Primary structure and functional expression of a *Drosophila* cyclic nucleotide-gated channel present in eyes and antennae

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Cyclic nucleotide-gated (CNG) ion channels serve as downstream targets of signalling pathways in vertebrate photoreceptors and olfactory sensory neurons. Whether CNG channels subserve similar functions in invertebrate photoreception and olfaction is unknown. We have cloned genomic DNA and cDNA encoding a cGMP-gated channel from *Drosophila*. The gene contains at least seven exons. Heterologous expression of cloned cDNA in both *Xenopus* oocytes and HEK 293 cells gives rise to functional ion channels. The *Drosophila* CNG channel is ~50-fold more sensitive to cGMP than to cAMP. The voltage dependence of blockage by divalent cations is different compared with the CNG channel of rod photoreceptors, and the Ca^{2+} permeability is much larger. The channel mRNA is expressed in antennae and the visual system of *Drosophila*. It is proposed that CNG channels are involved in transduction cascades of both invertebrate photoreceptors and olfactory sensillae.

Key words: calcium channel/*Drosophila*/olfactory transduction/photoreception/signal transduction

Introduction

Cation channels that are directly gated by cyclic nucleotides control the flow of ions across the plasma membrane of vertebrate photoreceptors and olfactory sensory neurons. These cyclic nucleotide-gated (CNG) channels serve as terminal targets of signalling pathways that employ either cAMP or cGMP as intracellular messengers. The sequence of events leading to light excitation of rod and cone photoreceptors of the vertebrate retina is well established. In darkness, the cGMP concentration is high and CNG channels are kept open by binding of cGMP. Light closes CNG channels and hyperpolarizes the cell by activating a highly amplifying enzyme cascade that hydrolyses cGMP (for recent reviews, see Kaupp and Koch, 1992; Lagnado and Baylor, 1992; Pugh and Lamb, 1993). Olfactory sensory neurons use a cAMP-signalling pathway to register odorants. Stimulation with odorants opens CNG channels by increasing the intracellular cAMP concentration and thereby depolarizes the membrane (Nakamura and Gold, 1987; Breer *et al.*, 1990).

In microvillar photoreceptors of invertebrates, light evokes a depolarization due to the opening of ion channels

in the plasma membrane. The enzymatic pathway(s) of excitation are poorly understood and the intracellular messenger(s), as well as the light-activated channel(s), have not been unequivocally identified. Substantial evidence implies a light-stimulated phosphoinositide enzyme cascade (for reviews, see Ranganathan *et al.*, 1991; Minke and Selinger, 1992; Hardie, 1993; Hardie and Minke, 1993). In this pathway, the enzyme phospholipase C (PLC) generates diacylglycerol (DAG) and inositol trisphosphate (IP_3) which mobilizes Ca^{2+} from intracellular stores (Brown *et al.*, 1984; Brown and Rubin, 1984; Fein *et al.*, 1984; Payne and Fein, 1987; Baumann and Walz, 1989).

There is also evidence for a second signalling pathway involving cGMP. When injected at specific sites of the rhabdom, cGMP activates *Limulus* ventral photoreceptors (Johnson *et al.*, 1986). Membrane patches excised from the light-sensitive lobe contain channels that can be opened by cGMP, but not by Ca^{2+} (Bacigalupo *et al.*, 1991). The electrical properties of these channels resemble those observed in cell-attached patches during a light stimulus.

Intriguing parallels exist between vision and the chemical senses of invertebrates. Pheromones stimulate the production of IP_3 (Breer *et al.*, 1990) and also cGMP (Ziegelberger *et al.*, 1990) in antennae of silkmoths. Olfactory sensillae contain various ion channels that can be activated by IP_3 (Fadool and Ache, 1992; Stengl, 1994), Ca^{2+} ions (Zufall *et al.*, 1991) and cyclic nucleotides (Zufall and Hatt, 1991; Michel and Ache, 1992). As with invertebrate photoreceptors, the sequence of intracellular events and the precise physiological functions these channels subserve in olfactory signal transduction are poorly understood.

To study the role of CNG channels in sensory organs of invertebrates, we have cloned cDNA encoding a CNG channel from *Drosophila*. The CNG channel gene is expressed in the visual and the olfactory system. The electrical properties of the cloned CNG channel resemble those of light-activated channels in *Drosophila* photoreceptors. In particular, the CNG channel is highly permeable to Ca^{2+} ions, suggesting that it mediates a light-induced Ca^{2+} entry into cells. Although these results do not identify the cloned CNG channel as the light- or odorant-activated channel in a rigorous sense, they support the notion that CNG channels are involved in the transduction of light and chemical stimuli by invertebrates.

Results

Cloning of a CNG channel from *Drosophila melanogaster*

In order to circumvent problems arising from tissue- and development-specific expression of CNG channel genes,

Fig. 1. Nucleotide and deduced amino acid sequence of DmCNGC. **(A)** Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue of the ATG initiation triplet and the preceding residues are indicated by negative numbers. The deduced amino acid sequence (in one-letter code) is shown above the nucleotide sequence. Amino acid residues are numbered beginning with the initiating methionine. The position of the last residue in each line is given on the right-hand side. The ATG triplet of the initiating methionine is doubly underlined. A nonsense codon preceding the ATG initiating triplet in the same reading frame and the stop codon are underlined. The putative transmembrane segments H1–H5, the voltage-sensor-like motif S4, the pore-forming region and the cyclic nucleotide-binding domain are overlined and labelled. The positions of the introns are marked by arrowheads. Potential *N*-glycosylation sites are indicated by open circles. **(B)** Alignment of the amino acid sequences of the pore regions of cyclic nucleotide-gated ion channels. DmCNGC: *D. melanogaster* CNG channel; BOVCONE: bovine cone photoreceptor CNG channel (Weyand *et al.*, 1994); BOVOLF: bovine olfactory CNG channel (Ludwig *et al.*, 1990); BOVROD: bovine rod photoreceptor channel (Kaupp *et al.*, 1989). Negatively charged amino acids are depicted as white letters against black, positively charged residues as black letters against grey. The positions of the amino acid residues are given at the left- and right-hand sides.

we initially screened a genomic *Drosophila* DNA library under reduced stringency with a bovine rod cDNA probe. A genomic clone thus isolated was used to screen 3×10^6 recombinants of a head-specific cDNA library. One clone (DmCNGC) was isolated that harbours the entire coding region. Figure 1A shows the nucleotide and deduced amino acid sequences of DmCNGC.

The *Drosophila* CNG channel cDNA consists of 2927 nucleotides. One long open reading frame was identified. The translation initiation site was assigned to the first triplet (positions 125–127 of cloned cDNA) that appears downstream of a nonsense codon in the same reading

frame. The nucleotide sequence TTTGTATGC flanking the initiating codon does not agree either with the consensus sequence for eukaryotic initiation sites (CCACCATGG; Kozak, 1984) or with that preferentially used in *Drosophila* genes (CCAA^A/CATGG; Cavener and Ray, 1991). An in-frame TAA translation termination codon is found at positions 1996–1998 (see Figure 1A). The cDNA contains a poly(dA) tract of 14 adenine residues which is preceded by a polyadenylation signal (AATAAA; Proudfoot, 1984). The size of the DmCNG transcript estimated by Northern blot analysis is larger (~3.6 kb; data not shown) than that of cloned cDNA. The deduced polypeptide sequence

consists of 665 amino acid residues with a calculated molecular mass of 75 993 Da.

Structural features of the *Drosophila* CNG channel

DmCNGC contains sequence motifs that are characteristic of the family of CNG channel proteins (for a review, see Eismann *et al.*, 1993): a central hydrophobic region that comprises six membrane-spanning segments, a cyclic nucleotide-binding domain within a partially hydrophobic region near the C-terminus, a pore-forming region and a residual voltage-sensor (S4) motif. The amino acid sequence of DmCNGC is similar to those of vertebrate CNG channels. Between the *Drosophila* and vertebrate CNG channels 61.9–64.1% (80.0–82.5%) of the aligned positions of these sequence motifs are occupied by identical or conserved (in parentheses) residues. The pore motif is highly conserved among CNG channels, and DmCNGC only contains a few non-conserved substitutions in the C-terminal third of this segment (Figure 1B). Whether these amino acid changes are responsible for the distinct interaction of divalent cations with the *Drosophila* CNG channel (see below) needs to be examined by future work. Furthermore, N-terminal to the S4-segment exists an insertion of 12 uncharged residues that is lacking in the vertebrate CNG channels.

Consensus sites for N-linked glycosylation are located in the first and third extracellular domain of DmCNGC (open circles in Figure 1A). Glycosylation in the third extracellular domain has been previously described for rod photoreceptor channels (Molday *et al.*, 1991; Wohlfart *et al.*, 1992).

Organization of the *DmCNGC* gene

We analysed the genomic organization of the *Drosophila* CNG channel by sequencing 7.5 kb of genomic DNA derived from overlapping recombinants. Comparison of genomic and cDNA nucleotide sequences revealed that the DmCNGC gene is composed of seven exons (Figure 2A). Consensus sequences of splice donor/acceptor sites are observed at most of the exon/intron boundaries. The genomic sequence differs in one position from the cDNA sequence, changing amino acid residue 73 from a glycine to an arginine. This variation is most likely due to a strain difference, because the genomic library was constructed from wild-type *Canton S* DNA, whereas cDNA was prepared from mRNA of wild-type *Berlin*. A stretch of 14 (da) residues located at the 3' end of the cDNA clone is absent in the genomic sequence and, therefore, represents the poly(A)⁺ tract of the native mRNA. Recently, the genomic organization of the human rod CNG channel has been reported. The N-terminal third of the polypeptide is encoded by seven exons, whereas the C-terminal two-thirds, beginning after H2, are encoded by a single large exon (Dhallan *et al.*, 1992). The exon/intron distribution in the *Drosophila* CNG channel gene is different; splicing occurs throughout the entire coding region. Functional domains are encoded by separate exons. Exon 3 codes for the H1/H2 segment, exon 4 for segments H3–H5 including the pore region and the voltage-sensor-like motif S4, and exon 5 encodes the cyclic nucleotide-binding site. Only the splice junction flanking the H2 segment (Figures 1A and 2A) is shared by the human and the *Drosophila* gene. Although the overall sequence similarity between

Fig. 2. Structure of the DmCNGC transcription unit. (A) Restriction map of part of the cloned genomic interval harbouring the DmCNGC gene. The distance is given in kbp. *EcoRI* = E; *HindIII* = H; *SacI* = S. Coding regions of the DmCNGC transcript are indicated by hatched boxes, non-translated regions are indicated by open boxes. Exons are numbered (1–7); the poly(A)⁺ tract is indicated by (AAA). The positions of the exon/intron boundaries are projected onto a schematic organization of the channel polypeptide. Functional domains are indicated. (B) Whole-genome Southern blot analysis with DmCNGC cDNA. *Drosophila melanogaster* genomic DNA was digested with *EcoRI* (lanes 1 and 4), *HindIII* (lanes 2 and 5), *SacI* (lanes 3 and 6), and bovine genomic DNA was digested with *EcoRI* (lanes 7 and 10), *HindIII* (lanes 8 and 12), *PstI* (lanes 9 and 13) and *SacI* (lanes 11 and 14). Fragments were separated on 0.75% agarose gels. Hybridizations performed under conditions of high stringency in buffer containing 50% formamide (lanes 1–3 and 11–14) and hybridizations performed under conditions of low stringency in buffer containing 30% formamide (lanes 4–6 and 11–14) are shown. Phage λ DNA digested with *EcoRI/HindIII* was used as a size marker.

vertebrate and *Drosophila* CNG channel genes is striking, the genomic organization has not been well conserved throughout evolution.

So far, three different vertebrate CNG channel genes have been identified that are expressed in rod and cone photoreceptors, olfactory sensory neurons, aorta, kidney and sperm (for a review, see Yau, 1994). In order to investigate whether more than one CNG channel gene does exist in the *Drosophila* genome, we hybridized whole-genome Southern blots with a DmCNGC cDNA probe. At high stringency, only fragments predicted from the restriction map of the DmCNGC gene were detected in *Drosophila* DNA (Figure 2B, lanes 1–3). When the

Fig. 3. PCR analysis of expression pattern of the DmCNGC gene. Total RNA was prepared from antennae, eyes, remaining heads and bodies of adult flies (wild-type strain: *Oregon R*). PCR amplification was performed with first-strand cDNA. PCR products of 332 bp length were obtained from antennae (lane 2), eyes (lane 3) and the remaining head fraction (lane 4), as well as from the positive control performed on clone pcDmCNGC (lane 7). A 1450 bp fragment was obtained from body-derived cDNA (lane 5) due to amplification of genomic DNA. RNA preparations from antennae (lane 8) and eyes (lane 9) treated with RNase before cDNA synthesis only gave rise to the 1450 bp genomic fragment in the eye preparation (lane 9). No PCR products were amplified in the water control (lane 6). Phage λ DNA digested with *EcoRI/HindIII* was used as a size marker (lanes 1 and 10).

stringency of hybridization was reduced no additional bands were detected (Figure 2B, lanes 4–6), whereas several signals were observed under these conditions in a Southern blot analysis of bovine DNA with the DmCNGC probe (Figure 2B, lanes 7–10). Most notably, when the blot of bovine genomic DNA was hybridized under conditions of high stringency no signals were detected (Figure 2B, lanes 11–14). We conclude from these results that *Drosophila* only contains a single CNG channel gene that is highly homologous to vertebrate CNG channel genes.

Expression pattern of DmCNGC gene

Distribution of channel-specific mRNA was studied by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification experiments. Because the DmCNGC clone was isolated from an adult head cDNA library, we restricted the analysis of expression pattern to this developmental stage. Fragments were amplified with a pair of primers that allow discrimination between PCR products amplified from cDNA and contaminating genomic DNA. Primer #1 anneals to the 3' end of exon 4 and primer #2 to the 5' end of exon 5. The expected length of the fragment amplified from cDNA is 332 bp, and for that from genomic DNA it is 1450 bp.

PCR products of 332 bp were amplified from first-strand cDNA of antennae and eyes from wild-type *Oregon R* (Figure 3, lanes 2 and 3). A faint 332 bp band was also amplified from the head fraction (Figure 3, lane 4). The body-derived cDNA only yielded the 1450 bp fragment (Figure 3, lane 5). A 332 bp product was also amplified from antennae and heads of the *Drosophila* mutant eyes absent (*eya*) (data not shown). From samples treated with RNase, the 1450 bp fragment, but not the 332 bp fragment, could be amplified (Figure 3, lane 9).

The nucleotide sequence of the 332 bp fragments is identical with the original DmCNGC sequence, except for a few nucleotide exchanges that are most likely due to

strain variations. Genomic DNA cloned from strain *Canton S* and cDNA cloned from strain *Berlin* both contain an *EcoRI* restriction site within the amplified nucleotide sequence. PCR fragments amplified from cDNA of strain *Oregon R* and the *eya* mutant are lacking this particular *EcoRI* restriction site due to a single nucleotide exchange. These findings prove that the amplified 332 bp PCR products are not the result of contamination by cloned cDNA.

Heterologous expression of DmCNGC

Expression of synthetic mRNA derived from clone pGEM-DmCNGC gave rise to cGMP-stimulated channel activity in *Xenopus* oocytes. Maximal current amplitudes (range 30–1300 pA/excised patch) at saturating cGMP concentrations were similar to those previously described for other CNG channels expressed in *Xenopus* oocytes (Kaupp *et al.*, 1989; Altenhofen *et al.*, 1991; Goulding *et al.*, 1992; Bönigk *et al.*, 1993; Weyand *et al.*, 1994).

A series of macroscopic current–voltage (*I*–*V*) recordings from inside-out patches at various cGMP concentrations is shown in Figure 4A. The relationship between normalized currents ($I/I_{\max}$) and cGMP or cAMP concentrations is shown in Figure 4B. Mean values of concentrations for half-maximal activation ($K_{1/2}$) and Hill coefficients (*n*) are summarized in Table I. cAMP activated the *Drosophila* channel at ~50-fold higher concentrations than cGMP. The $K_{1/2}$ value for the activation by cGMP of ~13 μ M is independent of membrane potential ($V_m \pm 80$ mV). The respective $K_{1/2}$ values of heterologously expressed DmCNGC are intermediate between those of mammalian photoreceptor and olfactory CNG channels (see triangles in Figure 4B).

Unlike the macroscopic *I*–*V* relationships of the heterologously expressed vertebrate CNG channels, which are either outwardly rectifying or exhibit a sigmoidal shape (Kaupp *et al.*, 1989; Dhallan *et al.*, 1990; Altenhofen *et al.*, 1991; Goulding *et al.*, 1992; Bönigk *et al.*, 1993; Weyand *et al.*, 1994), the *I*–*V* relationship of the *Drosophila* CNG channel was linear at all cGMP concentrations ($I_{-80 \text{ mV}}/I_{+80 \text{ mV}} = 1.02 \pm 0.09$; $n = 14$; for saturating cGMP). Figure 4C shows single-channel recordings from an inside-out membrane patch of HEK 293 cells transfected with pcDmCNGC. The single-channel *I*–*V* relationship was perfectly linear between +80 and –80 mV with a slope conductance of 36 pS (Figure 4D). For comparison, the moderately rectifying *I*–*V* relationship of the bovine cone photoreceptor CNG channel (Weyand *et al.*, 1994) is also shown in Figure 4D. The slope conductance of this CNG channel at 0 mV is ~15 pS. The shape of the macroscopic *I*–*V* relationship is determined by the voltage dependence of the open probability and by the ion permeation process itself. Because the macroscopic and the microscopic current–voltage relationships are linear, the open probability of DmCNGC is also expected to be independent of the membrane potential.

Selectivity for mono- and divalent cations

The selectivity for monovalent cations has been determined in inside-out patches of *Xenopus* oocyte membranes by measuring the reversal potential (V_{rev}) under symmetrical bi-ionic conditions with K^+ in the pipette (Figure 4E). Mean values for $V_{\text{rev}} \pm \text{SD}$ (number of experiments) are

Fig. 4. Electrophysiological properties of *Drosophila* CNG channel expressed in *Xenopus* oocytes and HEK 293 cells. **(A)** Series of current–voltage recordings from inside-out patches in the presence of different cGMP concentrations indicated at the respective current trace. Inset: current–voltage recordings at saturating cAMP (10 mM) and cGMP (1 mM) concentrations. **(B)** Ligand sensitivity of normalized currents $I/I_{\max}$ at -80 mV for cGMP (●) and cAMP (○). The smooth lines represent the fitted dose–response curves based on mean values of $K_{1/2}$ and n (see Table I). Circles represent data from single experiments. Triangles indicate concentrations of half-maximal activation ($K_{1/2}$) for cGMP (▼) and cAMP (▽) of the bovine olfactory CNG channel, and for cGMP (▲) and cAMP (△) of the bovine rod photoreceptor CNG channel. **(C)** Single-channel currents recorded from an excised inside-out patch of HEK 293 cells transfected with pcDmCNGC. $V_m = +50$ mV. The closed state of the channel is indicated by ‘c’. **(D)** Voltage dependence of single-channel currents of DmCNGC expressed in HEK 293 cells. Filled symbols represent two different experiments with DmCNGC recorded with $3 \mu\text{M}$ cGMP. From the straight line a conductance of 36 pS was calculated. Open symbols show single-channel currents of the bovine cone photoreceptor CNG channel expressed in *Xenopus* oocytes in the presence of $30 \mu\text{M}$ cGMP. **(E)** Selectivity for monovalent cations. Series of current–voltage recordings obtained from an excised inside-out patch under symmetrical bi-ionic conditions with K^+ in the pipette. cGMP concentration was $100 \mu\text{M}$.

Table I. Properties of heterologously expressed *Drosophila* CNG channel

	+80 mV	–80 mV
$I_{\text{cAMP}}/I_{\text{cGMP}}^a$	0.69 (9)	0.58 (9)
$K_{1/2}$ (cGMP) (μM)	13.1 ± 5 (14)	12.4 ± 3.9 (14)
$K_{1/2}$ (cAMP) (μM)	406.4 ± 64 (7)	507.5 ± 114 (7)
Hill coefficient (cGMP)	1.5 ± 0.6 (14)	1.7 ± 0.4 (14)
Hill coefficient (cAMP)	1.5 ± 0.3 (7)	1.4 ± 0.3 (7)
Single-channel conductance ^b (pS)	36	36
K_{Ca} (μM)	481.5 ± 130.1 (7)	352.3 ± 150.3 (7)
Relative ion permeability ^c	$\text{Ca}^{2+}:\text{Mg}^{2+}:\text{NH}_4^+:\text{K}^+:\text{Na}^+:\text{Li}^+:\text{Rb}^+:\text{Cs}^+ = 29.3:6.2:2.3:1:0.95:0.94:0.84:0.58$	
Divalent ion conductance ^d	$\text{Na}^+:\text{Ca}^{2+}:\text{Sr}^{2+}:\text{Ba}^{2+}:\text{Mg}^{2+} = 1:0.11:0.08:0.04:0.01$	

Mean values $\pm$ SD (number of experiments).

^aMaximum currents were determined with 500 – $1000 \mu\text{M}$ cGMP and 10 mM cAMP.

^bSingle-channel conductance was determined in 120 mM NaCl, 10 mM HEPES–NaOH and 10 mM EGTA–NaOH at pH 7.4 .

^cDetermined from V_{rev} .

^dDetermined from currents at $+100$ mV with 100 mM of the respective divalent cation.

NH_4^+ : -21.5 ± 2.5 mV (8); Na^+ : 1.0 ± 0.7 mV (7); Li^+ : 0.96 ± 0.7 mV (6); Rb^+ : 4.8 ± 0.4 mV (8); Cs^+ : 14.3 ± 0.7 mV (7). Relative ion permeabilities P_X/P_K derived from V_{rev} are given in Table I. Although small quantitative

differences exist, relative ion permeabilities are largely similar to those of cloned mammalian CNG channels.

A P_{Ca}/P_K ratio of 29.3 [$V_{\text{rev}} = -41.4 \pm 14.3$ mV (14)] demonstrates that the DmCNGC is much more permeable

Fig. 5. Blockage by divalent cations of DmCNGC. (A) Current–voltage recordings obtained from an outside-out patch of *Xenopus* oocyte membranes in the presence of various Ca^{2+} concentrations indicated at the respective current trace. cGMP concentration in the pipette solution was 1 mM. (B) Voltage dependence of current ratio $I/I_{\max}$ at various Ca^{2+} concentrations. (C) Dose dependence of Ca^{2+} blockage at -60 mV for DmCNGC and bovine rod CNG channel. Mean $K_i = 229.2 \pm 43.6$ μM , $n = 0.87 \pm 0.14$ (DmCNGC) and 4.1 ± 1.0 μM , $n = 0.85 \pm 0.15$ (BOVRD). (D) Voltage dependence of K_i constants for DmCNGC and BOVRD.

for Ca^{2+} ions than for monovalent cations. In fact, its relative Ca^{2+} permeability is almost 20-fold larger than that of the rod photoreceptor CNG channel [$P_{\text{Ca}}/P_{\text{K}} = 1.7$; $V_{\text{rev}} = -5.4 \pm 2.2$ mV (11)]. The permeability for Mg^{2+} ions determined under similar conditions was roughly five times smaller than for Ca^{2+} ions [$P_{\text{Mg}}/P_{\text{K}} = 6.2$; $V_{\text{rev}} = -21.8 \pm 5.3$ mV (5)].

Ca^{2+} blockage

An important feature of CNG channels is their ability to pass Ca^{2+} ions and thereby block the Na^{+} current in a voltage-dependent fashion. Ca^{2+} ions block the rod photoreceptor CNG channel by binding to sites within the channel pore (Root and MacKinnon, 1993; Eismann *et al.*, 1994). We examined the Ca^{2+} blockage of DmCNGC in outside-out patches of *Xenopus* oocyte membranes.

Ca^{2+} ions blocked the *Drosophila* CNG channel from the extracellular side in a strongly voltage-dependent fashion. Figure 5A shows a series of I – V recordings from an outside-out patch at various external Ca^{2+} concentrations. Without Ca^{2+} , the I – V relationship was linear, but became strongly out- and inwardly rectifying in the presence of external Ca^{2+} . Figure 5B shows the voltage dependence of the ratio of currents in the presence (I) and

absence ($I_{\max}$) of Ca^{2+} ($I/I_{\max}$). In the range from $+80$ to 0 mV, current inhibition by Ca^{2+} was weak at large positive potentials and increased when V_m was made less positive. Such behaviour is typical of a blocking ion that binds within the pore, with binding dependent on voltage. Blockage was maximal at a critical potential (mean value of $V_c \sim -2.5$ mV) and was relieved with further hyperpolarization (Figure 5B). The value of V_c was largely independent of the external Ca^{2+} concentration. For the *Drosophila* CNG channel, a complete relief of current inhibition at each negative V_m with respect to the corresponding positive V_m was observed at all Ca^{2+} concentrations, resulting in an almost symmetrical U-shaped voltage dependence. In contrast, the $I/I_{\max}$ versus V_m relationship of the rod CNG channel was highly asymmetrical and a partial relief of blockage with hyperpolarization was only observed at low Ca^{2+} concentrations (≤ 50 μM ; Eismann *et al.*, 1994).

We determined the concentration of half-maximal current inhibition (K_i) by a least-squares fit procedure using the Hill equation $I/I_{\max} = 1 - [\text{Ca}]^n / ([\text{Ca}]^n + K_i^n)$. Figure 5C shows the dependence of the relative current $I/I_{\max}$ on the external Ca^{2+} concentration. At -60 mV, the resting potential of *Drosophila* photoreceptors, DmCNGC

Fig. 6. Ca^{2+} conductance of DmCNGC. (A) Comparison of Na^+ currents with currents carried by divalent cations. I - V recordings from an inside-out patch of transfected HEK 293 cells. The pipette solution contained the impermeable cation NMDG. Currents were strictly outward rectifying and could only be carried by the cation tested. (B) Recordings from (A) displayed on an expanded current scale. (C) 8BrGMP-induced Ca^{2+} entry into HEK 293 cells transfected with pcDmCNGC. At the beginning of each trace, the $[\text{Mg}^{2+}]$ was lowered from 10 mM to the indicated values. (D) Dependence of the rate of Ca^{2+} entry on the external Mg^{2+} concentration. The rate of $\Delta[\text{Ca}^{2+}]_i$ was obtained from the slope of fluorescence traces measured within 20–40 s after changing $[\text{Mg}^{2+}]$. Normalized rates of Ca^{2+} from three different experiments: $K_{\text{Mg}} = 1.5 \pm 0.5$ mM, $n = 1.8$. For comparison, data for the bovine cone photoreceptor CNG channel are also shown ($K_{\text{Mg}} = 31 \pm 1.9$ μM , $n = 1.8$; taken from Weyand *et al.*, 1994).

was >50-fold less sensitive to Ca^{2+} blockage than the rod photoreceptor CNG channel (Figure 5C). Mean values for K_i were 229.2 μM for DmCNGC and 4.1 μM for the rod photoreceptor CNG channel. Although it was not possible to determine K_i of DmCNGC at exactly V_c , it is safe to say that it was ≤ 5 μM , and therefore largely similar to the minimum K_i value of the rod photoreceptor CNG channel (Figure 5D).

Ca^{2+} conductance

The relative conductance for divalent cations was measured in inside-out patches from HEK 293 cells transfected with pcDmCNGC. cGMP-activated currents were recorded with only one permeable cation at a time and an ion activity of 100 mM (Figure 6A and B). Relative conductances are shown in Table I. The Ca^{2+} current measured under these conditions was $11 \pm 1.8\%$ (6) of the current carried by Na^+ . Colamartino *et al.* (1991) reported that the relative Ca^{2+} conductance of the rod photoreceptor CNG channel is only 1.2%. This comparison

further illustrates the relatively high Ca^{2+} conductance of the *Drosophila* CNG channel.

To study Ca^{2+} permeation at physiological concentrations of divalent cations, we measured cGMP-induced Ca^{2+} entry into transfected HEK 293 cells by FURA-2 fluorescence imaging. In these experiments, 8BrGMP was used to activate DmCNGC in the presence of different extracellular Mg^{2+} concentrations.

The resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in HEK 293 cells was <100 nM. In the presence of 10 mM Mg^{2+} and 1 mM Ca^{2+} in the external medium, superfusion of pcDmCNGC transfected HEK 293 cells with 8BrGMP did not significantly increase $[\text{Ca}^{2+}]_i$. When the external Mg^{2+} concentration was rapidly lowered in the presence of 8BrGMP, $[\text{Ca}^{2+}]_i$ increased (Figure 6C). The 8BrGMP-induced rise in intracellular Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$) could be repetitively evoked when cells had recovered from each preceding stimulation after washout of cyclic nucleotides. The $\Delta[\text{Ca}^{2+}]_i$ was dependent on extracellular Ca^{2+} . When Ca^{2+} was removed

from the superfusing medium, 8BrGMP failed to evoke a $\Delta[\text{Ca}^{2+}]_i$. These results suggest that $\Delta[\text{Ca}^{2+}]_i$ does not originate from intracellular Ca^{2+} stores, but from Ca^{2+} entry through the *Drosophila* CNG channel. The rate of increase in $[\text{Ca}^{2+}]_i$ was dependent on the Mg^{2+} concentration. The initial rate of $\Delta[\text{Ca}^{2+}]_i$ (ΔF) was determined for each Mg^{2+} concentration within 40 s after changing $[\text{Mg}^{2+}]$ from 10 mM to the respective test concentration. The resulting dependence of ΔF on $[\text{Mg}^{2+}]$ is illustrated in Figure 6D. The solid line in Figure 6D represents a least-squares fit of the Hill equation $\Delta F/\Delta F_{\max} = 1 - [\text{Mg}^{2+}]^n / ([\text{Mg}^{2+}]^n + K_{\text{Mg}})$, where $\Delta F_{\max}$ is the initial rate of $\Delta[\text{Ca}^{2+}]_i$ in nominally Mg^{2+} -free solution. The concentration of half-maximal inhibition of the Ca^{2+} entry by Mg^{2+} (K_{Mg}) was 1.5 ± 0.5 mM (3). In comparison, Mg^{2+} blocked Ca^{2+} entry through the bovine cone photoreceptor CNG channel at much lower concentrations [$K_{\text{Mg}} = 31 \pm 1.9$ μM (5)]. The Hill coefficient of inhibition was 1.8 for both CNG channels. These results demonstrate that the *Drosophila* CNG channel carries a significant Ca^{2+} current that is only weakly sensitive to Mg^{2+} blockage at physiological ion concentrations.

Discussion

We have cloned cDNA from *Drosophila melanogaster* encoding a novel member of the CNG channel family that is expressed in eyes and antennae. The electrical properties of the heterologously expressed channel resemble those of the light-activated channel of *Drosophila* photoreceptors. The high Ca^{2+} permeability suggests that the CNG channel is involved in a cGMP-signalling pathway that might regulate the entry of Ca^{2+} into photoreceptors and olfactory sensillae.

Drosophila owns a single CNG channel gene

Three distinct genes have been previously identified that encode CNG channels from vertebrate rod and cone photoreceptors, and olfactory sensory neurons (Kaupp *et al.*, 1989; Dhallan *et al.*, 1990; Ludwig *et al.*, 1990; Goulding *et al.*, 1992; Bönigk *et al.*, 1993). CNG channels from kidney, aorta, retinal ganglion cells and testis are each encoded by one of these genes (Ahmad *et al.*, 1992, 1994; Biel *et al.*, 1993; Weyand *et al.*, 1994).

Photoreceptor and olfactory CNG channels differ from each other in several functional aspects. First, photoreceptor CNG channels are 30–50 times less sensitive to both cyclic nucleotides than olfactory CNG channels. Second, the difference in maximal currents ($I_{\max}$) activated by cAMP and cGMP is a characteristic feature that distinguishes photoreceptor from olfactory CNG channels (Tanaka *et al.*, 1989; Altenhofen *et al.*, 1991; Zufall *et al.*, 1991). For photoreceptor CNG channels, $I_{\max}$ activated by saturating cAMP concentrations is much smaller than $I_{\max}$ activated by cGMP ($I_{\text{cAMP}}/I_{\text{cGMP}} = 0.2$), whereas cAMP- and cGMP-activated currents through olfactory CNG channels are of equal amplitude. For the *Drosophila* CNG channel, the maximal cAMP-activated current was ~60% of the cGMP-activated current amplitude (Figure 4A; inset). Thus, the ligand sensitivity and the $I_{\text{cAMP}}/I_{\text{cGMP}}$ ratio of the *Drosophila* CNG channel are intermediate between those of the bovine olfactory and the photoreceptor channels. Moreover, the percentage of positions

in the aligned amino acid sequences of DmCNGC and vertebrate photoreceptor or olfactory CNG channels occupied by identical or conserved residues varies only between 80 and 82.5%. Therefore, the *Drosophila* CNG channel cannot be assigned to either one of the two channel subfamilies of vertebrates.

The absence of additional cross-hybridizing bands on genomic Southern blots probed under low stringency suggests that *Drosophila* may utilize a single type of vertebrate-like CNG channel. Two other cyclic nucleotide-sensitive channel species have been previously described in *Drosophila*. The *eag* gene encodes a voltage-dependent potassium-selective channel (Warmke *et al.*, 1991) whose activity is modulated by cAMP (Brüggemann *et al.*, 1993). Second, larval muscle contains a potassium channel that is directly activated by cAMP, but not cGMP (Delgado *et al.*, 1991). These cyclic nucleotide-sensitive potassium channels most likely belong to distantly related gene families and would not give rise to additional bands in a Southern blot analysis. Moreover, DmCNGC transcript was detected in the visual and the olfactory system of *Drosophila* by RT-PCR. These results argue that *Drosophila* utilizes a single type of CNG channel and that the distinct CNG channel genes of vertebrates evolved later in their phylogeny.

DmCNGC and light-activated channels share common properties

Several properties of heterologously expressed DmCNGC resemble those of the light-activated channels *in situ* from *Drosophila* and other invertebrates. Both DmCNGC and the light-activated channels of *Drosophila* (Hardie, 1991; Ranganathan *et al.*, 1991; Hardie and Minke, 1992) and of *Limulus* ventral photoreceptors (Brown and Mote, 1974) are cation selective and do not discriminate very well between alkali ions. Hardie (1991) reported a $P_{\text{Na}}/P_{\text{Cs}}$ ratio of 1:0.73 and Ranganathan *et al.* (1991) reported $P_{\text{Cs}} > P_{\text{K}} \sim P_{\text{Na}}$. The selectivity sequence given in Table I for DmCNGC is in good agreement with these *in situ* data.

The light-activated channel and the cloned CNG channel of *Drosophila* are both highly permeable to Ca^{2+} and Mg^{2+} ions. Most significantly, $P_{\text{Ca}}/P_{\text{Cs}}$ ratios between 25:1 and 40:1, and $P_{\text{Mg}}/P_{\text{Cs}}$ of 6:1, reported by Hardie (1991) for the light-activated channel *in situ*, are very similar to a $P_{\text{Ca}}/P_{\text{K}}$ ratio of ~29:1 and a $P_{\text{Mg}}/P_{\text{K}}$ ratio of ~6:1 determined for DmCNGC. Channels of other arthropod photoreceptors are also permeable to Ca^{2+} (Brown and Blinks, 1974; Ziegler and Walz, 1989).

The single-channel *I*–*V* relationships of DmCNGC, light-activated and cGMP-gated channels of *Limulus* ventral photoreceptors are linear with a slope conductance of 39–42 pS for the *Limulus* channel (Bacigalupo and Lisman, 1983; Bacigalupo *et al.*, 1991) and of 36 pS for DmCNGC (Figure 4D). *In situ* single-channel data from *Drosophila* photoreceptors have not yet been reported. While this comparison is suggestive, it does not prove that DmCNGC in fact represents the light-activated channel.

DmCNGC transcripts were detected in microdissected eyes by PCR amplification. As eyes contain several cell types, we cannot exclude that CNG channels are expressed in cells other than photoreceptors, and further experiments will be required to localize the channel polypeptide at the cellular level by immunocytochemical techniques.

Functional implications of high Ca^{2+} permeability

The most prominent feature that distinguishes DmCNGC from the rod photoreceptor CNG channel concerns the interaction between Ca^{2+} ions and the pore. First, the Ca^{2+} current carried by DmCNGC is ~10-fold larger than the Ca^{2+} current carried by the rod CNG channel. Second, DmCNGC is ~20-fold more selective for Ca^{2+} ions than its rod homologue. Third, the voltage dependence of the Ca^{2+} blockage is entirely different between the two channel species and, finally, the blockage of Ca^{2+} entry by external Mg^{2+} is 500-fold less efficient in DmCNGC than in the cone photoreceptor CNG channel.

Ca^{2+} ions are crucially important for light adaptation in both vertebrate and invertebrate photoreceptors (Lisman and Brown, 1972; Fain and Matthews, 1990), and also facilitate the light response of invertebrates (Payne *et al.*, 1986; Walz, 1992). The different Ca^{2+} permeability probably reflects an evolutionary adaptation to the specific ways by which light responses are generated in these photoreceptor cells.

CNG channels of vertebrate photoreceptors are open in darkness and allow Ca^{2+} to enter the cell. When these channels close in light, the intracellular $[\text{Ca}]$ decreases. The light-stimulated decrease in Ca^{2+} takes part in cellular pathways that control the light sensitivity and response recovery (Koch and Stryer, 1988; Hsu and Molday, 1993; Kawamura, 1993; Lagnado and Baylor, 1994). The rod CNG channel appears to be optimally designed to permit a small but steady entry of Ca^{2+} ions into the dark-adapted cell. At the same time, Ca^{2+} greatly diminishes the single-channel current carried by Na^+ ions and thereby enhances the signal-to-noise ratio of the response to single photons (Yau and Baylor, 1989). Channel blockage seems to be so important for the physiology of rod photoreceptors that it is maximal at exactly the resting potential (approximately -30 mV) and is not appreciably relieved during a hyperpolarizing light response (Eismann *et al.*, 1994).

In contrast to vertebrates, the light-activated channels of invertebrates are closed in darkness and open in light. DmCNGC would be well suited to serve as a light-activated channel. It is exquisitely permeable to Ca^{2+} ions, and the blockage of Ca^{2+} entry is much weaker. Most importantly, at $V_m = -60$ mV, the resting potential of a *Drosophila* photoreceptor cell, the Ca^{2+} blockage of current carried by monovalent cations is virtually absent. These features predestine DmCNGC to rush Ca^{2+} into the cell after opening.

The voltage dependence of Ca^{2+} blockage of DmCNGC would be also consistent with the pronounced Ca^{2+} and voltage dependence of the photoresponse kinetics measured *in vivo* (Hardie, 1991; Ranganathan *et al.*, 1991). When the external $[\text{Ca}^{2+}]$ is raised or as the cell is hyperpolarized, the photoresponse is accelerated. The accelerating effect of hyperpolarization is abolished in the absence of external Ca^{2+} . At more negative potentials and higher extracellular $[\text{Ca}^{2+}]$, more Ca^{2+} can enter the cell and this presumably determines the velocity of the photoresponse.

Ca^{2+} permeability and pore structure

A glutamate residue in the hairpin-like pore motif of the rod photoreceptor CNG channel is crucial for ion permeation and blockage by divalent cations (Root and

MacKinnon, 1993; Eismann *et al.*, 1994). The pore motif of DmCNGC also contains a glutamate residue at the same position (Figure 1B), consistent with a similar maximal potency by which Ca^{2+} ions block both CNG channels. Whereas the Ca^{2+} permeabilities of DmCNGC and the rod CNG channel are entirely different, the sequences of the pore-forming regions are strikingly similar. The first non-conserved residue occurs at the C-terminal end of the pore motif (Glu 320 in DmCNG) which probably furnishes the antechamber of the aqueous pore. We presume that amino acid residues in regions other than the pore motif are important determinants of the Ca^{2+} permeability and it will be interesting to elucidate the molecular basis of the Ca^{2+} selectivity of CNG channels.

Materials and methods

Screening of a genomic *Drosophila* DNA library

A genomic library (Maniatis *et al.*, 1978) was screened at low stringency according to Benton and Davis (1977). Hybridization was performed in $5\times\text{SSC}$ ($20\times\text{SSC}$ is 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), $5\times\text{Denhardt's}$ ($100\times\text{Denhardt's}$ contains 2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone), 0.1 mg/ml denatured herring testes DNA, 0.1% SDS, 30% formamide at 37°C for 14 h (McGinnis *et al.*, 1984). Qiabran filters (Diagen, Germany) were washed twice in $2\times\text{SSC}$, 0.1% SDS for 5 min at room temperature, followed by two washes for 30 min at 55°C . The probe was a 1627 bp *SauI* fragment from bovine rod CNG channel cDNA (Kaupp *et al.*, 1989) encompassing the coding region from transmembrane segment H3 to the 3' non-coding region. Labelled DNA probes were prepared with a DECAprime kit (Ambion, USA) following the manufacturer's instructions. Filters were exposed to X-ray films for ~16 h at -80°C .

Isolation of cDNA clones

A full-length cDNA clone was isolated from a $\lambda\text{gt}11$ -cDNA library prepared from head poly(A)⁺ RNA of *D. melanogaster* (gift of E. Buchner, Würzburg, FRG). The library was screened with a *Drosophila* genomic fragment encoding the cyclic nucleotide-binding domain and part of the C-terminus. Hybridization was in $5\times\text{SSC}$, $5\times\text{Denhardt's}$, 0.1 mg/ml denatured herring testes DNA, 0.1% SDS at 65°C for 14 h. Filters were washed in $1\times\text{SSC}$, 0.1% SDS at room temperature, followed by two washes at 65°C for 30 min. Autoradiography was for 12 h at -80°C .

Restriction maps and sequencing

Restriction maps were derived by a combination of single and double digests, followed by gel electrophoresis of fragments in 0.75% agarose gels. Restriction fragments were subcloned into pBluescript SK⁺ vectors (Stratagene, Germany) by standard cloning techniques (Sambrook *et al.*, 1989). Both strands of the DmCNG channel cDNA were sequenced according to the dideoxy nucleotide chain termination technique (Sanger *et al.*, 1977) using Sequenase Version 2.0 T7 DNA polymerase (US Biochemical). Exons of the DmCNGC gene were also sequenced on both strands, whereas the introns were only sequenced on one strand.

Analysis of genomic DNA

Genomic DNA was prepared from adult flies (wild type: *Oregon R*) and calf thymus tissue essentially as described in Brennan *et al.* (1984). Restriction enzyme-digested DNA was separated on 0.75% agarose gels and transferred to nylon membranes. Pre-hybridization was in $5\times\text{SSC}$, $5\times\text{Denhardt's}$, 0.1 mg/ml denatured herring testes DNA, 0.1% SDS, 50% formamide for 15 min at 42°C . Hybridization under conditions of high stringency was in the same buffer containing $\sim 1\times 10^6$ c.p.m./ml ^{32}P -labelled DmCNGC cDNA for 16 h at 42°C . Washing was in $1\times\text{SSC}$, 0.1% SDS twice for 35 min at 65°C . Hybridization under conditions of low stringency was in buffer containing 30% formamide for 16 h at 37°C . Washing was in $2\times\text{SSC}$, 0.1% SDS twice for 20 min at 52°C .

Construction of pcDmCNGC expression vector

A truncated version of DmCNGC cDNA containing the Kozak consensus sequence (Kozak, 1984) immediately 5' to the initiating methionine was generated by PCR. The following oligonucleotides were used: CGC-

AACGGATCCACCATGCGACATTTCAGTCAAAG and GCGTT-AGCGTCGACCAG corresponding to positions 1–22 and 912–928, respectively. The PCR product and the DmCNGC-pBluescript clone were digested with *Bam*HI and *Aat*II, gel purified and ligated. The resulting recombinant was digested with *Bam*HI and *Nsi*I, gel purified, and the modified cDNA fragment was subcloned into pcDNAI vector (Invitrogen, USA) using *Bam*HI and *Nsi*I restriction sites. The final recombinant was called pcDmCNGC.

Construction of pGEM-DmCNGC transcription vector

A *Bam*HI/*Xba*I fragment was isolated from the pcDmCNGC recombinant and ligated to pGEM-HE vector (gift of P. Tytgat, Leuven, Belgium) also digested with *Bam*HI and *Xba*I. The resulting recombinant was called pGEM-DmCNGC. For *in vitro* transcription (Melton *et al.*, 1984), 5 µg of the plasmid DNA were linearized with *Nhe*I and transcribed with T7 RNA polymerase (Promega, USA) using the manufacturer's buffer system.

PCR amplification of DmCNGC transcripts

Adult flies (wild type: *Oregon R*; mutant: *eya*) were etherized and decapitated. Antennae were microdissected with forceps, eyes were removed with a razor blade. Dissected tissue, as well as the remaining parts of the head and the body, were immediately frozen in liquid nitrogen. RNA was prepared by the urea–LiCl method (Zyskind and Bernstein, 1989) with minor modifications. Total RNA was reverse transcribed into first-strand cDNA using a cDNA synthesis kit from Gibco/Life Technologies (Germany). For PCR reactions, purified cDNA was amplified with the DmCNGC-specific primers #1 CCTAATCTT-CGCCACCATG and #2 GCTTGAGCTTCAGCACCAGGG corresponding to positions 1008–1027 and 1319–1339, respectively. Forty-five cycles were performed according to the following protocol: 1 min at 94°C, 1 min at 57°C, 1 min 15 s at 72°C. The PCR products were gel purified, subcloned into pBluescript vector and sequenced.

Functional expression in oocytes

Channel properties were studied with the patch-clamp technique in inside-out and outside-out patches after expression in *Xenopus* oocytes (Hamill *et al.*, 1981; Methfessel *et al.*, 1986; Stühmer *et al.*, 1987; Kushner *et al.*, 1989). mRNA specific for the *Drosophila* channel was synthesized from the pGEM-DmCNGC construct.

Ligand sensitivity was determined in inside-out-patches as described previously (Altenhofen *et al.*, 1991; Bönigk *et al.*, 1993). Pipette and bath solutions contained (mM): 100 KCl, 10 EGTA, 10 HEPES, pH 7.4 (KOH).

Relative permeabilities of monovalent ions were determined from the reversal potential (V_{rev}) of inside-out patches under symmetrical bi-ionic conditions with K^+ in the pipette and different monovalent cations (X^+) in the perfusion solution (mM): 100 XCl, 5 EGTA, 5 HEPES, pH 7.2 (NMDG). Currents were activated by 1 mM cGMP. Leak currents measured without cGMP were subtracted from each trace.

To measure the relative Ca^{2+} permeability of CNG channels in *Xenopus* oocytes, endogenous Ca^{2+} -activated Cl^- currents were eliminated by substituting gluconate for chloride. An intrapipette salt bridge (similar to Kleene, 1993) was used to shield the Ag/AgCl electrode from the chloride-free solution. Briefly, glass pipettes were coated with Sigmacote (Sigma, USA) from inside and filled with a potassium gluconate solution (mM): 100 KGlu, 5 HEPES, 5 EGTA, pH 7.2 (NMDG). This was followed by an agar bridge (0.1% agarose in the same solution). Finally, a potassium chloride solution [mM: 100 KCl, 10 HEPES, 10 EGTA, pH 7.2 (KOH)] was layered on top of the agar bridge. Pipette offset was corrected after patch excision into identical potassium gluconate solutions on both sides of the patch and in the presence of cGMP. Leak currents, I_{leak} , were recorded in the absence of cGMP with a perfusion solution containing (mM): 50 Ca(Glu)₂, 10 HEPES, pH 7.2 (NMDG). The seal resistance in the presence of calcium was increased and $V_{rev(leak)}$ differed considerably from the expected liquid junction potential (Barry and Lynch, 1991). This might be due to effects of calcium on membrane surface charges (cf. Vogel, 1974). Leak currents were subtracted from currents, I_{total} , measured in the same perfusion solution in the presence of cGMP to yield the currents, I_{CNGC} , mediated by the CNG channel: $I_{total} = I_{CNGC} + I_{leak} = g_{CNGC} (V_{command} - V_{rev(CNGC)}) + g_{leak} (V_{command} - V_{rev(leak)})$. The permeability ratio P_{Ca}/P_K was calculated from a modified Goldman–Hodgkin–Katz equation (Fatt and Ginsborg, 1958): $V_{rev} = RT/2F \cdot \ln (4P_{Ca} \gamma_{Ca} c_{Ca}/P_K \gamma_K c_K)$, where V_{rev} is the reversal potential, c is the concentration, P is the permeability and γ is the activity coefficient of the ions; F , R and T have their usual meaning. γ_K and γ_{Ca} (taken from Robinson and Stokes, 1959) were 0.773 and

0.35, respectively, where $\gamma_{Ca} = (\gamma_{CaCl_2})^2$ according to the Guggenheim convention (Shatkay, 1968), and $\gamma_{Ca} = \gamma_{Mg}$.

Blockage of current by external Ca^{2+} was determined in the outside-out configuration. Pipette and bath solutions were the same as in ligand sensitivity experiments with inside-out patches. The free Ca^{2+} concentration in Ca^{2+} buffers made from EGTA was calculated according to Fabiato (1988) using the association constants from Martell and Smith (1974). Free Ca^{2+} concentrations <100 µM were checked by a spectrophotometric assay using the Ca^{2+} indicator dye arsenazo III. The leak current was estimated by perfusing the patch with a solution containing (mM): 90 *N*-methyl-D-glucamine (NMDG), 10 KCl, 12 KOH, 5 EGTA, 5 HEPES, pH 7.4 (HCl). The current measured at $V_m = -46$ mV, the Nernst equilibrium potential for K^+ ions was taken as leak current. A linear extrapolation of the leak current at -46 mV was subtracted from each $I-V$ trace. Experiments were only analysed when the leak current did not exceed 10% of the cGMP-activated current in a Ca^{2+} -free solution. Because the leak current measured in control oocytes decreased with increasing Ca^{2+} concentrations, this method of leak correction underestimates the cGMP-activated currents at high external Ca^{2+} concentrations and may distort the foot of the dose–response relationship for high Ca^{2+} concentrations. Data were fitted to the Hill equation for each experiment separately. Mean values of K_i and Hill coefficient (n) were used to construct the fitted dose–response curves.

Functional expression in HEK 293 cells

HEK 293 cells were grown at 37°C (with 5% CO₂) in Minimal Essential Medium [supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM glutamine and 1% antibiotic/antimycotic solution (Gibco/Life Technologies, Germany)]. Cells were transfected for 24 h with pcDmCNGC by calcium phosphate co-precipitation (Chen and Okayama, 1987). For measurements, cells were transferred to polylysine-coated coverslips and allowed to grow for another 24 h. Coverslips were placed in an experimental chamber mounted on an inverted microscope. The bath solution contained (mM): 120 NaCl, 5 NaOH, 3 KCl, 1 CaCl₂, 3 MgCl₂, 50 glucose, 10 HEPES, pH 7.4.

Single-channel recordings. Patch-clamp experiments were conducted according to Hamill *et al.* (1981). The pipette solution contained (mM): 120 NaCl, 25 NaOH, 10 EGTA, 10 HEPES, pH 7.4. After sealing of the patch pipette to the cell membrane, CNG channel activity could be recorded from ~30% of the on-cell membrane patches. These patches were excised and exposed to various concentrations of cGMP in pipette solution. Single-channel conductance was derived from amplitude histograms constructed from single-channel current recordings of 30 s duration. Single-channel currents were sampled at 3 kHz and low-pass filtered at 1 kHz.

Recording of macroscopic currents. To achieve a higher density of expressed channels for macroscopic current measurements in inside-out patches, HEK 293 cells were co-transfected with pcDmCNGC and RSVTag (Dhallan *et al.*, 1990) plasmid. Cells were patched in divalent cation-free solution containing (mM): 120 NaCl, 25 NaOH, 3 KCl, 10 EGTA, 10 glucose, 10 HEPES, pH 7.4. The pipette solution contained (mM): 145 NMDG–HCl, 10 EGTA, 10 HEPES, pH 7.4. After excision, the cytoplasmic face of the inside-out patch was first exposed to a Na⁺ solution containing (mM): 110 NaCl, 25 NaOH, 10 EGTA, 10 HEPES, pH 7.4. The calculated ion activity was 100 mM. Subsequently, this solution was replaced by a solution containing different divalent cations (X^{2+}) (mM): 220 XCl₂, 5 NMDG, 10 HEPES, pH 7.4. Currents were recorded in the presence of 1 mM cGMP. Leak currents measured without cGMP for each ion species were subtracted.

Fluorescence imaging of cGMP-induced Ca^{2+} entry

The cGMP-induced rise in $[Ca^{2+}]_i$ was measured by fluorescence spectroscopy as previously described (Weyand *et al.*, 1994). Transfected HEK 293 cells were loaded with the Ca^{2+} -sensitive fluorescent dye FURA-2 (Grynkiewicz *et al.*, 1985) by incubation with 1 µM FURA-2/AM and 0.025% Pluronic 127 (Molecular Probes, USA) for 45 min at 37°C. The bath solution for FURA-2 loading contained (mM): 120 NaCl, 5 NaOH, 3 KCl, 1 CaCl₂, 3 MgCl₂, 50 glucose, 10 HEPES, pH 7.4. $[Ca^{2+}]_i$ was determined using a calcium imaging system (Applied Imaging, UK). Dye-loaded, pcDmCNGC-transfected HEK 293 cells were incubated with 2 mM 8Br-cGMP in the presence of 10 mM Mg²⁺ to block the ligand-activated Ca^{2+} entry. Subsequently, external Mg²⁺ was rapidly changed to a lower concentration. The time course of the ensuing change in $[Ca^{2+}]_i$ was recorded for 60 s after removal of Mg²⁺, normalized to the maximal rate of Ca^{2+} entry (recorded in a nominally

Mg²⁺-free solution) and used to construct the dose–response curve shown in Figure 4D. Complete exchange of solutions took 3–5 s. Higher [Mg²⁺] were tested first. Each test was completed within 1 min, after which the [Mg²⁺] was returned back to 10 mM to allow recovery of the resting cytosolic [Ca²⁺].

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