The activation of cyclic nucleotide-gated (CNG) channels is the final step in the transduction pathways in both vision and olfaction (1). The opening of olfactory and photoreceptor CNG channels relies on the binding of at least two molecules of cAMP or cGMP. These ubiquitous cyclic nucleotide second messengers bind at intracellular sites on the channel protein to activate a nonspecific cation conductance. This conductance has a significant permeability to calcium ions (1), and thus, CNG channel activation can lead not only to depolarization but also to Ca$^{2+}$ influx.

Native olfactory CNG channels are constructed from at least three different but highly homologous subunits, variously called CNG2 or a (original designation OCNC1 (2)), CNG5 or b (original designation OCNC2 (3, 4)), and CNG4.3 (5) or CNGβ1b (6) (for nomenclature of CNG channel subunits see Ref. 7). The different subunits of the native channel are thought to assemble in a heterotetrameric structure (8, 9). The gating of CNG channels following cyclic nucleotide binding has been the subject of intensive research (1, 8–18). These studies have identified several regions and residues that play an important role in channel activation. Several intracellularly located cysteine residues in or near the cyclic nucleotide (CN) binding region (see Ref. 7 for review and Fig. 1A for diagram) appear to affect the gating reaction either through subunit-subunit interactions or within single channel subunits (19, 20).

The gaseous messenger nitric oxide (NO) has been proposed to exert its gating effects on the CNG channel by a redox modulation of at least one of these intracellular cysteines by reactive nitrogen species that are downstream of NO itself (i.e. the nitrosonium ion, NO•) (21). This direct regulation of protein by NO, so-called “S-nitrosylation,” has been proposed to play a critical role in many processes such as blood pressure regulation, host defense, and neurotransmission (22, 23). Ion channel regulation has also been postulated to occur by S-nitrosylation (24–27). Among ion channels, only the CNG channel has been shown to be directly activated by NO (21).

Of the 8 cysteine residues distributed throughout the rat olfactory rCNG2 (a) channel (Fig. 1A), our previous biochemical evidence identified 1 residue located on the intracellular face of the channel as the putative target site for S-nitrosylation (21). We have therefore focused on the cysteines located on the intracellular face of the channel, and we generated a series of mutant subunit constructs in which each of these cysteines was changed to a serine residue. After expression in HEK 293 cells, we were able to test each of these channels for activation by cAMP and/or NO. We found that the cysteine in position 460, within the C-linker region just N-terminal to the CN binding region, is the critical residue in the reaction that leads to channel activation by NO.

**MATERIALS AND METHODS**

**Mutagenesis—**Cysteine to serine mutants of rCNG2 (a) were generated by substituting the specific cysteine residues with serines using the polymerase chain reaction-based mutagenesis described by Nelson and Long (28). Pfu polymerase (Stratagene, CA) was used to reduce the rate of contaminating mutations. The point mutants were designated as C460S, C484S, C520S, and C552S. All constructs were verified by sequencing.

**Channel Protein Expression—**Human embryonic kidney (HEK) 293 cells were grown at 37 °C in minimal essential medium supplemented with 10% horse serum and 1% gentamicin. A pCIS expression vector (Genentech, CA) containing either the wild type rat olfactory subunit rCNG2 (a) (29) or one of the four different types of mutant rCNG2 (a) channel was used to perform transient transfections using a standard calcium phosphate protocol (30). The cells were co-transfected with a vector containing the gene for the green fluorescent protein (GFP) (kind gift of M. Chalfie) at a 1:1 molar ratio. Patch clamp recordings were made 2–3 days after transfection. GFP was used as an indicator of transfection success (31, 32), efficiency, and probable expression of rCNG2 (a) wild type or mutants. GFP fluorescence was visualized in living cells without histological processing with a fluorescence microscope using fluorescein isothiocyanate filter sets that span the excitation wavelengths 450–500 nm.
Northern Blots—For Northern blot experiments, HEK 293 cells were harvested 2 days after transfection with rCNG2 (α) wild type or mutant cDNAs. The total RNA was extracted by TRIzol reagent (Life Technologies, Inc.). 15 μg of total RNA was size-fractionated on formaldehyde gel and blotted. The blot was hybridized at 42 °C with DIG-11-UTP labeled 500-bp cDNA fragment located at wild type rCNG2 (α) coding region 1251–1751 bp. The hybridized blot was detected by DIG nucleic acid detection kit (Roche Molecular Biochemicals).

Electrophysiological Recordings—Electrophysiological patch clamp recordings were made using the inside-out configuration (33). Electrodes were fabricated from thin-walled borosilicate glass (World Precision Instruments, TW150F-6) and fire-polished to tip resistances of 10 to 20 MΩ. The pipette solution was the same as the Ca2+-free solution (see below), so that both faces of the membrane patch were bathed in symmetrical solutions. The Ca2+-free solution contained (in mM) NaCl, 145; EGTA, 0.5; EDTA, 0.5; HEPES, 10; pH 7.6. Single channel currents were recorded using an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA) positioned close to the membrane patch. This rapid perfusion system was computer-controlled by the software Pulse (HEKA Elektronik, Lambrecht, Germany) and analyzed with the software Igor (Wavemetrics Inc., OR). Channel detection was by the 50% threshold protocol.

From experiments utilizing membrane-impermeant SH-modifying reagents to activate olfactory CNG channels in inside-out patches, we previously identified the probable NO target site as one of four intracellular cysteines of the rat olfactory CNG channel (21). As shown in Fig. 1, we activated olfactory CNG channels in inside-out patches, we previously identified the probable NO target site as one of four intracellular cysteines of the rat olfactory CNG channel (21). As shown in Fig. 1, we have utilized membrane-impermeant SH-modifying reagents to activate olfactory CNG channels in inside-out patches, we previously identified the probable NO target site as one of four intracellular cysteines of the rat olfactory CNG channel (21). As shown in Fig. 1, we have used co-transfection with the green fluorescent protein (GFP) as an indicator of transfection efficiency (32), we found variable levels of protein expression in different cells. This allowed us to obtain membrane patches containing from 1 to several hundred channels.

RESULTS

Localization of the S-Nitrosylation Site—From experiments utilizing membrane-impermeant SH-modifying reagents to activate olfactory CNG channels in inside-out patches, we previously identified the probable NO target site as one of four intracellular cysteine residues (21). As shown in Fig. 1, A and B, the four intracellular cysteines of the rat olfactory CNG channel rCNG2 (α) occur C-terminally to the sixth transmembrane region. Three of them (Cys-460, Cys-520, and Cys-552) are within the cyclic nucleotide binding domain, and the fourth (Cys-484) is in a region of the molecule that links the last transmembrane domain to the CN binding domain, known commonly as the C-linker region. In order to determine the possible contribution of each of these residues to the activation of the channel by NO, we prepared a series of mutant rCNG2 (α) channel subunits sequentially replacing the intracellular cysteine residues in positions 460, 484, 520, and 552 (Fig. 1, A and B) with serine residues by site-directed mutagenesis (28, 34). This is a conservative mutation in which the SH group of the cysteine, thought to be the redox site of NO action, is replaced by an OH group.

Northern blot analysis of each mutant construct showed uniformly high level of expression in HEK 293 cells (Fig. 1C). In all cases, functional channels were produced, as determined by cAMP-induced currents in transfected cells (see below). By using co-transfection with the green fluorescent protein (GFP) as an indicator of transfection efficiency (32), we found variable levels of protein expression in different cells. This allowed us to obtain membrane patches containing from 1 to several hundred channels.

Effects of Mutating Cysteine Residues—Exploring first the three channels with mutations in the CN binding region (C460S, C520S, and C552S), we were surprised to find that each of these mutants produced a functional channel with single channel properties not significantly different from those of wild type rCNG2 (α) channels (Fig. 2 and Table I). At a concentration of cAMP (50 μM), which approximates the Kd of the wild type rCNG2 (α) channel, the open probability, open and closed times, single channel conductances, and voltage dependence (data not shown) were similar in all three constructs. Although we did not test this further, cAMP binding did not appear to be affected by these single cysteine mutations.

Application of the NO donor S-nitrosothioproline (SNC) at 100 μM induced channel activity in these three mutants that was similar to that previously observed in the rCNG2 (α) wild type channel. In particular, the records were marked by the long openings characteristic of NO activation (Fig. 2). From these
data, we conclude that the cysteine residues located in the CN-binding site are not involved in the activation of the channel by NO.

In contrast to the results above, mutating the cysteine residue in the C-linker region (C460S) produced a channel that was completely insensitive to NO (Fig. 3). Concentrations of the NO donor SNC up to 1000 $\mu$M failed to elicit any channel activation, even after 30 min of continuous application. These single channel data could be reproduced with macroscopic current recordings in patches with hundreds of channels (data not shown). This result strongly implicated the Cys-460 as the only intra-cellular cysteine residue involved in the process of channel activation by NO.

As a further test, we made use of the cloned Drosophila melanogaster CNG channel (kind gift of Dr. U. B. Kaupp) which is the only known CNG channel that lacks this highly conserved cysteine residue, whereas it shares a 64% overall identity to the vertebrate CNG channels and does possess the three other cysteine residues in the CN-binding site (35). In agreement with Baumann et al. (35), we found that cGMP (50 $\mu$M) activated the cloned D. melanogaster channel expressed in HEK 293 cells. However, as we expected from our results with C460S, this channel was also found to be insensitive to NO (1–1000 $\mu$M) (Fig. 3). This observation further verified the unique involvement of this single cysteine residue in the NO activation process and confirmed the results obtained with the rat olfactory C460S mutant channel.

Interestingly, activation by cAMP (50 $\mu$M), at the single channel level, also appeared to be affected by the C460S mutation. The cAMP response was still present, confirming that this mutant channel was expressed, but a significant reduction in the open probability and an increase in the closed time constant of the channel were observed compared with the wild type rCNG2 ($\alpha$) channel (Table I). Meanwhile, the cAMP sensitivity was not affected. Thus, when we used membrane patches with large numbers of channels and recorded macroscopic currents in the presence of increasing concentrations of cAMP (1–1000 $\mu$M, Fig. 4), we obtained similar concentration-response curves for cAMP for the wild type rCNG2 ($\alpha$) channel and for the mutant C460S channels. The best fit of the Hill equation to the data gave a $K_{1/2}$ (half-maximal activation) of 64 ± 4 $\mu$M cAMP, a Hill coefficient ($n$) value of 2.1 for the rCNG2 ($\alpha$) channel, a $K_{1/2}$ of 55 ± 4 $\mu$M cAMP, and an n of 1.9 for the C460S mutant. These values were not significantly different, thus further confirming that this particular cysteine residue does not affect nucleotide binding but only the channel gating characteristics.

Concentration Dependence—As shown above, NO appears to interact with a single cysteine residue per channel subunit. A functional CNG channel is believed to be a tetramer composed of 2 or 3 different types of subunits (8, 9). Each of the subunits carries the conserved C-linker cysteine (Cys-460 for CNG2, Cys-350 for CNG5, and Cys-581 for the newly cloned subunit CNG4.3) providing four potential cysteine residues per channel that can undergo S-nitrosylation. This raises the question as to whether all four subunits must be nitrosylated for channel activation. Here we make use of the fact that the rCNG2 ($\alpha$) subunit alone will form a functional homomeric channel of four subunits, allowing us to investigate the concentration-response relation for SNC. For these experiments, we used patches with large numbers of channels and recorded macroscopic currents. Application of SNC in 10-s pulses at concentrations ranging from 1 to 1000 $\mu$M induced currents of 1 pA to 1 nA (Fig. 5A). The currents activated with a time course that was concentration-dependent (see below) and returned to base line upon removal of SNC.

Concentration-response relations were obtained by normal-
FIG. 3. Cys-460 is the nitric oxide target site. Comparison of single channel current recordings from channels composed of either wild type rCNG2 (α) subunits or rCNG2 (α) C460S mutant subunits expressed in HEK 293 cells. Channels were activated, respectively, by 50 μM cAMP and by 100 μM SNC, holding potential of −60 mV. In the case of the C460S mutant channel, no activation could be observed after cAMP treatment compared with the wild type channel. In inside-out patches from HEK 293 cells transfected with the cloned cGMP treatment compared with the wild type channel. In inside-out patches from HEK 293 cells transfected with the cloned Drosophila CNG channel (DmCNGC), treatment with 50 μM cGMP leads to immediate channel activation. However, treatment with the NO donor SNC (1–1000 μM) failed to induce channel activity. All recordings were performed at −60 mV holding potential.

Kinetics of Channel Activation—High levels of expression resulted in patches of membrane with hundreds of channels, and this allowed us to analyze the time course of the modification of Cys-460 by NO using a rapid perfusion system. Fig. 6A shows the onset of SNC-induced response at different concentrations on such an inside-out patch. The maximal current corresponded to the simultaneous activation of ~420 channels, as calculated from the 43-pS single channel conductance (Table I). Due to the cooperativity of the NO effect it is only possible to provide a qualitative analysis, but nonetheless the opportunity to analyze these effects through macroscopic currents provides a check on the single channel data. We focused on the rate of development of the current after a rapid change in SNC concentration (the On rate) and the observed rate constant of the exponential component required to describe the increase in current (the On relaxation). To analyze the responses shown in Fig. 6A, the rising phase of the currents induced by the application of SNC was fit separately with exponential functions. A single exponential component was sufficient to describe the increase of the current (for example see Fig. 6B). For SNC concentrations ranging from 1 to 1000 μM, τ varied from 248 (1 μM SNC) to 38 ms (1000 μM SNC). In Fig. 6C, the On rates (1/τ) obtained by the exponential fits were represented as a function of SNC concentration. The On rate value obtained for 1000 μM SNC was not taken into account since this measures not only reflects NO binding but was probably also influenced by the solution exchange time (~29 ms, see “Materials and Methods”).

The Nitric Oxide Target Site—Direct modulation of function by NO has now been demonstrated in a wide array of proteins (37, 38) and may represent an alternative pathway for protein modulation analogous to phosphorylation. In all of these cases
it is generally believed that a redox-like reaction proceeds at free SH groups on selected cysteine residues. However, in only a few cases have the specific cysteine residues been determined (39, 40). Among NO-sensitive ion channels the same mechanism has been postulated, but specific cysteine targets have not been identified. These currently include the N-methyl-D-aspartate receptor-channel complex (24), Ca\(^{2+}\)-activated K\(^+\) channels (25), Na\(^+\) channels in baroreceptors (26), cardiac Ca\(^{2+}\) release channels (27), and our own work on CNG channels (21, 31). In the present study, we have attempted to demonstrate conclusively the required involvement of a particular cysteine by utilizing olfactory CNG channels in which specific cysteine residues have been mutated to serine.

The main result of our study is that a single cysteine residue within the intracellular C-linker region in the rCNG2 (a) channel subunit is shown to be the NO target site, essential and capable of fully activating the channel, even in the absence of cyclic nucleotides. Other intracellularly located cysteines appear not to participate in the NO-mediated gating of the channel. It is not uncommon for several cysteine residues on a given protein to be candidates for nitrosylation. In the ryanodine receptor, out of a total of 364 cysteines, 84 provide free SH groups, but only 12 are thought to undergo nitrosylation (27). Whereas the precise parameters governing accessibility by NO are unknown, the existence of a consensus nitrosylation acid-base motif has been postulated based on large data base screenings (41). The proposed motif is $XY\_C\_Z$, where $X$ can be any of Gly, Ser, Thr, Cys, Tyr, Asn, or Gln; $Y$ can be Lys, Arg, His, Asp, or Glu; and $Z$ can be Asp or Glu. The most important element of the sequence is believed to be the Asp/Glu residues following the cysteine. Despite this rather degenerate motif, in the CNG channel only the Cys-460, identified by our biochem-

![A](image1.png)

**FIG. 5.** Concentration response of the nitric oxide donor SNC. A, family of current responses recorded at $+60$ mV in the presence of a 10-s pulse of increasing concentrations of the nitric oxide donor SNC (1–1000 \(\mu\)M) obtained on an inside-out patch from HEK 293 cells transfected with the wild type rCNG2 (a) channel and a vector containing the gene for the GFP. The high level of fluorescence allowed us to select a cell expressing hundreds of channels. The top trace represents the stimulus time course. B, SNC sensitivity of the mean normalized currents $I/I_{\text{max}}$ where $I_{\text{max}}$ is the mean of the currents in saturating cAMP (500 \(\mu\)M) (average of 6 experiments at each concentration). The continuous line is the best fit of the Hill equation to the data: $I_{\text{max}}(500 \mu\text{M cAMP}) = 1014 \pm 11 \text{ pA}, K_{50} = 75 \pm 3 \mu\text{M SNC}, n = 1.3$.

![B](image2.png)

**FIG. 6.** Kinetics of NO binding. A, SNC responses from Fig. 5 in enlarged time scale. Detail of the first 1.5 s of the family of current responses recorded at $+60$ mV in the presence of a 10-s pulse of increasing concentrations of the nitric oxide donor SNC (1–1000 \(\mu\)M). B, further enlarged response to SNC 100 \(\mu\)M from A. The solid line is a fit to the following: $I_{\text{max}}\exp(-kt)$, where $I_{\text{max}} = 542 \pm 12 \text{ pA}$, and $k$ is the reaction rate $= 6.7 \text{ s}^{-1}$. The bar indicated when SNC was present. The components ($k$) of the exponentials fitted to the current in the presence of SNC are those described as the On rate and plotted as functions of SNC concentration in C. C, plot of the observed rates of association (On rate, $k_{\text{on}} = 1/\tau$, in $\text{s}^{-1}$) versus SNC concentration. Solid line shows the linear regression fits to the data points. The slope of the regression line yields an estimate of $k_{\text{on}}$, and the intercept yields an estimate of $k_{\text{off}}$ according to the first-order kinetics: $1/\tau = k_{\text{on}}[\text{SNC}] + k_{\text{off}}$. The filled squares are the mean values from five experiments.
ical and mutation experiments as the NO target site, possesses the required motif (i.e., Gln, Asp, Cys, and Glu).

Since the functional channel is most probably made up of four subunits (8, 9), there are four potential nitrosylation sites per channel. However, factors other than those noted above may also determine the likelihood of NO activity at particular cysteines. Different degrees of accessibility to NO due to protein conformation, different reaction rates with NO at different cysteines due to redox status of the immediate environment, or cysteines in positions that may have no functional consequences upon nitrosylation could also account for the observation that in most proteins a relatively few free thiols are in fact involved in nitrosylation-induced activity (41). In the CNG channel, our concentration-response data indicate a Hill coefficient of less than 2, suggesting that as few as two of the four target cysteines may actually interact with NO. It does not, however, preclude activity at all four sites.

**Importance of the C-linker Region**—The activation of a CNG channel is a complex process comprising ligand binding and allosteric transitions from a closed to an open configuration. Mutagenesis studies have indicated that domains outside the CN-binding site, including the N terminus (12, 15) and the linker peptide of −90 amino acids (C-linker) that connects the CN-binding site to the last transmembrane domain, also influence channel activation. A histidine residue within this region that interacts with Ni²⁺ and other transition metal ions has been implicated in modulation of both rod and olfactory CNG channel gating (14). More recently, Zong et al. (42) have identified three residues in the C-linker region that confer the increased efficacy of cAMP observed in the olfactory CNG channel (11). These rate constants are all less (i.e., slower) than the expected value for a diffusion-limited reaction (48), and might suggest that the vast majority of encounters between the cysteine residues and a molecule of SH-modifying reagent fails to produce a successful modification. This also suggests that although NO is an effective activator of the channel, its overall slow channel-gating kinetics might serve to optimize the ability of the channel to operate under intermittent agonist conditions, which is as an integrator of pulsatile NO signals.

**Nitric Oxide and Olfaction**—It has been shown that NO is produced in olfactory neurons (49, 50), and therefore S-nitroso-cysteine or S-nitrosothiols are likely to be formed endogenously. The olfactory epithelium is highly vascularized, and NO produced in the blood vessels could also provide a source of NO that diffuses to sensory neurons. Moreover, normal respiration produces high concentrations of NO in the nasal lumen. Indeed, endogenous NO has been found in exhaled air from animals and humans (51, 52) with especially high concentrations in the upper airways of adult human (53–55) and newborn infants (56). Although many questions remain unanswered regarding the role of NO in nasal physiology and pathophysiology, situations in which heightened attention states increase blood flow or situations inducing a rapid sniffing behavior may increase the excitability of the epithelium through an NO pathway.

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A Single Intracellular Cysteine Residue Is Responsible for the Activation of the Olfactory Cyclic Nucleotide-gated Channel by NO

Marie-Christine Broillet

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