Disruption of an Intersubunit Interaction Underlies Ca^{2+} -Calmodulin Modulation of Cyclic Nucleotide-Gated Channels

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Cyclic nucleotide-gated channels are key molecular elements for olfactory transduction. Olfactory adaptation caused by repeated exposure to an odorant has been proposed to be mediated by the binding of Ca^{2+} -calmodulin to the NH_2 -terminal domain of the channel, breaking its interaction with the COOH -terminal domain and downregulating the channel. We used a fluorescence resonance energy transfer (FRET) approach to study the structural aspects of this domain–domain interaction under physiological conditions in real time. Fluorescent proteins enhanced cyan fluorescent protein and enhanced yellow fluorescent protein were genetically attached at sites adjacent to the NH_2 - and COOH -terminal interacting domains, respectively, allowing direct observation of molecular rearrangements in intact channels. FRET signals caused by the specific interdomain interaction were observed in both intact cells and excised patches. Comparison of the effective FRET efficiencies demonstrated that the interaction occurs specifically between subunits but not within the same subunit. Binding of Ca^{2+} -calmodulin caused a reversible decrease in FRET with the same time course as channel downregulation. These results suggest that a separation or reorientation of the interacting domains between subunits by Ca^{2+} -calmodulin leads to channel downregulation. The quaternary arrangement presents a structural framework for understanding the molecular mechanism of olfactory adaptation.

Key words: ion channel; Ca^{2+} -calmodulin; olfactory adaptation; signal transduction; GFP mutants; FRET; fluorescence

Introduction

The exposure of olfactory receptor neurons to odorant triggers an elevation of intracellular cAMP levels, and the opening of cyclic nucleotide-gated (CNG) channels in the olfactory cilia (Zagotta and Siegelbaum, 1996; Zufall and Munger, 2001). Opening of these cation-selective channels results in membrane depolarization, which initiates the electrical signal that propagates to the CNS. Permeation of Ca^{2+} ions through activated CNG channels also elevates intracellular Ca^{2+} levels. The Ca^{2+} ion, in turn, causes CNG channels to be subsequently downregulated, a negative feedback mechanism thought to mediate olfactory adaptation (Kurahashi and Menini, 1997).

CNG channels are tetrameric membrane proteins. The olfactory channel comprises three subunit types, CNGA2, CNGA4, and CNGB1b (Dhallan et al., 1990; Goulding et al., 1992; Bradley et al., 1994; Liman and Buck, 1994; Sautter et al., 1998; Bonigk et al., 1999). Each subunit contains six transmembrane segments, with the NH_2 - and COOH -terminal domains located intracellu-

larly, where they are accessible to cytosolic modulators (Zagotta and Siegelbaum, 1996). A mechanism for the downregulation of CNGA2 channels has been proposed previously (Varnum and Zagotta, 1997). The NH_2 -terminal domain contains a binding site for Ca^{2+} -calmodulin, an intracellular protein that inhibits CNG channels in both olfactory receptor neurons and photoreceptor neurons (Hsu and Molday, 1993; Chen and Yau, 1994; Liu et al., 1994; Gordon et al., 1995; Hackos and Korenbrot, 1997). Ca^{2+} -calmodulin has been proposed to disrupt an interaction between the NH_2 -terminal and the COOH -terminal domains that normally produces an autoexcitatory effect on CNGA2 channel activation, and its disruption causes channel inhibition (Varnum and Zagotta, 1997). However, how the NH_2 - and COOH -terminal domains interact within the quaternary structure of the channel protein and whether channel inhibition by Ca^{2+} -calmodulin results directly from the disruption of the interaction remain unclear.

To investigate the domain–domain interaction involved in CNG channel modulation, we used a fluorescence approach to monitor the proximity of the two domains in intact channels under physiological conditions. Enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP), two enhanced green fluorescent protein mutants used previously in “cameleon” Ca^{2+} indicators (Miyawaki et al., 1997), were genetically attached to the interacting domains. Proximity was assessed by the efficiency of fluorescence resonance energy transfer (FRET) between eCFP and eYFP. With FRET, light energy absorbed by a donor fluorophore (eCFP) is transferred nonradia-

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tively to a nearby acceptor fluorophore (eYFP), whose absorption spectrum overlaps the emission spectrum of the donor. The efficiency of energy transfer falls off with the sixth power of the distance between the donor and acceptor molecules. Because of this very strong dependence on the distance, FRET efficiency has been shown to be a sensitive reporter of proximity (Selvin, 1995; Tsien, 1998; Cha et al., 1999; Glauner et al., 1999; Makhina and Nichols, 2001; Zheng et al., 2002). The goals of this study were the following: (1) to validate the existence of an NH_2 -COOH interaction in functional CNG channels in intact membranes, (2) to determine whether this interaction is within a subunit or between subunits, and (3) to determine whether the disruption of this interaction has the time course expected if it underlies Ca^{2+} -calmodulin modulation.

Materials and Methods

Constructs and electrophysiology. In this study a chimeric CNG channel, termed CNGA21, was used that contained the CNGA2 (olfactory channel α -subunit, CNG2) NH_2 -terminal region up to the S1 transmembrane region and the remainder from CNGA1 (rod channel α -subunit, CNG1) (Gordon and Zagotta, 1995). eCFP was attached genetically to the NH_2 -terminal end of the calmodulin binding site (before amino acid P61 in CNGA2); eYFP was attached to the end of the C-helix of the cyclic nucleotide-binding domain (after amino acid D608 in CNGA1). eCFP and eYFP were fused either to different subunits, yielding eCFP-CNGA21 and CNGA21-eYFP, respectively, or to the same subunit, yielding eCFP-CNGA21-eYFP. These cDNAs were subcloned into a high-expression vector that contained the untranslated sequence of the *Xenopus* β -globin gene (Liman et al., 1992). A control construct, eCFP- Δ CNGA21, was made by deleting the calmodulin-binding domain (amino acids P61 to E90) from the eCFP-tagged construct. As a negative control, eYFP was also attached to the COOH terminal of the rat cannabinoid receptor CB1 after amino acid L473 after a short linker sequence ($G_4\text{EF}$). This construct, termed CB1-eYFP, was co-expressed with eCFP-CNGA21 to check for possible nonspecific FRET between molecules. An RNA ratio of 2:1 was used for the co-expression of eCFP and eYFP constructs.

Patch-clamp current recordings were made using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in conjunction with an ITC-16 board driven by Pulse (Heka Elektronik, Lambrecht/Pfalz, Germany). Pipettes with resistance of 200–400 K Ω were used to form giant patches. The pipette solution contained the following (in mM): 130 NaCl, 0.2 EDTA, and 3 HEPES, pH 7.2; the bath solution contained the following (in mM): 126 NaCl, 2 NTA- Na_2 , 3 HEPES, and 0.285 CaCl_2 , pH 7.2. The free Ca^{2+} concentration was 30 μM . cGMP was added to the bath solution to a final concentration of up to 1 mM. Calmodulin was added to the 50 μM cGMP solution to a final concentration of 250 nM. Statistical quantities are given as means $\pm$ SEM. Statistical significance was quantified using Student's *t* test.

Fluorescence recordings. Fluorescence signals from eCFP or eYFP-tagged channels in whole *Xenopus* oocytes were observed under a confocal microscope (Leica, Nussloch, Germany). All measurements were made using the animal hemisphere of the oocytes. Emission spectra of eCFP and eYFP were collected using laser excitation of 458 and 488 nm, respectively, and an emission window of 5 nm. The spectra closely matched the published spectra for these mutant green fluorescent protein (GFP) variants (Heim and Tsien, 1996), suggesting that the fluorescent proteins retained their fluorescence properties as fusion proteins with CNG channels. Under the same condition, uninjected oocytes yielded very low endogenous fluorescence with different spectral properties. A narrow range of photomultiplier tube gain was used to ensure linearity, which was checked by calculating FRET efficiency in a wavelength range covering both strong and weak eYFP emission (Zheng et al., 2002).

Fluorescence signals in excised inside-out patches were observed using the patch-clamp fluorometry (PCF) method (Zheng and Zagotta, 2000, 2003), with modifications. Membrane patches containing CNG channels

tagged with fluorescent proteins were observed under a 40 $\times$ oil-immersion objective [numerical aperture (NA), 1.3] on a Nikon (Tokyo, Japan) Diaphot 300 microscope. eCFP was excited with a monochromator (Cairn, Faversham, UK) at 440 nm. A dichroic mirror of 455 nm was used to separate fluorescence emission from the excitation light. Fluorescence emission was collected using a cooled CCD camera (Princeton Instruments, Trenton, NJ) in conjunction with two filter wheels (Sutter Instruments, Novato, CA). For emission spectra, 10 nm wide bandpass filters were used to cover the range from 460 to 540 nm. Emission filters 480AF30 and 535DF25 (Chroma Technology, Brattleboro, VT) were used to measure peak emission from eCFP and eYFP, respectively, for fluorescence ratio determination. Images were collected and processed with MetaMorph (Universal Imaging, Downingtown, PA).

Calculation of FRET efficiency. Quantification of FRET efficiency between eCFP and eYFP is complicated by the fact that, in a general case, emission at the acceptor wavelength contains three components: eYFP emission caused by FRET, eYFP emission caused by direct excitation, and eCFP emission. We chose a spectrum-based approach to remove contaminations caused by donor emission and direct excitation of the acceptor (see Fig. 3). This method also eliminated errors arising from the transfer function of the recording system, variation in the quantum yield of the acceptor, or variation in the concentration of total fluorescence molecules (Clegg, 1992; Selvin, 1995; Zheng et al., 2002). An eCFP spectrum collected from control oocytes expressing eCFP-tagged channels was recorded and used to subtract eCFP emission from spectra taken with 458 nm excitation from oocytes expressing eCFP/eYFP-tagged channels. This yielded an extracted eYFP emission spectrum, F_{458} , that had two components: that caused by direct excitation, F_{458}^{direct} , and that caused by FRET, F_{458}^{FRET} . F_{458} was normalized by total eYFP emission with 488 nm excitation, F_{488} . The resulting ratio, termed RatioA, can be expressed as:

$$\text{RatioA} = \frac{F_{458}}{F_{488}} = \frac{F_{458}^{\text{direct}}}{F_{488}} + \frac{F_{458}^{\text{FRET}}}{F_{488}} \quad (1)$$

The direct excitation component, $\frac{F_{458}^{\text{direct}}}{F_{488}}$, termed RatioA₀, was experimentally determined with oocytes expressing only eYFP-tagged channels.

Relative FRET efficiency can be quantified in two alternative ways. It can be calculated as the difference between RatioA and RatioA₀, (RatioA – RatioA₀), which is directly proportional to FRET efficiency:

$$\text{RatioA} - \text{RatioA}_0 = \frac{F_{458}^{\text{FRET}}}{F_{488}} \quad (2)$$

Alternatively, the ratio between RatioA and RatioA₀, termed FRET ratio or FR, is calculated as:

$$\text{FR} = \frac{\text{RatioA}}{\text{RatioA}_0} = 1 + \frac{F_{458}^{\text{FRET}}}{F_{458}^{\text{direct}}} \quad (3)$$

Like (RatioA – RatioA₀), FR is directly proportional to FRET efficiency. Because F_{458}^{FRET} and F_{458}^{direct} are measured under identical conditions, FRET ratio can be used to conveniently calculate the effective FRET efficiency, E_{eff} , as follows:

$$E_{\text{eff}} = \frac{\epsilon_{\text{eYFP}}}{\epsilon_{\text{eCFP}}}(\text{FR} - 1), \quad (4)$$

in which ϵ_{eYFP} and ϵ_{eCFP} are the molar extinction coefficients for eCFP and eYFP, respectively (Erickson et al., 2001). When only a single donor and acceptor are present E_{eff} equals the true FRET efficiency, E , from which the distance between the donor and the acceptor can be calculated as:

$$R = R_0 \left[\frac{1 - E}{E} \right]^{\frac{1}{6}}, \quad (5)$$

in which R_0 is the distance at which the FRET efficiency is 50%. However, when there are multiple donors, as is the case in our experiments, direct

Figure 1. Covalent linkage of the fluorescent protein does not change the function of the channel. *A*, Currents from CNGA21 channels (top) and eCFP–CNGA21–eYFP channels (bottom) recorded in 1 mM, 50 μ M, or 0 μ M cGMP solution. Voltage steps from a holding potential of 0 to -20 mV and then $+20$ mV were applied. *B*, cGMP dose–response relations for channels formed by both eCFP–CNGA21 and CNGA21–eYFP subunits before (filled squares) and after (filled circles) Ca^{2+} -calmodulin modulation, and after washing with Ca^{2+} -free solution (open circles). Curves are fits of the Hill equation:

$$\frac{I}{I_{\max}} = \frac{1}{1 + \left(\frac{K_d}{[\text{cGMP}]}\right)^n}$$

with parameters as follows: before Ca^{2+} -calmodulin, $K_d = 38.5 \mu\text{M}$, $n = 2.04$; after Ca^{2+} -calmodulin, $K_d = 111.4 \mu\text{M}$, $n = 1.94$; after wash, $K_d = 39.7 \mu\text{M}$, $n = 2.01$.

Table 1. Gating properties of the channel constructs

	$I_{50\mu\text{M cGMP}}/I_{1\text{mM cGMP}}$	$(I_{\text{before}} - I_{\text{CaM}})/I_{\text{before}}$
CNGA21	0.67 ± 0.07 ($n = 5$)	0.74 ± 0.12 ($n = 3$)
eCFP–CNGA21	0.68 ± 0.04 ($n = 3$)	0.75 ± 0.07 ($n = 6$)
CNGA21–eYFP	0.64 ± 0.06 ($n = 3$)	0.65 ± 0.22 ($n = 3$)
eCFP–CNGA21–eYFP	0.62 ± 0.06 ($n = 5$)	0.65 ($n = 1$)
CNGA21–eYFP + eCFP–CNGA21	0.72 ± 0.02 ($n = 24$)	0.66 ± 0.08 ($n = 8$)
CNGA21–eYFP + eCFP– Δ CNGA21	0.24 ± 0.05 ($n = 4$)	0.33 ± 0.11 ($n = 3$)

Fractional activation was calculated as the ratio of current in 50 μM cGMP ($I_{50\mu\text{M cGMP}}$) to that in 1 mM cGMP ($I_{1\text{mM cGMP}}$). Fraction of current inhibition by Ca^{2+} -calmodulin was calculated as the ratio of the change in current ($I_{\text{before}} - I_{\text{CaM}}$) to the total current before modulation (I_{before}). Statistical quantities are given as means $\pm$ SEM. Numbers of measurements are given in parentheses.

conversion to R becomes nontrivial. In our experiments eCFP-tagged subunits were expressed in excess relative to eYFP-tagged subunits. The excess eCFP-tagged subunits ensured that each eYFP-tagged subunit was next to an eCFP-tagged subunit and contributed to FRET during energy transfer. The fluorescence emission from eCFP was subtracted when F_{458} was calculated.

One potential error in our analysis comes from eCFP emission in the range of eYFP peak emission when excitation light for the acceptor eYFP was used to measure F_{488} . This contaminating eCFP signal was quite small, however; we estimated that in the worst case $\sim 5\%$ of F_{488} came from eCFP, causing an underestimate of RatioA by that amount.

In patch-clamp fluorometry experiments, changes in FRET efficiency were quantified as changes in the ratio of fluorescence intensity of peak eYFP emission to peak eCFP emission with excitation at 440 nm. A decrease in the ratio indicates a decrease in eYFP emission and/or an

increase in eCFP emission, which occurs when the FRET pair moves apart and the FRET efficiency drops.

Measurement of anisotropy. Anisotropy was measured from the animal pole of oocytes expressing channel–eYFP fusion constructs using a fluorescence microscope with a $10\times$ objective (NA, 0.25), and from inside-out patches using PCF with a $40\times$ objective (NA, 1.3). An excitation polarizer was placed right before the excitation filter in a horizontal position; two emission polarizers, in a parallel ($I_{\parallel}$) and a perpendicular ($I_{\perp}$) position, respectively, were placed underneath the filter cube on a sliding holder. The steady-state anisotropy, A , was calculated using the equation:

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (6)$$

The intrinsic polarization properties of the recording system were assessed by measuring anisotropy of tetramethylrhodamine maleimide (TMRM) dissolved in glycerol, which has an expected anisotropy of 0.38 (Cha and Bezanilla, 1998). We measured an anisotropy value of 0.386 with the $10\times$ objective and 0.428 with the $40\times$ objective, from which correction factors of 1.034 and 1.144, respectively, were calculated and used to calibrate measurements of channel anisotropy values.

Results

Spectrum analysis of FRET efficiency

In this study a chimeric CNG channel was used that contained the CNGA2 (olfactory channel α -subunit, CNG2) NH_2 -terminal region up to the S1 transmembrane region and the remainder from CNGA1 (rod channel α -subunit, CNG1) (Gordon and Zagotta, 1995). This channel, termed CNGA21 hereafter, exhibits over 90% sequence similarity to CNGA2. In addition, homomeric CNGA21 channels exhibit Ca^{2+} -calmodulin modulation indistinguishable from CNGA2 channels. CNGA21 channels were chosen because at high levels, they express a requirement for our fluorescence studies. For these experiments, eCFP and eYFP were genetically attached to the channel subunit just proximal to the calmodulin-binding site in the NH_2 -terminal region and distal to the cyclic nucleotide-binding region in the COOH-terminal domain, respectively. Channels tagged with eCFP and/or eYFP retained normal function. The apparent affinities of these channels for cGMP, deduced from the fractional activation at 50 μM cGMP, were similar to that of untagged channels (Fig. 1A and Table 1). In addition, the apparent affinity of fluorescent protein-tagged channels for cGMP was reduced by Ca^{2+} -calmodulin and subsequently recovered by washing with Ca^{2+} -free solution (Fig. 1B) in the same manner as for untagged channels (Table 1).

Fluorescence signals from labeled channels expressed in *Xenopus* oocytes were measured using confocal microscopy (Fig. 2). Fluorescence intensities were measured only from the surface membrane where mature, properly assembled channels were located. Confocal microscopy measurement of oocytes has the additional advantage that any autofluorescence from cytosolic sources was eliminated. The emission spectra from channel-attached eCFP and eYFP were collected (Fig. 2). They closely

matched the published spectra for these mutant GFP variants (Heim and Tsien, 1996).

FRET was measured as enhanced emission of the acceptor (eYFP) during donor (eCFP) excitation. However, because of overlap in eCFP and eYFP spectra the measured eYFP emission caused by FRET is always contaminated by both direct excitation of eYFP and by eCFP emission in the eYFP range. To overcome these problems, we quantified FRET efficiency using spectrum measurements (Fig. 3) (Zheng et al., 2002). This approach eliminated these contaminations as well as errors attributable to differences in channel density across experiments (Clegg, 1992; Selvin, 1995). The total emission spectrum from channels containing both eCFP and eYFP was collected (Fig. 3A, red line). The eYFP emission spectrum was extracted by subtracting a scaled eCFP spectrum collected from control oocytes expressing only eCFP-tagged channels (blue line). The ratio of the extracted spectrum (F_{458} , green line) to the eYFP spectrum with direct excitation (F_{488} , black line) was calculated as RatioA. Because RatioA is not dependent on wavelength, it was used to check conveniently for linearity of the recording system as well as significant contaminations by other fluorescence sources (Zheng et al., 2002). The RatioA component caused by the direct excitation of eYFP (denoted RatioA₀) was measured directly from control oocytes expressing only eYFP-tagged channels (Fig. 3B). The difference between RatioA and RatioA₀ (RatioA – RatioA₀), as well as the ratio RatioA/RatioA₀ (FR), both directly proportional to FRET efficiency, were determined. When the eCFP–CNGA21 subunit and the CNGA21–eYFP subunit were co-expressed, the eCFP–CNGA21 subunit was expressed in excess to ensure that each eYFP-tagged subunit was next to an eCFP-tagged subunit. The fluorescence emission from eCFP was subtracted when F_{458} was calculated.

Intersubunit interaction in CNG channels

If specific interdomain interactions occur in intact CNG channels under physiological conditions, it would bring the attached eCFP and eYFP molecules close enough to allow FRET to occur. We first tested channels composed of eCFP–CNGA21–eYFP subunits. These channels, containing a donor and an acceptor fluorophore on each subunit, produced robust FRET (Figs. 3A and 4, lane 1). We then co-expressed eCFP–CNGA21–eYFP subunits with an excess of CNGA21 subunits without fluorescence tags at an RNA ratio of 1:10 so that most channels contained no more than one eCFP–CNGA21–eYFP subunit. Limiting eCFP and eYFP to a single subunit in a channel almost completely eliminated FRET (Fig. 4, lane 2). If FRET occurs only between fluorescent proteins attached to the same subunit, the two experiments should yield identical FRET efficiency. These results suggest that, instead, the FRET shown in Figure 4, lane 1, occurred between eCFP and eYFP on neighboring subunits. To test this possibility directly we co-expressed eCFP–CNGA21 and CNGA21–eYFP, yielding channels that allowed only intersubunit FRET. As shown in Figure 4, lane 3, this configuration produced appreciable FRET. These results suggest that in intact channels the NH₂- and

Figure 2. Fluorescence properties of channel-tagged fluorescent proteins. Confocal images of oocytes expressing CNGA21 (A), eCFP–CNGA21 (B), CNGA21–eYFP (C), or eCFP–CNGA21–eYFP (D) channels, with 458 nm (left) and 488 nm (right) laser excitation. Emission spectra for each construct were collected with excitations of 458 nm (red) and 488 nm (black).

Figure 3. Quantification of FRET efficiency with emission spectra. A, Quantification of RatioA with eCFP–CNGA21–eYFP channels. B, Quantification of RatioA₀ with CNGA21–eYFP channels. Arrows indicate excitation wavelengths at 458 and 488 nm. Emission spectra are color coded as follows: red, 458 nm excitation; black, 488 nm excitation; blue, eCFP emission spectrum; green, extracted eYFP spectrum (difference between red and blue).

Figure 4. Specific intersubunit interaction revealed by FRET. Mean values and SEMs of both FR (top axis) and (RatioA – RatioA₀) (bottom axis) are shown for each channel type, with the number of measurements given in parentheses. The expected subunit arrangement for each channel is also shown.

the COOH-terminal-interacting domains from different subunits are in close proximity.

Is the observed FRET caused by a specific intersubunit interaction between the NH₂- and COOH-terminal domains, or is it simply attributable to close packing of the two domains? If the FRET is caused by a specific interaction, removal of one of the interacting domains should prevent FRET. We deleted the calmodulin-binding domain from the eCFP-tagged subunit (amino acids P61 to E90). We co-expressed this subunit, termed eCFP-ΔCNGA21, and the CNGA21-eYFP subunit. This deletion has been shown previously to disrupt the interaction between the NH₂- and COOH-terminal domains *in vitro* and to remove the autoexcitatory effect in the intact olfactory CNG channel (Varnum and Zagotta, 1997). These channels showed lower apparent affinity for cGMP than channels with intact calmodulin-binding domains (Table 1), confirming that the autoexcitatory interaction was indeed disrupted by the deletion. As predicted, FRET efficiency was significantly reduced ($p < 0.01$, Student's *t* test) (Fig. 4, lane 4). Furthermore, co-expression of eCFP-CNGA21 channels with eYFP-tagged cannabinoid receptors (CB1-eYFP) did not give appreciable FRET (Fig. 4, lane 5), confirming that nonspecific FRET did not occur appreciably under our experimental conditions. Taken together, our results provide direct evidence that a specific interaction between the NH₂- and COOH-terminal regions of olfactory CNG channels exists under physiological conditions. In addition, our data with intact channels reveals, for the first time, that this interdomain interaction occurs only between subunits but not within the same subunit.

In these experiments, we took advantage of the steep sensitiv-

ity of FRET efficiency to changes in distance between the fluorescent proteins. However, FRET efficiency is also known to show a less steep dependence on the relative orientation of the pair of fluorophores. The orientation dependence is less a problem when fluorophores do not have a preferable orientation, or are wobbling during fluorescence emission (Lakowicz, 1999). There is evidence that some covalently attached GFP molecules are highly mobile under physiological conditions (Hink et al., 2000). To confirm the mobility of our channel-attached fluorescent proteins, we measured anisotropy from eYFP attached to the NH₂-terminal end, either just before or just after the Ca²⁺-calmodulin-binding domain (eYFP-CNGA21 and eYFP-ΔCNGA21, respectively), and to the COOH-terminal end, either immediately after the cyclic nucleotide binding domain (CNGA21-eYFP) or at the COOH terminal of the rod CNG channel (CNGA1-eYFP) (Fig. 5). The anisotropy measured from all four channels was ~0.2, intermediate between immobile (anisotropy of 0.4, e.g., TMRM in glycerol) and highly mobile (anisotropy of 0). Therefore, eYFP attached to all the sites showed reasonable flexibility, confirming that FRET faithfully reported proximity instead of orientation of the fluorescent proteins.

Quaternary rearrangements during

Ca²⁺-calmodulin modulation

Ca²⁺-calmodulin inhibits CNG channel current by binding to the same sequence in the NH₂-terminal domain that is involved in the interdomain interaction shown here (Liu et al., 1994; Varnum and Zagotta, 1997). Although Ca²⁺-calmodulin has been shown to disrupt the interdomain interaction *in vitro*, it remains to be shown that channel inhibition results directly from disruption of the interaction. Using the eCFP-eYFP pair as a proximity sensor, the disruption of the interaction can be recorded directly as real-time FRET changes. To record FRET efficiency under physiological conditions the PCF technique was used (Zheng and Zagotta, 2000, 2003). Excised, inside-out membrane patches containing fluorescence protein-tagged channels were voltage-clamped and both fluorescence emission and ionic current were recorded simultaneously (Fig. 6A). PCF measures fluorescence signals from isolated membrane patches, thus eliminating autofluorescence from cytosolic sources. As shown in Figure 6B, spectra from PCF experiments (filled circles) closely matched those from confocal microscopy from intact oocytes (lines). This experimental design allowed changes in channel structure to be measured with fluorescence, and changes in channel function to be measured with current, while Ca²⁺-calmodulin was applied to the intracellular side of the membrane.

PCF allowed the direct observation of structural rearrangements of the interacting domains upon binding of Ca²⁺-calmodulin. During the application of Ca²⁺-calmodulin, a reduction in the CNG channel current was observed (Fig. 7A, top), indicating that the binding of Ca²⁺-calmodulin removed the au-

Figure 5. Channel-attached fluorescent proteins retain mobility. Mean values and SEMs of the anisotropy measurements from TMRM in glycerol and eYFP attached to various positions of the CNG channel, with the number of measurements given in parentheses. Anisotropy values were measured from whole oocytes (black bars) as well as from patches before (hatched bars) and after (white bars) Ca^{2+} -calmodulin modulation.

toexcitatory effect exerted by the NH_2 -terminal domain. At the same time, a decline in FRET efficiency, measured as the ratio of the eYFP emission to the eCFP emission, was observed (Fig. 7A, bottom). The decrease in FRET indicated separation or reorientation of the interacting domains. Several lines of evidence suggest that these structural changes upon Ca^{2+} -calmodulin binding are linked directly to channel inhibition. First, the time course of FRET reduction (69.5 ± 11.2 sec, $n = 4$) closely matched the time course of current reduction (62.8 ± 13.5 sec, $n = 4$), suggesting that the change in FRET efficiency tracked the same physical process underlying current reduction. Second, both the reduction of FRET efficiency and the inhibition of current could be reversed by washing with Ca^{2+} -free solution, which removed Ca^{2+} -calmodulin from the channel (Fig. 7A). After recovery, both FRET efficiency and current could be reduced again by adding Ca^{2+} -calmodulin (data not shown). We noticed that sometimes the fluorescence ratio did not recover completely, which might be because of photobleaching or photochemical transformation of eYFP during the course of fluorescence recording (Dickson et al., 1997). Third, binding of Ca^{2+} -calmodulin caused quaternary rearrangements in the channel without directly altering the fluorescence properties or the mobility of individual fluorescent proteins (see below). Thus, the separation or reorientation of the NH_2 - and COOH-terminal interacting domains tracks the time course of Ca^{2+} -calmodulin modulation, suggesting that this rearrangement underlies channel inhibition.

To test the possibility that Ca^{2+} -calmodulin directly altered the fluorescence properties of eCFP and eYFP, we did two control experiments in which fluorescent proteins were present but there was no FRET involved. The first experiment used a co-expression

Figure 6. Simultaneous monitoring of channel modulation with current and fluorescence using patch-clamp fluorometry. *A*, Fluorescence recordings from a membrane patch containing channels formed by both eCFP–CNGA21 and CNGA21–eYFP subunits. Left, Bright field; right, fluorescence image. The inset indicates the area from which fluorescence intensity was measured. *B*, Emission spectra recorded with PCF (filled circles) and from whole oocytes recorded with a confocal microscope (lines).

of CNGA21–eYFP subunits and eCFP– Δ CNGA21 subunits (whose Ca^{2+} -calmodulin-binding domain has been deleted) (Fig. 7B). In these channels, the untagged NH_2 - and COOH-terminal domains were still available to interact. Such interaction produced a small, slow Ca^{2+} -calmodulin modulation (Fig. 7B and Table 1). The second control experiment used eCFP–CNGA21 channels that contained only eCFP (Fig. 7C). In either case, binding of Ca^{2+} -calmodulin caused no change in the fluorescence intensity or the fluorescence ratio. A comparison between Ca^{2+} -calmodulin effects on FRET in experimental and control channels is shown in Figure 7D.

An alternative explanation for the decrease in FRET associated with Ca^{2+} -calmodulin binding is that Ca^{2+} -calmodulin might immobilize the fluorescent proteins in unfavorable orientations. To rule out this possibility, we measured anisotropy from eYFP

Figure 7. Binding of Ca^{2+} -calmodulin separates or reorients the interacting domains. *A*, Current (top) and fluorescence ratio (bottom) recorded from channels containing both eCFP-CNGA21 and CNGA21-eYFP subunits. Stable current in response to 50 μ M cGMP was first recorded in 30 μ M Ca^{2+} solution, indicated by a gray bar above the current trace. The duration of application of Ca^{2+} -calmodulin (with 30 μ M Ca^{2+}) and Ca^{2+} -free solution are indicated by a white bar and a black bar, respectively. The time courses of current and fluorescence ratio are fitted with single-exponential functions (dotted lines) with time constants of 65.9 and 61.7 sec, respectively. *B*, Similar experiment using channels containing eCFP- Δ CNGA21 and CNGA21-eYFP subunits. *C*, Similar experiment using channels containing only eCFP-CNGA21 subunits. *D*, Summary of the change in fluorescence ratio after the application of Ca^{2+} -calmodulin.

attached to either the NH_2 -terminal of the Ca^{2+} -calmodulin-binding domain, the COOH-terminal of the cyclic nucleotide binding domain, or the COOH-terminal of the channel before and after Ca^{2+} -calmodulin modulation. Binding of Ca^{2+} -calmodulin to the channel was monitored with current recordings. Comparison of anisotropy from channels with and without Ca^{2+} -calmodulin bound showed no significant difference (Fig. 5). These results, together with those shown in Figure 7*B–D*, confirmed that binding of Ca^{2+} -calmodulin per se did not affect the fluorescence properties of eCFP and eYFP.

Discussion

The present study focused on a specific interdomain interaction in CNG channels that is the target of Ca^{2+} -calmodulin modulation. We took advantage of FRET between eCFP and eYFP that are genetically attached to CNG channel subunits as a distance

sensor to investigate the structural aspects of this interaction. This experimental design allowed us to record static structure as well as structural rearrangements in intact, functional channels. The ability to record channels in native membrane with the fluorescence approach also enabled us to correlate structural rearrangements (indicated by changes in FRET efficiency) with functional changes (indicated by current recordings). Previously, the NH_2 -terminal/COOH-terminal domain-domain interaction has been studied in solution as isolated fragments using a biochemical approach (Varnum and Zagotta, 1997). The fluorescence approach reported here allowed us to address three important issues: (1) to validate the existence of an NH_2 -COOH interaction in functional CNG channels in intact membranes, (2) to determine whether this interaction is within a subunit or between subunits, and (3) to determine whether the disruption of this interaction has the time course expected if it underlies Ca^{2+} -calmodulin modulation.

In this study we used a spectrum-based method that allowed accurate quantification of FRET efficiency between eCFP and eYFP, whose emissions are known to overlap significantly (Tsien, 1998). Because FRET efficiency is determined with relative fluorescence intensity (RatioA and RatioA₀) the method is insensitive to differences in channel density across experiments, distortion of fluorescence signals caused by the transfer function of the recording system, or possible variation in the quantum yield of the acceptor (Clegg, 1992; Selvin, 1995; Zheng et al., 2002). Other approaches to extract FRET signals from contaminating fluorescence emissions have been developed (Gordon et al., 1998). For example, a simple epifluorescence microscope-based approach termed "three-cube (3^3) FRET" has been applied to eCFP-eYFP FRET quantification (Erickson et al., 2001). In this approach,

emissions from eCFP and eYFP were measured at their peak position, and the FRET component was separated nicely with the combination of three filter cubes. A useful feature of the spectrum-based approach we used here is that both RatioA and RatioA₀ are independent of wavelength. When calculated over a range of high- and low-emission wavelengths, RatioA and RatioA₀ can be used to check conveniently for linearity of the recording system as well as significant contamination of other fluorescence sources (Zheng et al., 2002).

Our experiments provide direct evidence for a specific, dynamic, interdomain, intersubunit interaction in CNG channels (Fig. 8). In the resting state of the channel there is a specific interaction between the NH_2 -terminal domain and the COOH-terminal domain of a neighboring subunit. This interaction promotes channel opening by cyclic nucleotides. Activation of CNG

Figure 8. Molecular rearrangements of the quaternary structure of olfactory CNG channels during Ca^{2+} -calmodulin modulation. Two of the four subunits are shown. cGMP molecules are shown bound to the intracellular cyclic nucleotide-binding domains. Top, Specific interactions between the NH_2 -terminal domain and the COOH-terminal domain of another subunit. Bottom, Separation of the interacting domains when Ca^{2+} -calmodulin molecules bind to the NH_2 -terminal domains.

channels leads to elevated intracellular concentrations of Ca^{2+} ions, which activate calmodulin. Binding of Ca^{2+} -calmodulin to the NH_2 -terminal interacting domain of the channel is shown in this study to separate or reorient this domain physically relative to the COOH-terminal domain. The COOH-terminal interacting domain includes the cyclic nucleotide-binding domain and the C-linker region that joins the cyclic nucleotide-binding domain and the pore (Varnum and Zagotta, 1997). It has been suggested that the C-linker acts as a lever arm to couple ligand binding to the gating conformational changes in the pore (Gordon and Zagotta, 1995; Gordon et al., 1997; Brown et al., 1998; Zong et al., 1998; Paoletti et al., 1999; Zheng and Zagotta, 2000; Johnson and Zagotta, 2001). When the COOH-terminal domain is not interacting with the NH_2 -terminal domain, opening of the channel is energetically less favorable. The channel spends less time open, decreasing Ca^{2+} influx, completing the negative feedback loop underlying olfactory adaptation.

Our results with homomeric channels present a structural framework for understanding the regulation of native CNG channels during sensory transduction. CNG channels in olfactory neurons are formed by three types of subunits, CNGB1, CNGA2, CNGA4, and CNGB1b, of which both CNGA2 and CNGB1b subunits contain a Ca^{2+} -calmodulin binding domain in the NH_2 -terminal (Dhallan et al., 1990; Goulding et al., 1992; Bradley et al., 1994; Liman and Buck, 1994; Liu et al., 1994; Sautter et al., 1998; Bonigk et al., 1999; Grunwald et al., 1999). Experiments using knock-out mice (Munger et al., 2001) and *in vitro* expression (Bradley et al., 2001) have shown that the rate of current inhibition by Ca^{2+} -calmodulin varies greatly depending on sub-

unit composition. It is tempting to speculate that the difference in Ca^{2+} -calmodulin effects results from specific interactions between subunits like those we have observed here. Indeed, the most profound sequence differences among the three subunits reside in the NH_2 -terminal domains. CNG channels in the rod photoreceptor cells also exhibit specific interdomain interactions that are subject to Ca^{2+} -calmodulin modulation (Trudeau and Zagotta, 2002). Similar to what we report here, in rod channels the interaction is only intersubunit, occurring between the COOH-terminal domain of CNGA1 and the NH_2 -terminal domain of CNGB1 subunits. However, unlike the olfactory channels, this interaction appears to involve the COOH-terminal domain after the cyclic nucleotide-binding domain.

References

- Bonigk W, Bradley J, Muller F, Sesti F, Boekhoff I, Ronnett GV, Kaupp UB, Frings S (1999) The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. *J Neurosci* 19:5332–5347.
- Bradley J, Li J, Davidson N, Lester HA, Zinn K (1994) Heteromeric olfactory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP. *Proc Natl Acad Sci USA* 91:8890–8894.
- Bradley J, Reuter D, Frings S (2001) Facilitation of calmodulin-mediated odor adaptation by cAMP-gated channel subunits. *Science* 294:2176–2178.
- Brown RL, Snow SD, Haley TL (1998) Movement of gating machinery during the activation of rod cyclic nucleotide-gated channels. *Biophys J* 75:825–833.
- Cha A, Bezanilla F (1998) Structural implications of fluorescence quenching in the Shaker K^+ channel. *J Gen Physiol* 112:391–408.
- Cha A, Snyder GE, Selvin PR, Bezanilla F (1999) Atomic scale movement of the voltage-sensing region in a potassium channel measured via spectroscopy. *Nature* 402:809–813.
- Chen TY, Yau KW (1994) Direct modulation by Ca^{2+} -calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. *Nature* 368:545–548.
- Clegg RM (1992) Fluorescence resonance energy transfer and nucleic acids. *Methods Enzymol* 211:353–388.
- Dhallan RS, Yau KW, Schrader KA, Reed RR (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* 347:184–187.
- Dickson RM, Cubitt AB, Tsien RY, Moerner WE (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature* 388:355–358.
- Erickson MG, Alseikhan BA, Peterson BZ, Yue DT (2001) Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. *Neuron* 31:973–985.
- Glauner KS, Mannuzzu LM, Gandhi CS, Isacoff EY (1999) Spectroscopic mapping of voltage sensor movement in the Shaker potassium channel. *Nature* 402:813–817.
- Gordon GW, Berry G, Liang XH, Levine B, Herman B (1998) Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J* 74:2702–2713.
- Gordon SE, Zagotta WN (1995) Localization of regions affecting an allosteric transition in cyclic nucleotide-activated channels. *Neuron* 14:857–864.
- Gordon SE, Downing-Park J, Zimmerman AL (1995) Modulation of the cGMP-gated ion channel in frog rods by calmodulin and an endogenous inhibitory factor. *J Physiol (Lond)* 486:533–546.
- Gordon SE, Varnum MD, Zagotta WN (1997) Direct interaction between amino- and carboxyl-terminal domains of cyclic nucleotide-gated channels. *Neuron* 19:431–441.
- Goulding EH, Ngai J, Kramer RH, Colicos S, Axel R, Siegelbaum SA, Chess A (1992) Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* 8:45–58.
- Grunwald ME, Zhong H, Lai J, Yau KW (1999) Molecular determinants of the modulation of cyclic nucleotide-activated channels by calmodulin. *Proc Natl Acad Sci USA* 96:13444–13449.
- Hackos DH, Korenbrot JJ (1997) Calcium modulation of ligand affinity in the cyclic GMP-gated ion channels of cone photoreceptors. *J Gen Physiol* 110:515–528.

- Heim R, Tsien RY (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr Biol* 6:178–182.
- Hink MA, Griep RA, Borst JW, van Hoek A, Eppink MH, Schots A, Visser AJ (2000) Structural dynamics of green fluorescent protein alone and fused with a single chain Fv protein. *J Biol Chem* 275:17556–17560.
- Hsu YT, Molday RS (1993) Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature* 361:76–79.
- Johnson Jr JP, Zagotta WN (2001) Rotational movement during cyclic nucleotide-gated channel opening. *Nature* 412:917–921.
- Kurahashi T, Menini A (1997) Mechanism of odorant adaptation in the olfactory receptor cell. *Nature* 385:725–729.
- Lakowicz J (1999) *Principle of fluorescence spectroscopy*, ed 2. New York: Plenum.
- Liman ER, Buck LB (1994) A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. *Neuron* 13:611–621.
- Liman ER, Tytgat J, Hess P (1992) Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron* 9:861–871.
- Liu M, Chen TY, Ahamed B, Li J, Yau KW (1994) Calcium-calmodulin modulation of the olfactory cyclic nucleotide-gated cation channel. *Science* 266:1348–1354.
- Makhina EN, Nichols CG (2001) Mutant GFP-based FRET analysis of K channel organization. In: *Ion channel localization methods and protocols* (Lopatin AN, Nichols CG, eds), pp 261–274. Totowa, NJ: Humana.
- Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* 388:882–887.
- Munger SD, Lane AP, Zhong H, Leinders-Zufall T, Yau KW, Zufall F, Reed RR (2001) Central role of the CNGA4 channel subunit in Ca²⁺-calmodulin-dependent odor adaptation. *Science* 294:2172–2175.
- Paoletti P, Young EC, Siegelbaum SA (1999) C-linker of cyclic nucleotide-gated channels controls coupling of ligand binding to channel gating. *J Gen Physiol* 113:17–34.
- Sautter A, Zong X, Hofmann F, Biel M (1998) An isoform of the rod photoreceptor cyclic nucleotide-gated channel beta subunit expressed in olfactory neurons. *Proc Natl Acad Sci USA* 95:4696–4701.
- Selvin PR (1995) Fluorescence resonance energy transfer. In: *Methods in enzymology*, pp 300–334. New York: Academic.
- Trudeau MC, Zagotta WN (2002) Mechanism of calcium/calmodulin inhibition of rod cyclic nucleotide-gated channels. *Proc Natl Acad Sci USA* 99:8424–8429.
- Tsien RY (1998) The green fluorescent protein. *Annu Rev Biochem* 67:509–544.
- Varnum MD, Zagotta WN (1997) Interdomain interactions underlying activation of cyclic nucleotide-gated channels. *Science* 278:110–113.
- Zagotta WN, Siegelbaum SA (1996) Structure and function of cyclic nucleotide-gated channels. *Annu Rev Neurosci* 19:235–263.
- Zheng J, Zagotta WN (2000) Gating rearrangements in cyclic nucleotide-gated channels revealed by patch-clamp fluorometry. *Neuron* 28:369–374.
- Zheng J, Zagotta WN (2003) Patch-clamp fluorometry recording of conformational rearrangements of ion channels. *Sci STKE*, April 1:PL7.
- Zheng J, Trudeau MC, Zagotta WN (2002) Rod cyclic nucleotide-gated channels have a stoichiometry of three CNGA1 subunits and one CNGB1 subunit. *Neuron* 36:891–896.
- Zong X, Zucker H, Hofmann F, Biel M (1998) Three amino acids in the C-linker are major determinants of gating in cyclic nucleotide-gated channels. *EMBO J* 17:353–362.
- Zufall F, Munger SD (2001) From odor and pheromone transduction to the organization of the sense of smell. *Trends Neurosci* 24:191–193.