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

Jie Zheng, Michael D. Varnum, and William N. Zagotta

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 $\text{Ca}^{2+}$-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 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 $\text{Ca}^{2+}$-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 $\text{Ca}^{2+}$-calmodulin leads to channel downregulation. The quaternary arrangement presents a structural framework for understanding the molecular mechanism of olfactory adaptation.

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

Introduction

The exposure of olfactory receptor neurons to odorant triggers an elevation of intracellular AMP 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 $\text{Ca}^{2+}$ ions through activated CNG channels also elevates intracellular $\text{Ca}^{2+}$ levels. The $\text{Ca}^{2+}$ 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 intracellularly, 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 $\text{Ca}^{2+}$-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). $\text{Ca}^{2+}$-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 $\text{Ca}^{2+}$-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" 2$^{+}$-calmodulin 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-
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; Glauer 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 NH2–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 Ca2+–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) NH2-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 NH2-terminal end of the calmodulin binding site (after amino acid P61 in CNGA2); eYFP was attached to the end of the G-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 an high-expression vector that contained the untranslated sequence of the Xenopus β-globin gene (Liman et al., 1992). A control construct, eCFP–ACNGA21, 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 termini of the rat cannabinoid receptor CB1 after amino acid L473 after a short linker sequence (G6EF). 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 kΩ 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-Na2, 3 HEPES, and 0.285 CaCl2, pH 7.2. Pipettes with resistance of 200–400 kΩ 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-Na2, 3 HEPES, and 0.285 CaCl2, pH 7.2. The free Ca2+ concentration was 30 μM. cGMP was added to the bath solution to a final concentration of up to 1 μM. Calmodulin was added to the 50 μM cGMP solution to a final concentration of 250 μM. Statistical quantities are given as means ± 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× 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.* Relative FRET efficiency is quantified by the decrease in the emission at the acceptor wavelength caused by the presence of the donor. This decrease can be monitored as a decrease in the primary parameter, the quantum yield of the acceptor, or variation in the concentration of total fluorescence being excited, or variation in 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 $R_{458}$, can be expressed as:

$$R_{458} = \frac{F_{458}^{direct}}{F_{458}^{FRET}} + \frac{F_{488}^{FRET}}{F_{488}}.$$  

(1)

The direct excitation component, $F_{488}^{direct}$, $R_{458}$ 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 $R_{458}$ and $R_{458}^{0}$, which is directly proportional to FRET efficiency:

$$R_{458} - R_{458}^{0} = \frac{F_{458}^{FRET}}{F_{458}^{direct}}.$$  

(2)

Alternatively, the ratio between $R_{458}$ and $R_{458}^{0}$, termed FRET ratio or FR, is calculated as:

$$FR = \frac{R_{458}}{R_{458}^{0}} = 1 + \frac{F_{458}^{FRET}}{F_{458}^{direct}}.$$  

(3)

Like ($R_{458} - R_{458}^{0}$), 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, $F_{458}^{eff}$, as follows:

$$E_{458} = \frac{F_{458}^{FRET}}{F_{458}^{direct}}(FR - 1),$$  

(4)

in which $F_{458}^{FRET}$ and $F_{458}^{direct}$ are the molar extinction coefficients for eCFP and eYFP, respectively (Erickson et al., 2001). When only a single donor and acceptor are present $E_{458}$ equals the true FRET efficiency, $E$, from which the distance between the donor and the acceptor can be calculated as:

$$R = R_{p}\left[1 - \frac{E}{E_{0}}\right],$$  

(5)

in which $R_p$ is the distance at which the FRET efficiency is 50%. However, when there are multiple donors, as is the case in our experiments, direct
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_{458}. This contaminating eCFP signal was quite small, however; we estimated that in the worst case 5% of F_{458} 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× objective (NA, 0.25), and from inside-out patches using PCM with a 40× 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_g) and a perpendicular (I_p) position, respectively, were placed underneath the filter cube on a sliding holder. The steady-state anisotropy, A, was calculated using the equation:

\[
A = \frac{I_p - I_g}{I_p + 2I_g}.
\]

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 Bezania, 1998). We measured an anisotropy value of 0.386 with the 10× objective and 0.428 with the 40× 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) NH2-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 Ca2+-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 NH2-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 Ca2+-calmodulin and subsequently recovered by washing with Ca2+-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

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**Table 1. Gating properties of the channel constructs**

<table>
<thead>
<tr>
<th>Channel Construct</th>
<th>I_{Ggest}/I_{memb,Ca}</th>
<th>I_{before} - I_{CaM}/I_{before}</th>
</tr>
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<tbody>
<tr>
<td>CNGA21</td>
<td>0.67 ± 0.07 (n = 5)</td>
<td>0.74 ± 0.12 (n = 3)</td>
</tr>
<tr>
<td>CNGA21–eYFP</td>
<td>0.68 ± 0.04 (n = 3)</td>
<td>0.75 ± 0.07 (n = 6)</td>
</tr>
<tr>
<td>CNGA21–eCFP</td>
<td>0.64 ± 0.06 (n = 6)</td>
<td>0.65 ± 0.22 (n = 3)</td>
</tr>
<tr>
<td>CNGA21–eYFP + eCFP–CNGA21</td>
<td>0.62 ± 0.06 (n = 5)</td>
<td>0.65 (n = 1)</td>
</tr>
<tr>
<td>CNGA21–eYFP + eCFP–CNGA21, I_{CaM}/I_{before}</td>
<td>0.72 ± 0.02 (n = 24)</td>
<td>0.66 ± 0.08 (n = 8)</td>
</tr>
<tr>
<td>CNGA21–eYFP + eCFP–CNGA21, I_{CaM}/I_{before}</td>
<td>0.24 ± 0.05 (n = 4)</td>
<td>0.33 ± 0.11 (n = 3)</td>
</tr>
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Fractional activation was calculated as the ratio of current in 50 μM cGMP (I_{Ggest}) to that in 1 mM cGMP (I_{before}). Fraction of current inhibition by Ca2+-calmodulin was calculated as the ratio of the change in current (I_{before} - I_{CaM}) to the total current before modulation (I_{before}). Statistical quantities are given as means ± SEM.
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 $\text{RatioA}$. 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 $\text{RatioA}_0$) was measured directly from control oocytes expressing only eYFP-tagged channels (Fig. 3B). The difference between RatioA and $\text{RatioA}_0$ ($\text{RatioA} - \text{RatioA}_0$, as well as the ratio $\text{RatioA}/\text{RatioA}_0$ (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 NH$_2$- and
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 (Zheng 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 of 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 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-
to excitatory effect exerted by the NH2-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 Ca2+-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 Ca2+-free solution, which removed Ca2+-calmodulin from the channel (Fig. 7A). After recovery, both FRET efficiency and current could be reduced again by adding Ca2+-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 Ca2+-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 NH2- and COOH-terminal interacting domains tracks the time course of Ca2+-calmodulin modulation, suggesting that this rearrangement underlies channel inhibition.

To test the possibility that Ca2+-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 of CNGA21–eYFP subunits and eYFP–ΔCNGA21 subunits (whose Ca2+-calmodulin-binding domain has been deleted) (Fig. 7B). In these channels, the untagged NH2- and COOH-terminal domains were still available to interact. Such interaction produced a small, slow Ca2+-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 Ca2+-calmodulin caused no change in the fluorescence intensity or the fluorescence ratio. A comparison between Ca2+-calmodulin effects on FRET in experimental and control channels is shown in Figure 7D.

An alternative explanation for the decrease in FRET associated with Ca2+-calmodulin binding is that Ca2+-calmodulin might immobilize the fluorescent proteins in unfavorable orientations. To rule out this possibility, we measured anisotropy from eYFP
attached to either the NH2-terminal of the Ca2+-calmodulin-binding domain, the COOH-terminal of the cyclic nucleotide binding domain, or the COOH-terminal of the channel before and after Ca2+-calmodulin modulation. Binding of Ca2+-calmodulin to the channel was monitored with current recordings. Comparison of anisotropy from channels with and without Ca2+-calmodulin bound showed no significant difference (Fig. 5). These results, together with those shown in Figure 7B–D, confirmed that binding of Ca2+-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 Ca2+-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 NH2-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 NH2–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 Ca2+-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., 1992). For example, a simple epifluorescence microscope-based approach termed “three-cube (33) FRET” has been applied (Tsien, 1998). 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 NH2-terminal domain and the COOH-terminal domain of a neighboring subunit. This interaction promotes channel opening by cyclic nucleotides. Activation of CNG
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 NH$_2$-terminal domain. 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, 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 subunit 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).

**References**


