaM (Figure 3, open bar), followed by an association phase when CaM was present in the running buffer (Figure 3, black bar), and a dissociation phase after removal of CaM (Figure 3, open bar). Each association–dissociation cycle was followed by a regeneration phase during which the flow cell was flushed with 2 mM EGTA/ 0 Ca^{2+} to remove CaM bound to the peptide (Figure 3, hatched bar). Among P4–P11, peptides P6 and P10 gave rise to the largest signals with saturating amplitudes. P6 was excluded as a potential CaM-binding site because (i) deletion of the P10 motif abolished CaM-binding almost entirely (Figure 2B, FP β CT3 Δ CaM2, lane 5), and (ii) fusion constructs that contain P6 but lack P10 (e.g. FP β CT4; Figure 2B, lane 3) do not bind CaM. Other peptides gave no CaM-induced signals (data not shown). P10 entails a reasonably good consensus sequence for CaM-binding (amino acids 1242–1255).

Fig. 4. Quantitative analysis of CaM binding to N-terminal targets. (A) Sensorgrams recorded with peptides P1–P3. Peptides were immobilized at similar densities on the sensor chip surface 0.5 – 1 ng peptide/ mm^2 . Only P1 and P2 peptides showed significant CaM-binding. (B) Sensorgrams recorded with peptide P2 and CaM. The recording protocol was identical to that in Figure 3. The CaM concentrations in the running buffer were: 0 , 30 , 60 , 150 , 300 , 450 , 600 and 900 nM. Right panel: $K_D = 283$ nM. (C) Sensorgrams recorded with the fusion protein BNT and CaM. The CaM concentrations in the running buffer were: 30 , 60 , 150 , 300 , 450 , 600 , 1200 and 6000 nM. Measuring conditions were as described in (B). Right panel: $K_D = 437$ nM. Bottom trace: sensorgram recorded with immobilized FP β NTCaM1 and 1200 nM CaM. (D) Sensorgrams recorded with peptide P α_{olf} and CaM. Measuring conditions were as in Figure 3. Right panel: $K_D = 10.5$ nM. The CaM concentrations in the running buffer were 0 , 30 , 60 , 150 , 300 , 450 , 600 and 900 nM.

To quantify the binding of CaM to peptides or fusion constructs, the plateau amplitude R_{eq} at various CaM concentrations was analysed in a Scatchard plot (Figures 3 and 4, right panels). The K_D constants were determined as the negative reciprocal of the slope of R_{eq}/C versus R_{eq} (see Equation 3 in Material and methods). Figure 3A shows a set of sensorgrams with peptide P10 and CaM concentrations ranging from 30 to 900 nM. CaM bound reversibly to P10 in a concentration-dependent and saturable fashion. The CaM-dependence of the P10 signal shown in Figure 3A resulted in a curvilinear relationship (right panel). We interpret the curved nature of the Scatchard plot to indicate that immobilized P10 forms a heterogeneous population of CaM targets on the chip surface. Curvilinear Scatchard plots do not permit the determination of reliable K_D constants (Klotz, 1989); therefore, in this particular case, we determined an EC_{50} value of 552 nM.

The C-terminal fusion construct FP β CT3 was immobilized via an additional N-terminal cysteine residue. FP β CT3 bound CaM in a concentration- and Ca^{2+} -dependent fashion with a mean K_D value of 207 nM

Table I. Summary of dissociation constants K_D (nM)

P2/CaM	240 ± 44 (4)
S-CaM/P2	524 (1)
FPβNT/CaM	504 ± 98 (4)
S-CaM/FPβNT	21 (1)
bCaM/βNT	4 ± 0 (2)
Pα _{olf} /CaM	12.4 ± 0.5 (4)
P2/Rec	503 ± 110 (4)
P10/CaM	560 ± 68* (3)
FPβCT3/CaM	207 ± 21 (2)

*EC₅₀ value; the first abbreviation in the left panel symbolizes the immobilized binding partner. S-CaM, spinach CaM. Number in parentheses indicate the number of independent duplett determinations.

(Figure 3B). Because the deletion of the CaM2 site in the β-subunit did not abolish modulation of the heterologously expressed channel (see below, Figure 8), we did not characterize the CaM2 site any further.

Identification of a CaM-binding site in the N-terminal region

Many CaM-binding sites encompass short segments of ~14 amino acid residues which are characterized by long-chain aliphatic or aromatic residues at positions 1 and 14 of the motif and by the propensity to adopt a basic amphipathic α-helix (Crivici and Ikura, 1995). A CaM-binding motif which conforms to these features has been identified in the N-terminus of the α-subunit of the rat olfactory CNG channel (Liu *et al.*, 1994). However, similar sites were not detected in the N-terminal sequence of the rod β-subunit.

A search for less conserved motifs led us identify a segment (designated CaM1) that is remotely reminiscent of other CaM-binding sites (Figure 1A, see Discussion for details). Binding to CaM1 and adjacent sequences was examined using three overlapping peptides P1–P3. Superfusion of immobilized peptide P2 with CaM gave rise to a signal of ~1200 RU that reversed after removal of CaM (Figure 4A). Two peptides, P1 and P3, whose sequences partially overlap with the N- and C-terminus of the P2 peptide, respectively (Figure 1A), have been employed to determine the boundaries of this target site. Peptide P1 also bound CaM, whereas peptide P3, which was lacking the long-chain aliphatic Leu⁶⁸², showed no CaM binding (Figure 4A). This result suggested that CaM1 is largely confined to the N-terminal half of P2. The K_D constant of CaM binding to P2 was determined from the concentration dependence of the binding signal using CaM concentrations of 30–900 nM (Figure 4B). Data from four experiments with seven CaM concentrations each yielded a mean K_D of 240 nM (Table I).

The K_D constant of CaM-binding to P2 is at least 100-fold larger than the CaM concentrations needed to control the native cGMP-gated channel in suspensions of rod outer segments (EC₅₀ ≈ 1–2 nM; Hsu and Molday, 1993; Bauer, 1996) or the heteromeric channel composed of α/β' subunits (EC₅₀ ≤ 5 nM; see Figure 8C). We therefore examined the possibility that the N-terminal domain comprises more than one CaM-binding site or that P2 does not represent the complete CaM target. Two fusion proteins were constructed between MBP and the entire N-terminus up to transmembrane segment S1 either

with (FPβNT) or without (FPβNTΔCaM1) the binding motif in P2 (Figure 1B). Following cleavage from the MBP part and purification, the N-terminal domain, βNT, was immobilized by means of NH₂-groups; CaM binding was examined by SPR spectroscopy. Figure 4C shows a series of sensorgrams recorded at different CaM concentrations. Scatchard analysis of sensorgrams yielded a mean K_D value of 437 nM (Figure 4C, right panel). Thus, the K_D values for P2 and the N-terminal domain are largely similar within a factor of two (Table I). The uncleaved purified fusion protein yielded similar results. The CaM-binding signal was abolished entirely in the N-terminal construct that is lacking the CaM1 site (Figure 4C, bottom trace). These experiments argue that other regions in the N-terminus neither enhance binding of CaM to P2 nor carry another CaM-binding site in addition to CaM1.

Next we considered whether local electrostatic fields or steric constraints impair CaM binding to immobilized targets in the dextran matrix and thereby lead to high K_D values. We chose the peptide Pα_{olf}, a high-affinity CaM-binding site in the N-terminal region of the olfactory α-subunit (Liu *et al.*, 1994), to test for this possibility. The time course of sensorgrams with Pα_{olf} was entirely different to sensorgrams with P2 and P10. Dissociation of CaM from Pα_{olf} proceeded almost 100 times slower than for P2 or P10, suggesting that Pα_{olf} has a much higher CaM affinity than P2 and P10 (Figure 4D). A Scatchard analysis led to a mean K_D of 12.4 nM. This value agrees reasonably well with a K_D of 3.4 nM determined by fluorescence measurements with dansyl-CaM (Liu *et al.*, 1994). Previously it has been demonstrated that the K_D value of CaM-binding to neuronal NOS peptides as determined by SPR spectroscopy is in excellent agreement with values obtained by other techniques (Zocher *et al.*, 1996). Thus, the binding affinity of at least two CaM targets is not altered by surface immobilization.

Effects of surface immobilization were also examined by using a reversed geometry on the sensor chip. CaM was immobilized using three different methods: spinach CaM was immobilized by thiol-coupling via a single Cys residue at position 26; biotinylated bovine CaM was coupled via streptavidin; bovine CaM was coupled via NH₂-groups. The binding affinities of several constructs were largely similar for each of the three configurations. In another control experiment, we ascertained that immobilized CaM and CaM in solution bound to calcineurin with similar affinity (Takano *et al.*, 1994; data not shown). Moreover, analysis of sensorgrams recorded with immobilized CaM at different P2 concentrations gave a K_D of 524 nM, i.e. a similar affinity to that observed with immobilized P2 and CaM in the mobile phase. These results suggest that immobilization of CaM does not alter binding to its targets. However, a much higher binding affinity was observed for FPβNT in the mobile phase compared with immobilized FPβNT (compare Figures 4C and 5A). A K_D of 21 nM was determined using the extrapolated R_{eq} values from Figure 5A. The response amplitudes of sensorgrams obtained with the deletion mutant FPβNTΔCaM1 were reduced dramatically, but not completely abolished (compare Figure 5A and B), and the K_D value of residual binding was increased 15-fold (K_D = 307 nM). These results suggest that the N-terminal domain in fact carries a binding site of nanomolar affinity and

Fig. 5. Analysis of CaM-binding with a reversed geometry. (A) Sensorgrams recorded with FPβNT. Calmodulin from spinach was immobilized via the cysteine at position 26. The density on the chip was 2–3 ng proteins/mm². The FPβNT concentrations in the running buffer were: 1.5, 7.5, 15, 30, 75, 105, 150, 300 and 750 nM (filled bar). (B) Sensorgrams recorded with FPβNTΔCaM1. The recording protocol is identical to that in (A). Traces 2 and 3 are indistinguishable from each other. FPβNTΔCaM1 was tested with the same flow cell on the sensor chip. Note the identical scaling in (A) and (B). (C) Binding of P2 and βNT to immobilized CaM. The first part of the sensorgram was recorded with 1.6 μM P2 (first filled bar); after two regeneration pulses with EGTA, 50 nM purified βNT (second filled bar) were injected.

that immobilization of FPβNT severely impairs the affinity for CaM. The residual binding signal might indicate that other regions can bind CaM with considerably lower affinity. A direct comparison between P2- and FPβNT-signals is shown in Figure 5C. CaM was immobilized, and P2 and FPβNT were injected in series. The sensorgrams illustrate the large difference in the kinetics of binding signals which reflect the difference in CaM affinity. The kinetics of P2 binding and dissociation were fast and not significantly different to kinetics measured in the other configuration (compare Figures 4A and 5C). In contrast, dissociation of βNT from immobilized CaM proceeded much more slowly (Figure 5C). In fact, the βNT signals are similar to signals recorded with the high-affinity Pα_{olf} peptide. In summary, these data show that P2 encompasses the essential CaM-binding target, but that other factors which reside in the complete N-terminal domain are required to achieve a high affinity interaction with CaM (see Discussion).

Probing CaM-binding by an enzyme-linked assay

The ability of peptides and fusion proteins to bind CaM in solution was tested by an assay using neuronal NO synthase (NOS). Preparations of photoreceptor outer/inner segments that contain neuronal NOS and a soluble guanylyl cyclase, which is the target of NO, were incubated with increasing concentrations of peptide or fusion protein. The inhibition of Ca²⁺–CaM-dependent NOS activity was monitored via cGMP production. Pα_{olf}, P2, P10, FPβNT and FPβCT3 inhibited NOS activity in a concentration-dependent manner (Figure 6). No inhibition was observed with P3, in agreement with the SPR measurements of Figure 4A. Half-maximal inhibition was observed at

Fig. 6. Semi-quantitative analysis of CaM binding to peptides and fusion proteins in solution. Inhibition of photoreceptor NOS activity by CaM-binding peptides or fusion proteins. NOS activity was monitored by endogenous soluble guanylyl cyclase (sGC) activity. Concentrations of half-maximal inhibition IC₅₀ were 0.37 μM (Pα_{olf}, ▼), 0.85 μM (FPβCT3, △), 3.6 μM (P10, ○), 4.7 μM (FPβNT, ▲), and 11.5 μM (P2, ●). No inhibition was observed with peptide P3 (▽).

concentrations (IC₅₀) of ~0.37 μM Pα_{olf}, 3.6 μM P10 and 11.5 μM P2. The IC₅₀ values for FPβNT and FPβCT3 were 4.7 μM and 0.85 μM, respectively.

These results confirm that P2 and P10 harbour CaM-binding sites and that the N- and C-terminal fusion constructs show a higher affinity than the corresponding peptides. These semi-quantitative measurements, in agreement with the overlay experiment of Figure 2, also indicate that the C-terminus displays a higher affinity for CaM than the N-terminus.

Ca²⁺ sensitivity of CaM binding

The free [Ca²⁺] in dark-adapted rod photoreceptors is 200–500 nM and falls to much lower levels upon

Fig. 7. Ca^{2+} dependence of CaM binding. (A) Left panel: Sensorgrams of CaM binding to immobilized P2 at $[\text{Ca}^{2+}]$ ranging from 50 nM to 10 μM ; 300 nM CaM. All other conditions as described in Figure 3. Free $[\text{Ca}^{2+}]$ was adjusted by 2 mM Ca^{2+} –EGTA buffer. Right panel: Dose–response relation of CaM-binding. Data point and error bars represent the mean and SD from three experiments, respectively. The curve was fitted to the data using the Hill equation. $K_{1/2}^{\text{Ca}} = 117$ nM; $n = 2.8$. (B) Binding of CaM to immobilized FP β NT; 600 nM CaM; all other conditions as in (A). $K_{1/2}^{\text{Ca}} = 124$ nM; $n = 2.6$. (C) Binding of CaM to immobilized FP β CT3; 600 nM CaM; all other conditions as in (A). $K_{1/2}^{\text{Ca}} = 270$ nM; $n = 2.3$.

illumination (Korenbrodt and Miller, 1989; Lagnado *et al.*, 1992; Gray-Keller and Detwiler, 1994; McCarthy *et al.*, 1994). We therefore addressed the question of whether CaM binds to immobilized targets within the physiological range of $[\text{Ca}^{2+}]$ and whether both CaM-binding sites have similar or different Ca^{2+} sensitivities. The left-hand panel of Figure 7A shows a series of sensorgrams obtained with immobilized P2 and 300 nM CaM at various $[\text{Ca}^{2+}]$. At 50 and 100 nM Ca^{2+} , no binding signals were observed and at ~ 1 μM Ca^{2+} the signal saturated. The Ca^{2+} dependence of binding was satisfactorily fitted by the Hill equation with $K_{1/2}^{\text{Ca}} = 117$ nM and a Hill coefficient $n = 2.8$ (Figure 7A, solid line). Similar results were obtained with FP β NT and FP β CT3; the respective binding data were $K_{1/2}^{\text{Ca}} = 124$ nM, $n = 2.6$ for FP β NT (Figure 7B) and $K_{1/2}^{\text{Ca}} = 270$ nM, $n = 2.3$ for FP β CT3 (Figure 7C). The $K_{1/2}^{\text{Ca}}$ depends on the total CaM concentration, $[\text{CaM}]$, according to $K_{1/2}^{\text{Ca}} = K_{\text{Ca}} (K_{\text{CaM}}/[\text{CaM}])^{1/n}$ (see Bauer, 1996), wherein K_{Ca} and n represent the equilibrium constant and Hill coefficient, respectively, of Ca^{2+} binding to CaM, and K_{CaM} the equilibrium constant of CaM binding to the target. The total CaM concentration in a rod outer segment is of the order of 3–4 μM (Kohnken *et al.*, 1981), whereas 300 nM CaM was

used in the experiments of Figure 7. If the majority of intracellular CaM is available for binding to the channel, then the $K_{1/2}^{\text{Ca}}$ of channel modulation in an intact rod may be ~ 3 -fold lower, i.e. 40–50 nM.

This estimate of the $K_{1/2}^{\text{Ca}}$ and Hill coefficient of CaM binding matches closely the $K_{1/2}^{\text{Ca}}$ of Ca^{2+} modulation of the channel measured in truncated rods ($K_{1/2}^{\text{Ca}} = 48$ –55 nM; $n = 1.4$ –1.6) (Nakatani *et al.*, 1995; Sagoo and Lagnado, 1996). In summary, binding of CaM to immobilized targets occurs exactly within the dynamic range of $[\text{Ca}^{2+}]$ prevailing during the rod photoresponse.

Specificity of CaM-binding sites

The unusual features of the CaM1 and CaM2 sites prompted us to examine whether other retinal Ca^{2+} -binding proteins, recoverin and guanylyl cyclase-activating protein (GCAP1), also recognize these binding sites. Recoverin bound to P2 in a Ca^{2+} - and concentration-dependent fashion. The K_{D} values were similar to those of CaM binding (~ 300 –500 nM, Table I). Signals were completely reversed by EGTA and control peptides gave no binding signal with recoverin (data not shown). Recoverin did not bind to the C-terminal construct FP β CT3. GCAP1 gave no specific binding signals with P2. These results demonstrate that recoverin can bind promiscuously to the CaM1 site, but not to the CaM2 site, and that GCAP1 does not bind to CaM1.

Functional expression of wild-type and mutant channels

We addressed the following questions by heterologous expression of α - and β -subunits in *Xenopus* oocytes and recording of cGMP-activated currents from inside-out patches as described by Körschen *et al.* (1995). Do α/β heteromeric channels reproduce essential features of CaM modulation of native channels? Is the P2 or the P10 site or both required for CaM modulation? Does recoverin modulate channel activity *in vitro*?

Co-expression of α - and β -subunits in *Xenopus* oocytes often results in mixtures of homo- and hetero-oligomeric channels. Channel species can be distinguished by their different sensitivities to blockage by *L-cis*-diltiazem (Chen *et al.*, 1993). *L-cis*-diltiazem at 10 μM blocks the α -subunit only weakly, but almost entirely suppresses the current through hetero-oligomeric channels (Körschen *et al.*, 1995). Only experiments which showed a profound blockage by *L-cis*-diltiazem were included in the analysis (Figure 8A and D, lane 5). In the presence of saturating cGMP concentrations (500 μM), no difference was observed between current recordings with or without 300 nM CaM in the perfusion bath (Figure 8A and D, traces 1 and 2). When the cGMP concentration roughly equalled the $K_{1/2}$ value of channel activation (40 μM), the current was attenuated in the presence of Ca^{2+} –CaM (Figure 8A and D, compare traces 3 and 4). The modulation by Ca^{2+} –CaM was reversible. When the membrane patch was perfused with a solution containing EGTA and no CaM, the current amplitude completely recovered.

The effect of CaM on the rod CNG channel in broken ROS is half-maximal at 1.5–3.5 nM CaM (Hsu and Molday, 1993; Bauer, 1996). To examine the possi-

Fig. 8. Calmodulin and recoverin sensitivity of heterologously expressed CNG channels. **(A)** The effect of Ca^{2+} -CaM on the cGMP-activated current of hetero-oligomeric channels ($\alpha + \beta'$) was measured in excised inside-out patches of *Xenopus* oocyte membranes. The bath solutions (traces 1–4) contained 100–120 mM K-gluconate, 10 mM HEPES, 2 mM NTA- Ca^{2+} buffer (50 μM free Ca^{2+}) adjusted with KOH to pH 7.4, and the following additions: 500 μM cGMP (trace 1); 500 μM cGMP and 300 nM CaM (trace 2); 40 μM cGMP (trace 3); 40 μM cGMP and 300 nM CaM (trace 4). The bath solution for trace 5 contained 10 μM *L*-cis-diltiazem and 500 μM cGMP. **(B)** Dependence of the decrease in cGMP-activated current on three different CaM concentrations. The current in the presence of 40 μM cGMP at time $t = 0$ was 270 pA at $V_m = -40$ mV. **(C)** Calmodulin modulation of CNG channels containing mutant β -subunit that are lacking either the CaM1 site ($\alpha + \beta\Delta\text{CaM1}$; upper trace) or the CaM2 site ($\alpha + \beta\Delta\text{CT}$; lower trace). The short transient deflections in the lower current trace represent artefacts caused by a change in solutions. All other conditions as in (B). Initial current amplitude was 200 pA (upper trace) and 150 pA (lower trace). **(D)** cGMP-activated currents of hetero-oligomeric channels consisting of wt α - and β' -subunits in the presence of recoverin (300 nM, traces 2 and 4) and its absence (traces 1 and 3). All other conditions as in (A). **(E)** Modulation of normalized cGMP-activated currents (40 μM cGMP) by CaM (50 nM) in the absence and presence of recoverin (1 μM). The current in the presence of 40 μM cGMP at time $t = 0$ was 60 pA at $V_m = -40$ mV.

bility that an unknown factor present in rod photoreceptors is required for this high CaM sensitivity we estimated the $K_{1/2}$ value for the Ca^{2+} -CaM modulation of hetero-oligomeric wild-type (wt) channels by recording the decrease of the cGMP-activated current (40 μM cGMP) at 5, 12 and 50 nM CaM (Figure 8B). Although the decline in current proceeded much more slowly at 5 nM CaM than at 50 nM CaM, a maximal decrease of ~60% was achieved at CaM concentrations as low as 5 nM (Figure 8B). This result demonstrates that both the extent and sensitivity of the CaM modulation in the native channel (Hsu and Molday, 1993; Bauer, 1996) and heterologously expressed α - and β -subunits are rather similar. Therefore, the high CaM sensitivity is intrinsic to the heteromeric ($\alpha + \beta$) channels, and no other photoreceptor-specific component(s) must be invoked.

Figure 8C (upper trace) shows an experiment with hetero-oligomeric channels built from wt α -subunit and a mutant β -subunit that lacks residues 682–696 ($\beta\Delta\text{CaM1}$, Figure 1B). In ($\alpha + \beta\Delta\text{CaM1}$) channels, the Ca^{2+} -CaM modulation was abolished entirely. These results demonstrate that Ca^{2+} -CaM modulation involves a segment in the β -subunit that is represented by the CaM1 site. In contrast, deletion of a large C-terminal portion entailing the CaM2 site ($\Delta 1203$ –1394, $\beta\Delta\text{CT}$) did not abolish CaM modulation in ($\alpha + \beta\Delta\text{CT}$) channels.

While the maximal suppression of current by CaM was somewhat smaller (~40%) than in the wt channel (compare Figures 8B and C), a half-maximal effect was achieved at ~10 nM CaM. This result demonstrates that binding of CaM to the CaM2 site is not necessary for the CaM regulation of ligand sensitivity of the rod CNG channel.

Considering that recoverin binds to the CaM1 site with an affinity similar to that of CaM, we examined whether recoverin can also modulate channel activity. Purified native recoverin (300 nM) had little or no effect on the cGMP-activated currents in excised patches (Figure 8D). If recoverin bound to the CaM site but was unable to modulate channel activity, then recoverin might be expected to antagonize the modulation by CaM. However, the decrease of current caused by 50 nM CaM was not affected markedly by the presence of 1 μM purified recoverin (Figure 8E). This result suggests that recoverin is unlikely to modulate the rod CNG channel *in vivo* and that the affinity of the native channel for recoverin must be significantly lower than that for CaM.

Discussion

CaM-binding domains of many proteins are confined to ~20 residue fragments. CaM binds as tightly to these peptide targets as to the intact protein. Furthermore, a

reasonably good correspondence between efficacy for CaM modulation (EC_{50}) and binding (K_D) to target peptides has been reported for several CaM-dependent enzymes, including adenylyl cyclases ($EC_{50} = 15\text{--}20\text{ nM}$; $K_D = 2\text{ nM}$; Tang *et al.*, 1991; Vorherr *et al.*, 1993), calcineurin ($EC_{50} = 16\text{--}100\text{ nM}$, $K_D = 16\text{--}24\text{ nM}$; Hubbard and Klee, 1987; Takano *et al.*, 1994), and NOS-I ($EC_{50} = 3.5\text{ nM}$, $K_D = 5\text{ nM}$; Schmidt *et al.*, 1991; Zoche *et al.*, 1996). Sequences of many CaM target peptides are characterized by aromatic residues at positions 1 and 14 of the motif and additional hydrophobic residues at positions 5 and 8. Several positively charged residues are interspersed between the hydrophobic residues. In the α -helical wheel representation, basic and hydrophobic residues segregate on opposite sites of the helix. These observations form the basis for the amphipathic α -helical peptide model of CaM recognition (O'Neil and DeGrado, 1990; Crivici and Ikura, 1995). In addition, Ca^{2+} -free CaM can bind to another motif, called IQ motif, with a consensus sequence like IQxxxRGxxxR (Cheney and Mooseker, 1992; Rhoads and Friedberg, 1997).

The peptide target of the olfactory α -subunit completely conforms to the amphipathic α -helical model. Furthermore, values for EC_{50} of modulation (21 nM; Chen and Yau, 1994) and K_D of binding (4–12 nM; Liu *et al.*, 1994; this paper) are of the same order. In contrast, the CaM1 site of the β -subunit from rod photoreceptors appears to be unique among CaM targets: (i) at position 14, the motif comprises a positively charged residue instead of a hydrophobic or aromatic residue; (ii) a conspicuously large number of Glu/Asp residues exists within the motif and shortly before or after the motif; consequently, (iii) an α -helical wheel representation of the peptide displays no amphipathic character; (iv) the motif is in part similar to an IQ motif ($^{682}\text{LQELVKLFKERTK}^{695}$). However, binding of CaM to this site is clearly Ca^{2+} -dependent (Figure 7), which is untypical for a conventional IQ-binding motif.

As might be expected from the unconventional motif, the affinity of CaM-binding to this target peptide is low, in strong contrast to the extremely high CaM sensitivity of channel regulation. Because the EC_{50} values for CaM modulation are largely similar for heterologously expressed channels consisting of α - and β -subunits and for native channels from rod photoreceptors, the cause of the high sensitivity must lie in the channel subunits themselves.

The difference in the ability of an agonist to bind to a receptor site on the channel polypeptide and its potency to change the open state is not troublesome in principle. Simple models show that the agonist action can be divided up into two steps: the initial binding of a ligand and the subsequent isomerization between shut and open states, i.e. gating. Because of the principle of reciprocity, binding and gating are intimately coupled with each other and it is difficult to clearly separate the two processes experimentally (for discussion see Jackson, 1992; Amin and Weiss, 1993; Colquhoun and Farrant, 1993; Goulding *et al.*, 1994; Liu *et al.*, 1994). In fact, the decrease in the apparent cAMP sensitivity of the olfactory channel can be accounted for by an action of CaM on channel gating (Liu *et al.*, 1994). However, with respect to the rod CNG channel, the difference between EC_{50} for CaM activation

and K_D for binding to the peptide seems to be too large to be solely accounted for by gating. We sought to resolve this discrepancy. In fact, the binding affinity of the free N-terminal construct for immobilized CaM is 20-fold higher than the affinity determined in the reversed geometry and close to the EC_{50} value. Sensorgrams obtained with native CNG channel preparations and immobilized CaM also showed a high-affinity binding (data not shown). However, sensorgrams did not allow the same rigorous data evaluation as with the fusion constructs due to the presence of phospholipid liposomes.

In the following, we discuss two variants of a mechanism that could account for these observations. By interacting with other parts of the correctly folded N-terminal region, the CaM1 site might adopt a high-affinity conformation. This extended CaM target might become disturbed upon immobilization, resulting in a conformation similar to the P2 peptide. Alternatively, N-terminal domains might interact with each other to produce a high-affinity target for CaM. Do our experimental results favour one of these possibilities, i.e. interaction within a single domain or between domains? Disruption of the conformation of the N-terminus or CaM upon immobilization is unlikely for the following reasons: first, the CaM affinity of immobilized FP β NT is largely the same irrespective whether the construct was coupled via NH_2 -groups or cysteine residues at the far end of the N-terminal region. Secondly, experiments with immobilized fusion construct FP β NT and cleaved and purified β NT yielded similar results. Furthermore, the K_D values obtained with either one of the two immobilization geometries were largely similar for two CaM targets, P2 and calcineurin. For two other CaM targets, P α_{olf} and a CaM-binding peptide from NOS, the K_D values determined by SPR and by other techniques that do not involve surface immobilization, were also of the same order (Liu *et al.*, 1994; Zoche *et al.*, 1996; this paper). Thus, these observations suggest that two interacting N-terminal domains form a high-affinity target for CaM. In the functional channel, the N-terminal domain of the β -subunits may undergo additional contacts with the α -subunit. Within this framework, binding of CaM to the CaM1 site could disrupt this network of interactions and thereby control the apparent cGMP sensitivity by an allosteric mechanism. In fact, specific interaction between the N-terminus and the C-terminus of the CNG channel α -subunit from olfactory neurons has recently been reported to underly channel activation (Varnum and Zagotta, 1997). Interaction involved the CaM target on the N-terminus of the olfactory α -subunit and CaM was found to interfere with this interaction (Varnum and Zagotta, 1997).

Whereas modulation of the rod CNG channel by Ca^{2+} -CaM is well established *in vitro* (Hsu and Molday, 1993; Gordon *et al.*, 1995; Nakatani *et al.*, 1995; Bauer, 1996), efforts to demonstrate that the rod photoreceptor is using that mechanism *in vivo* have yielded mixed results (Gray-Keller and Detwiler, 1995; Nakatani *et al.*, 1995; for review see Molday, 1996; Detwiler and Gray-Keller, 1996; Koutalos and Yau, 1996; Sagoo and Lagnado, 1996), and even an unidentified endogenous factor has been implied (Gordon *et al.*, 1995). In particular, the small shift in the $K_{1/2}$ for cGMP suggested that Ca^{2+} -CaM modulation of the channel contributes minimally to photoreceptor

Table II. Positions and sequences of peptides

P α_{olf}	(amino acids 60–85)	QQRGGFRRI ARLVGVLEW AYRNFR
P1	(amino acids 667–692)	ATSTASQNSA IINDRLQELV KLFKER
P2	(amino acids 676–701)	AIINDRLQEL VKLFKERTK VKEKLI
P3	(amino acids 683–708)	QELVKLFKER TEKVEKLID PDVTSD
P4	(amino acids 1133–1162)	LVTLKAGSVF GEISLLAVGG GNRRTANVVA
P5	(amino acids 1153–1182)	GNRRTANVVA HGFTNLFIELD KKDLNEILVH
P6	(amino acids 1173–1202)	KKDLNEILVH YPESQKLLRK KARRMLRNNN
P7	(amino acids 1192–1217)	KKARRMLRNN NKPKEKSVLI LPPRAG
P8	(amino acids 1203–1228)	KPKEKSVLIL PPRAGTPKLF NAALAA
P9	(amino acids 1223–1248)	NAALAAAGKM GAKGGRGGRL ALLRAR
P10	(amino acids 1236–1261)	GGRGGRLALL RARLKELAAAL EAAARQ
P11	(amino acids 1252–1277)	LAALAAARQ QQLLEQAKSS EDAAVG

adaptation and recovery. However, Koch *et al.* (1987) working with broken rod outer segments described two forms of the channel that differed in cGMP sensitivity by at least 10-fold. Likewise, Sagoo and Lagnado (1996) observed a ≤ 20 -fold modulation of channel current by Ca^{2+} in rod photoreceptors shortly after establishing the truncated configuration; the modulation faded away with recording time. Both observations support the hypothesis that cGMP sensitivity can vary by more than one order of magnitude and that this modulation requires an elusive factor. That factor might indeed represent the physiologically authentic ligand of the CaM1 site. CaM may act as partial agonist that binds to this site but is unable to change the channel activity to the same extent. However, the extremely high sensitivity of the rod channel for CaM sets a practical limit for any other Ca^{2+} binding protein to serve as a modulatory molecule. For such a protein being relevant physiologically, it must either be significantly more abundant or display an even higher affinity than CaM. Other known Ca^{2+} -binding proteins in photoreceptors, i.e. recoverin and GCAP, either do not bind to the CaM1 or CaM2 sites or do not modulate channel activity. Alternatively, this factor might control the apparent cGMP affinity through binding to the CaM2 site. Recently, Müller *et al.* (1998) reported that phosphorylation of a Ser residue adjacent to the CaM-binding site of the olfactory α -subunit sensitizes the channel for cAMP, but leaves unaffected the cAMP sensitivity in the presence of Ca^{2+} -CaM, thereby enlarging the dynamic range of sensitivity regulation by Ca^{2+} -CaM. A similar mechanism may exist in rod CNG channels. In fact, Gordon *et al.* (1992) and Molokanova *et al.* (1997) reported that the cGMP sensitivity of the rod CNG channel is affected by phosphorylation. Future work will be required to show whether the extent of channel modulation by CaM depends on the channel's phosphorylation state.

Materials and methods

Synthesis and immobilization of peptides and fusion proteins

Peptides were synthesized, purified and characterized as described previously (Zoche *et al.*, 1996). The purity of the peptides was $\geq 95\%$. Peptides were immobilized on a BIAcore sensor Chip CM5 by means of an additional cysteine residue at the N-terminus of the peptide. The C-terminus was in the amide form. The nomenclature and sequences of peptides are given in Table II.

The following fusion proteins between MBP and various N- and C-terminal segments were constructed. Construct FPBNT contained the entire N-terminal domain of the β' -part (A574–D763); FPBNTACaM1

contained the N-terminal domain except for the CaM1 site (Δ L682–V696); FPBCT1 (M993–K1269) contained almost the entire C-terminal domain beginning shortly after transmembrane segment S6, including the linker region and the entire cGMP-binding motif; FPBCT2 (M993–S1146) contained the linker region, helix A, and sheets β_1 to β_6 of the β role of the cGMP-binding site (for review see Finn *et al.*, 1996). FPBCT3 (K1130–K1269) carried sheets β_5 to β_8 and helices B and C. An N-terminal cysteine was introduced in front of K1130 to allow immobilization via an SH-group; FPBCT3ACaM2 lacked the CaM2 site (L1242–E1256); FPBCT4 (K1130–N1202) carried β_5 to β_8 and helices B and C, but lacked the C-terminal half of FPBCT3. All fusion proteins were purified via an amylose column; purity $\geq 90\%$. In addition fusion construct FPBNT was cleaved from the MBP and subsequently purified to yield BNT. Constructs of N-terminal and C-terminal domains were immobilized either via SH- or NH_2 -groups. Immobilization of C-terminal fusion constructs via NH_2 -groups proved difficult; in the few successful experiments, perfusion with CaM gave rise to small, poorly reproducible SPR signals or no signal. We interpret this result to indicate that residues that are important for CaM recognition have been modified by the immobilization. We therefore introduced an additional cysteine at the NH_2 -terminus to immobilize these constructs via a thiol group.

CaM was immobilized by three different means: biotin-labelled bovine CaM via streptavidin on the chip surface; bovine CaM via NH_2 -groups; and spinach CaM (Watterson *et al.*, 1980; Mills *et al.*, 1988) via thiol-coupling at Cys 26. Control experiments with cleaved fusion constructs and purified channel domains or MBP demonstrated that MBP neither binds CaM itself nor does it interfere with CaM binding to channel constructs.

The following constructs were used for functional expression in oocytes. The construct β wt (amino acids 572–1394) lacked the GARP-part; construct $\beta\Delta$ CaM1 lacked the CaM1 site (Δ L682–V696); construct $\beta\Delta$ CT lacked part of the C-terminal region (Δ I203–1394).

Surface plasmon resonance (SPR) measurements

In the following, we use a new terminology recently introduced by Schuck (1997). Interaction between a 'receptor' and a 'ligand' was studied by SPR spectroscopy using the BIAcore system. One of the reactants, designated 'receptor', was covalently attached to the sensor surface. In the association phase of the experiment, a second reactant, designated 'ligand', was introduced in the mobile buffer phase flowing above the sensor surface. In most experiments, the receptor was a peptide derived from the amino acid sequence of the β -subunit from CNG channels of bovine rod photoreceptors (Körschen *et al.*, 1995), the α -subunit from bovine olfactory neurons (Ludwig *et al.*, 1990), a fusion protein (see above), or CaM. The ligand was calmodulin (CaM), native recoverin or recombinant guanylyl cyclase-activating protein (GCAP1). The association of Ca^{2+} -binding proteins with, and their dissociation from, immobilized target sites cause a change in refractive index near the surface of the thin gold film on the sensor chip; this optical change correlates with changes in resonance units. Recordings of changes in refractive index with time are referred to as sensorgrams. For a discussion of the physics of this technique and the definition of resonance units (RU) see Garland (1996) and Schuck (1997). The density of peptides and fusion proteins on the sensor chip surface ranged between 0.4 and 16 ng/mm². Immobilization was monitored using SPR spectroscopy. CaM was purchased from Sigma (Munich; purity 98%). Native recoverin and recombinant guanylyl cyclase-activating protein (GCAP1) were purified as described previously (Frins *et al.*, 1996; Lange and Koch, 1997).

The running buffer contained: 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EGTA, 3 mM CaCl₂, 10 mM MgCl₂, and 0.005% (v/v) Tween-20. The complex formation between Ca²⁺-binding proteins and the immobilized target was measured with ~30–6000 nM of ligand in the running buffer. A sample volume of 20–40 µl was injected into the flow cell. Dissociation of bound ligand was initiated by injecting running buffer without ligand. After each measuring cycle, the sensor chip was regenerated by injection of 5–15 µl running buffer containing 2 mM EGTA and no added CaCl₂ to dissociate all ligand from the immobilized receptor.

The Ca²⁺ buffer solutions used to measure the Ca²⁺ dependence of CaM-binding were identical to the running buffer solution except for the respective Ca²⁺ additions. Free [Ca²⁺] were calculated using the Chelator programme (Schoenmakers *et al.*, 1992) and verified with the Ca²⁺ indicator dye Fura-2. Measured [Ca²⁺] were a maximum of 2-fold lower than calculated [Ca²⁺]. Measured [Ca²⁺] are plotted in Figure 7.

Analysis of sensorgrams

The dissociation constant K_D of receptor–ligand interaction can be determined either from the dependence of the equilibrium plateau signal on the concentration of free ligand in the mobile phase or from kinetic rate constants. Kinetic data are commonly analysed by assuming a reversible bimolecular reaction between the immobilized receptor and the mobile ligand that can be described by the following rate equation:

$$dR/dt = k_a C (R_{\max} - R_t) - K_D R_t \quad (1)$$

where dR/dt is the rate of formation of the complex between the ligand C and the receptor R ; $R_{\max}$ represents the total number of accessible binding sites expressed as resonance units (RU); R_t represents the number of occupied binding sites at time t and, consequently $(R_{\max} - R_t)$ represents the number of free binding sites remaining at time t ; k_a and k_d refer to the chemical rate constants of association and dissociation, respectively. From the change in resonance units with time, dR/dt , k_a and k_d as well as the dissociation constant $K_D = k_d/k_a$ can be calculated. The binding kinetics are often confounded by mass transport of ligand into and within the dextran matrix (Schuck and Minton, 1996; Schuck, 1997). As a consequence, K_D values derived from kinetic data can be seriously in error (Schuck and Minton, 1996; Schuck, 1997). Alternatively, K_D constants can be determined by a Scatchard analysis from the plateau signals at thermodynamic equilibrium, when $dR/dt = 0$, according to

$$dR/dt = k_a C (R_{\max} - R_{eq}) - k_d R_{eq} = 0 \quad (2)$$

Rearrangement of equation 2 and substituting the association constant K_A for k_a/k_d yields

$$R_{eq}/C = K_A R_{\max} - K_A R_{eq} \quad (3)$$

Plotting R_{eq}/C versus R_{eq} , allows determination of K_A (or K_D) and the $R_{\max}$ from the slope and intercept, respectively. This method does not rely on rate constants and therefore K_D values are not compromised by limited mass transport. In a few experiments with high-affinity targets the R_{eq} values were obtained by extrapolation using a curve fitting program. In this paper, K_D values are exclusively derived from R_{eq} measurements.

Inhibition of CaM-regulated neuronal NOS by synthetic peptides and fusion proteins

The ability of synthetic peptides or fusion proteins to bind CaM was also studied by an enzyme-linked *in vitro* assay using neuronal NO-synthase (NOS). Activity of NOS was monitored via the activation by NO of a soluble guanylyl cyclase (sGC) which produced cGMP *in vitro* (Koch *et al.*, 1994; Zoche and Koch, 1995):

As a source of neuronal NOS we used a preparation of photoreceptor outer segments attached to parts of the inner segment (OS-IS) which contain NOS and sGC activities. Formation of cGMP was quantified by HPLC. Increasing amounts (10^{-8} to 10^{-4} M) of CNG channel peptides or fusion proteins were used to bind endogenous CaM and thus prevent NO synthesis and subsequent formation of cGMP.

Overlay assay

Fusion proteins were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore). Filters were blocked by incubation for 1 h with 1% (w/v) milk powder in Tris-buffered saline (TBS): 137 mM NaCl, 3 mM KCl and 5 mM Tris, pH 7.5, followed by incubation for 45 min

with 1 µg/ml biotinylated CaM (bCaM), 0.05% Tween-20, 1 mM CaCl₂ and 0.1% milk powder in TBS. Filters were washed twice for 10 min in the same buffer without bCaM and milk powder and then incubated for 30 min with Extravidin-HRP (1:15 000, Sigma) in the same buffer used for bCaM. Finally, filters were first washed twice for 10 min in the same buffer without milk powder followed by two washes for 5 min in TBS/1 mM CaCl₂. Labelling of polypeptides was detected using the ECL system (Amersham). CaM was labelled with biotin by incubation for 2 h at 25°C in coupling buffer containing: 150 mM NaCl, 100 mM HEPES-Na, pH 7.4, 9 mM NHS-LC-Biotin (Pierce), 1 mM CaCl₂ and 0.05 mM CaM. The reaction was terminated by addition of 50 mM Tris-HCl, pH 7.4. Biotinylated CaM was separated from the coupling reagent by gel filtration using a NAP-5 column (Pharmacia).

Electrophysiology

The α - and β -subunits of the rod CNG channel were expressed heterologously in *Xenopus* oocytes. For co-expression, a short form of the β -subunit was used; this form was lacking the GARP-part of the complete β -subunit (amino acids 572–1394, designated the β' -part; Körschen *et al.*, 1995). There is no apparent difference in the CaM sensitivity between the native channel and hetero-oligomeric channels that lacked the GARP-part of the β -subunit. A mutant β' -subunit, lacking the putative CaM-binding site (amino acids 682–696) was constructed using the polymerase chain reaction. The deletion of the CaM-binding site was verified by sequencing. The final construct was designated $\beta\Delta\text{CaM1}$. *In vitro* transcription of cloned cDNA, injection of cRNA into oocytes and preparation of oocytes for patch-clamp experiments was carried out as described previously (Bönigk *et al.*, 1993). The solution in the pipette and the perfusion medium contained: 100–120 mM K-gluconate, 10 mM HEPES-KOH (pH 7.4), and the indicated additions of cGMP, EGTA, nitrilotriacetic acid (NTA)-Ca²⁺-buffer and CaM. Leak currents recorded in the absence of cGMP were subtracted from currents measured in the presence of the ligand.

The modulation of cGMP-activated currents by CaM was determined in excised inside-out patches. After excision, the membrane patch was perfused for 1–2 min with a solution containing 10 mM EGTA to remove all endogenous CaM that might have adhered to the channel in the oocyte. Subsequently, leak currents were recorded in a solution containing 50 µM free Ca²⁺ (0.8 mM Ca²⁺-gluconate and 2 mM NTA). Finally, current–voltage (I – V) relations were recorded in 50 µM free Ca²⁺ with and without CaM (0.3–0.5 µM) at saturating (500 µM) and roughly half-maximal (40 µM) cGMP concentrations. The formation of hetero-oligomeric channels was tested with 10 µM *L-cis*-diltiazem as described in Körschen *et al.* (1995). In experiments, in which the kinetics of CaM action were investigated, the patch was continuously clamped at –40 mV and superfused with 40 µM cGMP and the indicated concentration of CaM. Current amplitudes were normalized to current levels in the absence of CaM and plotted versus time.

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