

Movement of the C-Helix during the Gating of Cyclic Nucleotide-Gated Channels

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ABSTRACT Movements within the cyclic nucleotide-binding domain of cyclic nucleotide-gated channels are thought to underlie the initial phase of channel gating (Tibbs, G. R., D. T. Liu, B. G. Leybold, and S. A. Siegelbaum. 1998. *J. Biol. Chem.* 273:4497–4505; Zong, X., H. Zucker, F. Hofmann, and M. Biel. 1998. *EMBO J.* 17:353–362; Matulef, K., G. E. Flynn, and W. N. Zagotta. 1999. *Neuron*. 24:443–452; Paoletti, P., E. C. Young, and S. A. Siegelbaum. 1999. *J. Gen. Physiol.* 113:17–33; Johnson, J. P., and W. N. Zagotta. 2001. *Nature*. 412:917–921). To investigate these movements, cysteine mutation was performed on each of the 28 residues (Leu-583 to Asn-610), which span the agonist-binding domain of the α -subunit of the bovine rod cyclic nucleotide-gated channel. The effects of Cd^{2+} ions, 2-trimethylammonioethylmethane thiosulfonate (MTSET) and copper phenanthroline (CuP) on channel activity were examined, in excised inside-out patches in the presence and in the absence of a saturating concentration of cGMP. The application of 100 μM Cd^{2+} in the presence of saturating concentration of cGMP caused an irreversible and almost complete reduction of the current in mutant channels E594C, I600C, and L601C. In the absence of cGMP, the presence of 100 μM Cd^{2+} caused a strong current reduction in all cysteine mutants from Asp-588 to Leu-607, with the exception of mutant channels A589C, M592C, M602C, K603C, and L606C. The selective effect of Cd^{2+} ions was very similar to that observed when adding the oxidizing agent CuP to the bath medium, except for mutant channel G597C, where CuP caused a stronger current decrease ($67 \pm 7\%$) than Cd^{2+} ($23 \pm 4\%$). In the absence of cGMP, MTSET caused a reduction of the current by $>40\%$ in mutant channels L607C, L601C, I600C, G597C, and E594C, whereas in the presence of cGMP only mutant channel L601C was affected. The application of MTSET protected many mutant channels from the effects of Cd^{2+} and CuP. These results suggest that, when CNG channels are in the open state, residues from Asp-588 to Leu-607 are in an α -helical structure, homologous to the C-helix of the catabolite gene activator protein (Weber, I. T., and T. A. Steitz. 1987. *J. Mol. Biol.* 198:311–326). Furthermore, residues Glu-594, Gly-597, Ile-600, and Leu-601 of these helices belonging to two different subunits must be in close proximity. In the closed state the C-helices are in a different configuration and undergo significant fluctuations.

INTRODUCTION

Cyclic nucleotide-gated (CNG) channels require the direct binding of cAMP or cGMP to open (Kaupp et al., 1989; Dhallan et al., 1990) and are responsible for the current that underlies sensory transduction in vertebrate photoreceptors and in olfactory sensory neurons (Cook et al., 1987; Nakamura and Gold, 1987; Yau and Baylor, 1989; Altenhofen et al., 1991; Menini, 1995; Zimmerman, 1995; Kaupp, 1995; Biel et al., 1995; Finn et al., 1996; Zagotta and Siegelbaum, 1996; Zagotta, 1996).

All CNG channels are activated to some extent by both cAMP and cGMP. In rods and cones, CNG channels are more potently activated by cGMP than by cAMP (Kaupp et al., 1989; Varnum et al., 1995), while channels in chemosensory cilia, such as in olfactory mucosal epithelium, respond equally well to both ligands (Dhallan et al., 1990). These channels are believed to be tetramers composed of several homologous subunits, usually referred to as CNGA and CNGB subunits (Körschen et al., 1995; Shammatt and Gordon, 1999; He et al., 2000; Bradley et al., 2001). The

CNGB1 channel from bovine rods (BROD) is composed of 690 residues (Kaupp et al., 1989). Each subunit encodes for a cyclic nucleotide-binding (CNB) domain composed of ~ 125 amino acids in the cytoplasmic C-terminal end (Kaupp et al., 1989; Molday et al., 1991; Henn et al., 1995; Liu et al., 1996).

The CNB domain of the BROD CNGB1 channel shares $\sim 20\%$ sequence identity with that of other cyclic nucleotide (CN) binding proteins and in particular with two proteins for which crystal structures are now available: the catabolite gene activator protein (CAP) (Weber and Steitz, 1987; Passner et al., 2000) and the cAMP-dependent protein kinase (PKA) (Su et al., 1995). The topography of CNB domain in CAP and PKA is common and consists of a short N-terminal α -helix, referred to as the A-helix, which precedes an eight-stranded antiparallel β roll and is followed by two α -helices, referred to as B- and C-helix, respectively. Recently, a low-resolution (7 Å) structure of a BROD/CAP chimera in complex with cAMP (in *anti*-conformation) has been obtained (Scott et al., 2001) co-expressing the CNB domain of the CNG channel and the DNA binding domain of CAP. This structure suggests that the CNB domain of CNG channels is composed of two dimers, each of which having a folding very similar to that of CAP, in which the two C-helices are at the interface between the two monomers. This configuration, however, may be introduced in the chimera by the presence of the CAP DNA binding

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domain. For this reason, it is crucial to obtain direct evidence both for the existence of a helical structure in the C-terminal part of the CNB domain of CNG channels (analogous to the C-helix of CAP) and for the dimeric folding of the whole domain.

Several electrophysiological experiments have shown that residues in the CNB domain move, following binding of cGMP in the binding pocket (Sun et al., 1996; Scott and Tanaka, 1998; Tibbs et al., 1998; Li and Lester, 1999; Matulef et al., 1999). These experiments, based on cysteine scanning mutagenesis, have shown that residues such as Gly-597 and Cys-505 have a different accessibility in the presence or in the absence of a saturating concentration of cGMP.

Many of these experimental observations were recently embodied in a detailed molecular model (Punta et al., in press) of the CNB domain, based on comparative modeling. In this model, the CNB domain is shown as a dimer, with a tertiary structure similar to that of CAP. In the CNB domain model there are two C-terminal α -helices, B and C, for which no direct experimental structural evidence is available.

The present manuscript has several aims: first, to verify with electrophysiological experiments the structure of the CNB domain (as proposed by Scott et al., 2001 and Punta et al., in press), with particular focus on the existence of an α -helix in the BROD CNGA1 channel analogous to the C-helix of CAP; second, to verify the proposed dimeric nature of the CNB domain and to identify residues in close contact in the open state, i.e., in the presence of a saturating cGMP concentration; third, to experimentally investigate global movements of the CNB domain during gating, i.e., following the binding of cGMP.

In the open state, our results confirm that residues from Asp-588 to Leu-607 have an α -helical configuration (C-helix). Moreover, residues Leu-601, Ile-600, Gly-597, and Glu-594 of two C-helices are in close contact. These results strongly suggest that in the open state the CNB domain is a double dimer, in agreement with the recent low-resolution structure of the CNG channel obtained by Higgins et al., 2002, and homologous to the tertiary structure of CAP. In the closed state, our results suggest that C-helices are in a different position and may undergo significant structural rearrangements (Matulef et al., 1999; Punta et al., in press).

METHODS

Molecular biology

The clone of the BROD CNGA1 channel, consisting of 690 residues, was mutated using the QuickChange site-directed Mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Wild-type (wt) and mutant RNAs were synthesized in vitro by using the mCAP RNA Capping kit (Stratagene). Sequences were verified with the DNA sequencer LI-COR (4000L). Cysteines were introduced in the stretch from Leu-583 to Asn-610 in the wt channel and in position 597, 600, 602, and 605 of the cysteine-free CNGA1 channel, kindly supplied by William Zagotta.

Oocyte preparation and chemicals

The wt or mutant channel cRNAs were injected into *Xenopus laevis* oocytes (Rettilli, Varese, Italy). Oocytes were prepared as previously described (Nizzari et al., 1993). Injected eggs were maintained at 19°C in a Barth solution supplemented with 50 μ g/ml gentamycin sulfate and containing (in mM): 88 NaCl, 1 KCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 2.4 NaHCO₃, 5 TRIS-HCl. During the experiments, oocytes were kept in Ringer's solution containing (in mM): 150 NaCl, 2.5 KCl, 1 CaCl₂, 1.6 MgCl₂, 10 HEPES-NaOH. The solutions were maintained at pH 7.4 (buffered with NaOH). The MTS compounds were purchased from Toronto Research Chemicals (Ontario, Canada). All the other chemicals were from Sigma Chemicals (St. Louis, MO).

Recording apparatus

cGMP-gated currents from excised patches (Hamill et al., 1981) were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments Inc., Foster City, CA), 1–5 days after RNA injection, at room temperature (20–22°C). The perfusion system was as previously described (Sesti et al., 1995) and allowed complete solution changing within 1 s. Borosilicate glass pipettes had resistances of 3–5 M Ω in symmetrical standard solution. The current traces obtained in the inside-out patch-clamp configuration, soon after patch excision, used for obtaining steady-state current-voltage relations were the difference between currents in the presence and absence of 1 mM cGMP. The patch potential was stepped up from 0 to +60 mV and from 0 to –60 mV. Currents were low-pass filtered at 1 kHz and acquired on-line (at 5 kHz). pClamp hardware and software (Axon Instruments) and Origin software (Microcal Software, Inc., Northampton, MA) were used for data acquisition and analysis.

Application of sulfhydryl-specific reagents

Sulfhydryl-specific reagents are useful tools for studying the proximity of specific regions in ion channels. Here, to study the conformational changes between the open and closed state of CNG channels, we analyzed the effects of sulfhydryl-specific reagents on mutant channel activity (Cd²⁺, MTSET, and CuP) in the presence and in the absence of cGMP. In the presence of 1 mM cGMP the open probability is close to 1 (Bucossi et al., 1997), meaning that the channels are most frequently in the liganded and open state. Therefore, sulfhydryl-specific reagents were applied in the presence and in the absence of 1 mM cGMP. The Cd²⁺ effect was tested by perfusing the intracellular side of the patch with a standard solution devoid of EDTA (to avoid partial Cd²⁺ chelation; Gordon and Zagotta, 1995) supplemented with 1 mM cGMP and/or 100 μ M CdCl₂ for 5 min. The only effect of the EDTA withdrawal is the activation of a background offset current due to the well-known presence of Ca²⁺-dependent Cl[–] channels in *Xenopus* oocytes (Miledi et al., 1984). This current, however, reached the steady state soon after the change of solution, as reported also in previous work (Becchetti and Roncaglia, 2000), therefore, no Cl[–] channel blockers were used during these experiments. The effect of MTS compounds was tested at a concentration of 2.5 mM, in standard solution with EDTA, as previously described (Becchetti et al., 1999). To study the effect of the probe in the closed state, patches were exposed to the appropriate reagent for 5 min, in the absence of CNs. After wash-out, cGMP was applied to measure the residual current. To study the effect in the open state, sulfhydryl-specific reagents were applied in the presence of 1 mM cGMP. All effects of sulfhydryl reagents on channel activity described in this study were obtained after washing-out reagents and in the presence of a steady cGMP-gated current, for at least 10 min. All currents were measured at the steady state, i.e., after the effect of sulfhydryl reagents had developed fully. The wt—i.e., the BROD CNGA1—channel current is not irreversibly reduced by 100 μ M Cd²⁺ either in the closed or in the open state (Roncaglia and Becchetti, 2001). Similarly, the addition

of 1 μM or 1 mM CuP to the bathing medium did not lead to any permanent decrease of the cGMP-gated current (data not shown, see also Matulef and Zagotta, 2002). As already shown (Sun et al., 1996; Roncaglia and Becchetti, 2001; Becchetti et al., 1999) MTSET does not reduce the current of the wt in the open state, but in the closed state a decrease of $\sim 20\%$ of the cGMP-gated current is observed. Therefore, current reduction by Cd^{2+} , CuP, and to some extent also by MTSET, can be attributed to an action on the exogenous introduced cysteines. To check whether the observed decrease of the cGMP-activated current was caused by a change of the maximal cGMP-activated current or by a shift in the half-activating constant K_m , the value of K_m was measured before and after the application of sulfhydryl reagents in mutant channels L607C, M602C, G597C, and K598C. In all of these mutant channels the value of K_m was not significantly affected by sulfhydryl reagents and was always between 70 and 150 μM . In the absence of sulfhydryl reagents, mutant channels L601C, I600C, G597C, and E594C in the presence of 1 mM cGMP exhibited a rundown of the cGMP-activated current varying between 5 and 23% within 120 min. No rundown of the cGMP-activated current was observed in the wt channel. In this case, the value of the maximal current before application of sulfhydryl reagents was taken as the average over 5 min before their application and the value of the maximal current after application of sulfhydryl reagents was taken as the average over 5 min after their washing out. All points in Fig. 1, *E* and *F*, Fig. 2 *E*, Fig. 3, *C–E*, and Fig. 4, *C–E* were obtained from at least five patches excised from at least two different oocytes.

RESULTS

Each residue from Leu-583 to Leu-607 and Asn-610 of the BROD CNGA1 channel (Kaupp et al., 1989) were mutated one by one into cysteine, and the effects of sulfhydryl reagents such as MTSET, Cd^{2+} , and CuP on channel activity were investigated. These mutant channels will be referred to as cysteine mutants, and the sensitivity of a given residue (such as Leu-607) to sulfhydryl reagents implies the sensitivity of the corresponding cysteine mutant (i.e., L607C). All results presented in this manuscript, for each mutant, were obtained in at least five different patches excised from at least two different oocytes.

As it will be shown, MTSET and Cd^{2+} act very differently on cysteine mutants in the CNB domain because one molecule of MTSET forms a covalent bond with the thiol group of a single cysteine (Akabas et al., 1992; Karlin and Akabas, 1998), whereas one Cd^{2+} ion usually binds to two or even more cysteines (Beniath et al., 1996; Holmgren et al., 1998; Loussouarn et al., 2000). If the same effect produced by Cd^{2+} can be induced by applying the oxidizing agent CuP, known to be able to enhance the formation of disulfide bonds between neighboring cysteines (Glusker, 1991; Hastrup et al., 2001), the current modification caused by Cd^{2+} is very likely due to its binding to two cysteines in close contact. Therefore, the inhibition of the maximal cGMP-activated current in the tested mutants by MTSET and Cd^{2+} reflects specific physical mechanisms involving both the accessibility of the different residues to these sulfhydryl-specific reagents and their mutual distance in neighboring subunits.

The present analysis indicates that residues from Leu-583 to Asn-610 can be grouped in four distinct regions accord-

ing to their Cd^{2+} and CuP sensitivity: residues in the C-terminal end (from Lys-603 to Leu-607) and in the N-terminal end (from Pro-587 to Met-592) are blocked by Cd^{2+} and CuP in the closed state but not in the open state; residues in the middle (from Leu-593 to Met-602) are blocked by Cd^{2+} both in the open and closed states, and residues downstream (from Leu-583 to Pro-587) are never blocked in the open or in the closed state by Cd^{2+} and CuP.

Residues from Lys-603 to Asn-610

cGMP-gated currents were recorded in all cysteine mutants from Lys-603 to Leu-607, with the exception of mutant channel D604C. In the closed state, as shown in Fig. 1, *A* and *C*, the application of Cd^{2+} and MTSET reduced the current of mutant channels L607C and L606C, whereas in the open state (see panels *B* and *D*), no significant reduction of the current was noticed and a small potentiation was observed in mutant channel L606C. The data of Fig. 1 *E* show that in the closed state, the current of the mutant channels L607C and G605C was strongly reduced by Cd^{2+} , by $83 \pm 2.4\%$ and $88 \pm 3\%$ respectively, while the current of the mutant channels L606C and K603C was decreased only by 18.6 ± 2.9 and $19.3 \pm 2.3\%$, respectively. None of these mutant channels exhibited a permanent current reduction by Cd^{2+} in the open state, as shown in Fig. 1, *B*, *D*, and *E*.

MTSET applied in the closed state decreased by $77.5 \pm 2.5\%$ the current in mutant channel L607C and by $30 \pm 7\%$ and $23 \pm 2\%$ in mutant channels G605C and L606C, respectively. On the contrary, mutant channel K603C was slightly potentiated. When MTSET was applied in the open state no current decrease was observed (as in the wt channel), while in the case of mutant channels L606C and K603C a potentiation of $\sim 30\%$ was observed. Similar results were obtained when the smaller sulfhydryl reagent MTSEA was used.

The addition of 1 μM CuP to the bathing medium in the presence of 1 mM cGMP for 2 min only weakly reduced cGMP-gated current in mutant channels G605C and K603C. This induced current reduction was irreversible and was at most 10% (see Fig. 1 *E*). In the absence of cGMP, exposure to 1 μM CuP for 2 min reduced the cGMP-gated current by $80 \pm 4\%$ in mutant channel G605C and by $75 \pm 25\%$ in L607C. The decrease induced by the exposure of the patch to 1 μM CuP for 2 min was very similar to that caused by exposure to 1 mM CuP for just 10 s (see Fig. 3 in the case of mutant channel I600C). Also, Asn-610 was mutated into a cysteine and the current of this mutant channel N610C was not reduced by any of the tested sulfhydryl reagents either in the open or in the closed states.

Similar experiments were also repeated in the mutant channel G605C of the cysteine-free CNG channel (Matulef et al., 1999). In the open state, Cd^{2+} and CuP had effects similar to those observed in the single mutant G605C. In the closed state,

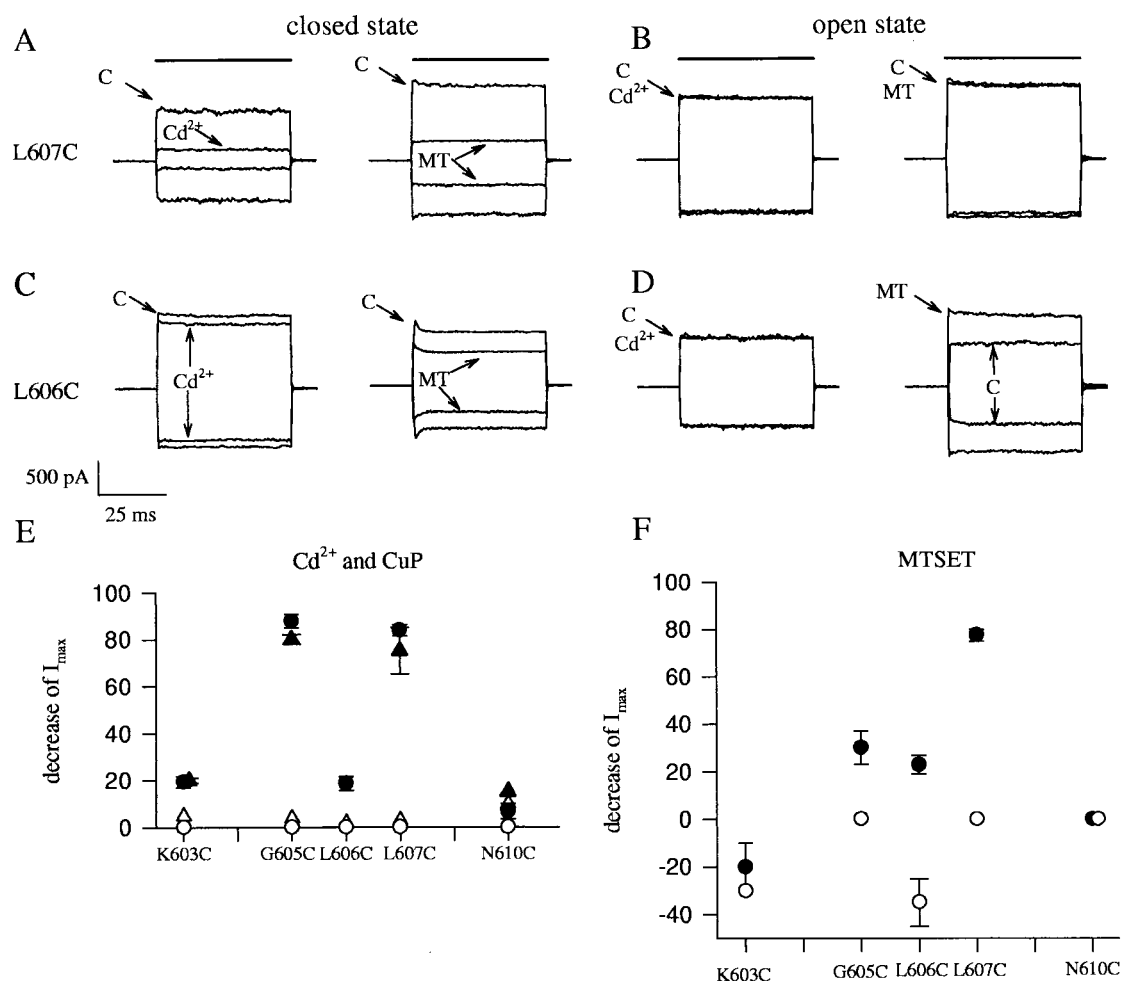


FIGURE 1 The effect of Cd^{2+} and MTSET on mutant channels L607C and L606C in the presence and absence of cGMP. (A) Irreversible reduction of the cGMP-gated current caused by $100\ \mu\text{M}$ Cd^{2+} (left) and $2.5\ \text{mM}$ MTSET (right) added for 5 min in the absence of cGMP in mutant channel L607C. (B) As in A, but with sulfhydryl reagents added in the presence of $1\ \text{mM}$ cGMP. (C) As in A, but for mutant channel L606C. (D) As in C, but in the presence of $1\ \text{mM}$ cGMP. In A–D, voltage steps from 0 to $\pm 60\ \text{mV}$. Each trace is the average of 10 individual trials. The cGMP-gated current was obtained as the difference of the current in the presence and in the absence of $1\ \text{mM}$ cGMP. The solid horizontal lines over current traces indicate the time of the voltage command. C indicates the control current, Cd^{2+} the current after exposure for 5 min to $100\ \mu\text{M}$ Cd^{2+} , MT the current after exposure for 5 min to $2.5\ \text{mM}$ MTSET. (E) Percentage of $100\ \mu\text{M}$ Cd^{2+} and $1\ \mu\text{M}$ CuP irreversible current reduction in the absence of cGMP (closed circles for Cd^{2+} and closed triangles for CuP) and in the presence of $1\ \text{mM}$ cGMP (open circles for Cd^{2+} and open triangles for CuP) for cysteine mutants from Lys-603 to Leu-607. (F) As in E, but for MTSET. In E and F error bars indicate the standard deviation. Each point was obtained from at least five patches excised from at least two different injected oocytes.

however, Cd^{2+} and CuP did not produce any significant current decrease, i.e., any reduction larger than 10%.

Residues from Leu-593 to Met-602

Cysteine mutants from Leu-593 to Met-602 displayed a different sensitivity to Cd^{2+} , as current in these mutants was reduced also in the open state. As shown in Fig. 2, the current of mutant channels L601C (A and B) and I600C (C and D) was strongly reduced by the application of $100\ \mu\text{M}$ Cd^{2+} both in the closed (A and C) and in the open states (B and D).

The effect of MTSET on these two mutant channels was rather different. MTSET significantly reduced ($\sim 100\%$) the current of mutant channel L601C both in the open and in the closed state, while the current of mutant channel G597C was reduced ($\sim 100\%$) only in the closed state (Matulef et al., 1999). Current reduction induced by MTSET on the other cysteine mutants is reported in Fig. 2 E. MTSET applied to the patch in $1\ \text{mM}$ cGMP resulted in the potentiation of mutant channels M602C and Q599C. In the closed state, MTSET reduced the current of the mutant channels M602C, L601C, I600C, G597C, and E594C by $35 \pm 5\%$, 100% , $55 \pm 5\%$, 100% , and $55 \pm 2\%$, respectively.

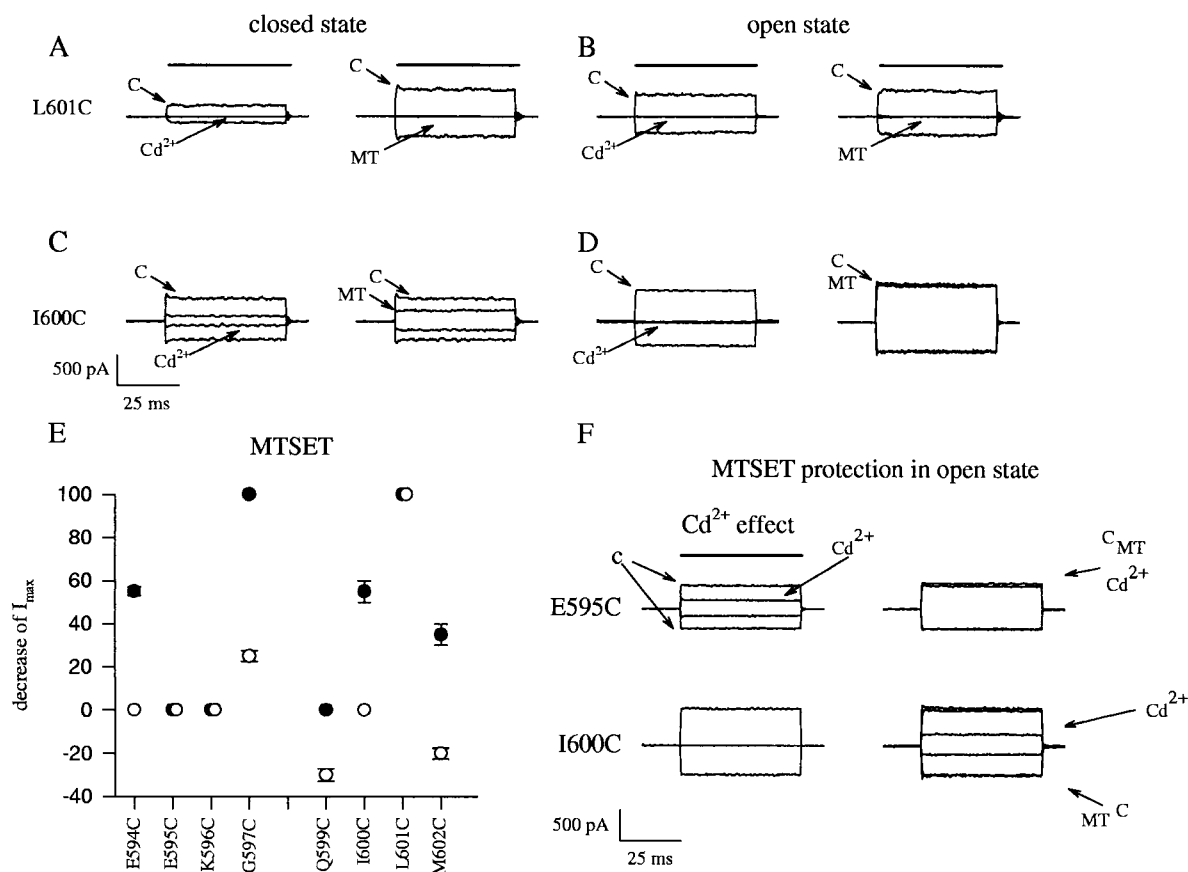


FIGURE 2 The effect of Cd^{2+} and MTSET on mutant channels L601C and I600C in the presence and absence of cGMP. (A) Irreversible reduction of the cGMP-gated current caused by 100 μM Cd^{2+} (left) and 2.5 mM MTSET (right) added for 5 min in the absence of cGMP in mutant channel L601C. (B) As in A, but with sulfhydryl reagents added in the presence of 1 mM cGMP. (C) As in A, but for mutant channel I600C. (D) As in C, but in the presence of 1 mM cGMP. In A–D voltage steps are as in Fig. 1. (E) Percentage of 2.5 mM MTSET irreversible current reduction in the absence of cGMP (closed circles) and in the presence of 1 mM cGMP (open circles) for cysteine mutants from Glu-594 to Met-602. (F) Protection by MTSET of Cd^{2+} current reduction in mutant E595C and I600C. Left: current traces before and after the addition of 100 μM Cd^{2+} for 5 min in the open state. Right: effect of 100 μM Cd^{2+} in the open state before and after the application of 2.5 mM MTSET.

The small effect of MTSET on most of these cysteine mutants, illustrated in Fig. 2 E, does not imply the inaccessibility of these residues to MTSET. In fact, when mutant channels E595C and I600C were initially exposed to MTSET in the open state, a significantly smaller current reduction by Cd^{2+} was observed (Fig. 2 F). After pretreatment by MTSET the percentage of current reduction induced by Cd^{2+} in mutant channels E595C and I600C in the open state decreased from ~50% and 100% to ~10% and $58 \pm 4\%$, respectively. A significantly smaller current reduction induced by Cd^{2+} and by the oxidizing agent CuP was observed, after pretreatment by MTSET, also in mutant channels Q599C and K596C, but not in mutant channel E594C, both in the closed and in the open states. Therefore, residues Leu-601, Ile-600, Gln-599, Arg-596, and Glu-595 are accessible to MTSET in both states of the channel.

As shown in Fig. 3 A, application of CuP in the closed state caused an irreversible suppression of the cGMP-gated

current in mutant channel I600C. This decrease was observed within 2 min in the presence of 1 μM CuP and within 10 s in the presence of 1 mM CuP. A similar effect was observed also in the open state for low and high CuP concentrations (see Fig. 3 B). The time course of this decrease is shown in Fig. 3 C: both in the open and closed states, CuP completely suppressed the cGMP-gated current within 2 min. A comparison between the current decrease induced by exposure for 5 min to 100 μM Cd^{2+} and for 2 min to 1 μM CuP is shown in Fig. 3, D and E. In the open state, application of 100 μM Cd^{2+} to the mutant channels M602C, L601C, I600C, Q599C, G597C, K596C, E595C, and E594C caused a reduction of the current equal to $30 \pm 3\%$, 100%, 100%, $\pm 4\%$, $20 \pm 5\%$, $55 \pm 4\%$, and 100%, respectively. In the open state, the current decrease induced by the two sulfhydryl reagents was almost identical, except in mutant channel G597C. In this case, the current reduction induced by CuP was $67 \pm 7\%$, considerably higher than that

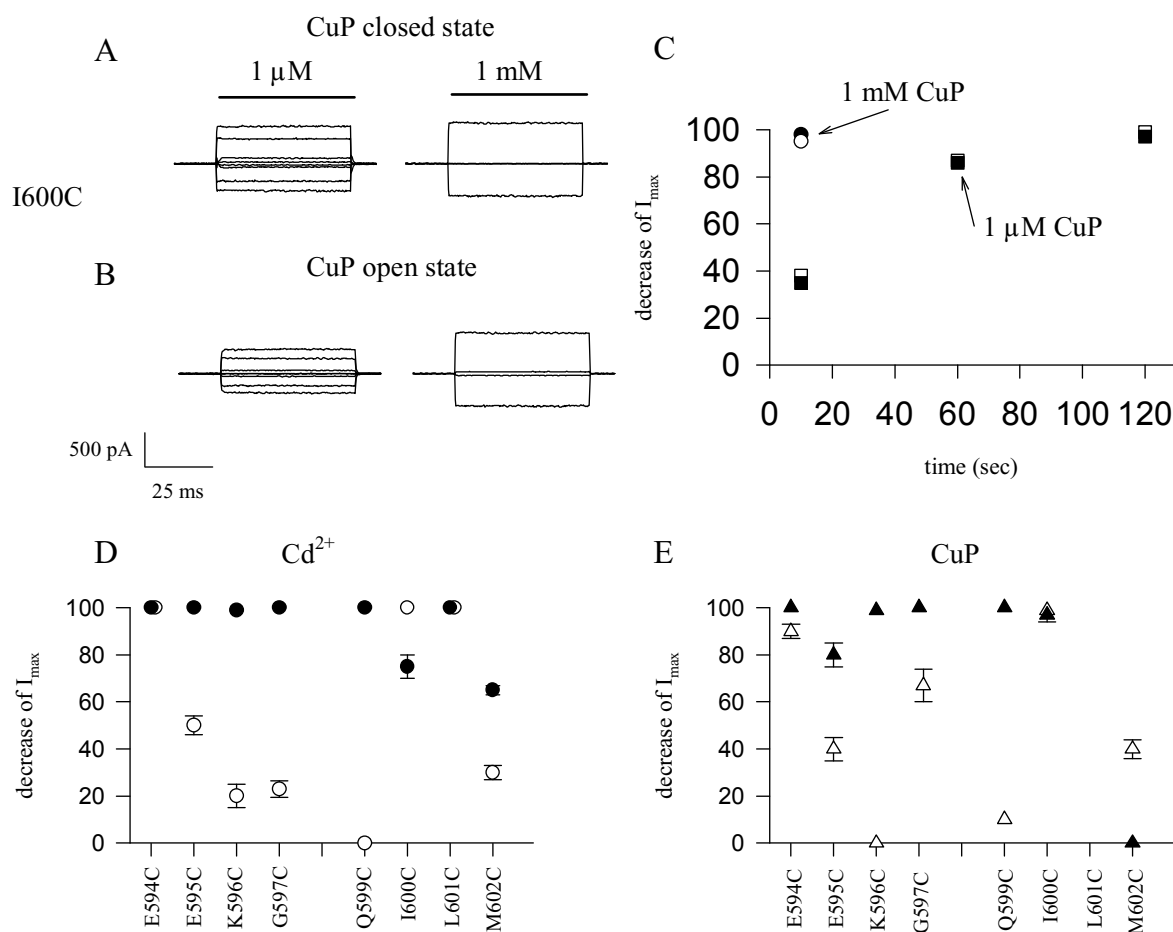


FIGURE 3 The effect of CuP on cysteine channels from Glu-594 to Met-602. (A) Irreversible reduction of the cGMP-gated current, in the closed state, caused by an addition of 1 μ M CuP and 1 mM CuP for mutant channel I600C. (B) As in A, but in the open state. In A and B voltage steps are as in Fig. 1. The four traces shown in the left panel of A and B were obtained before application of 1 μ M CuP and after 10, 60, and 120 s. On the right panel of A and B only the trace obtained before application of 1 mM CuP and after 10 s are shown. (C) Time course of current reduction in the open and closed state, in the presence of 1 mM and 1 μ M CuP. (D) Percentage of 100 μ M Cd²⁺ irreversible current reduction in the absence (closed circles) and in the presence (open circles) of 1 mM cGMP for cysteine mutants from Glu-594 to Met-602. (E) Percentage of 1 μ M CuP irreversible current reduction in the absence (closed triangles) and in the presence (open triangles) of 1 mM cGMP for cysteine mutants from Glu-594 to Met-602.

induced by Cd²⁺. This difference could be explained if in G597C the cysteines were close to each other, but so deeply buried that they would be inaccessible to Cd²⁺.

In the closed state, the application of 100 μ M Cd²⁺ to the mutant channels M602C, L601C, I600C, Q599C, G597C, K596C, E595C, and E594C caused a reduction of the current equal to 65 \pm 2%, 100%, 75 \pm 5%, 100%, 100%, 100%, 100%, and 100%, respectively. The application of CuP had similar but not identical effects: in mutant channels M602C, I600C, and E595C CuP induced a current reduction equal to 0%, 97 \pm 3%, and 80 \pm 5%, respectively.

A cysteine was also introduced in positions 597, 600, and 602 of the cysteine-free CNG channel (Matulef et al., 1999). In these mutant channels, neither Cd²⁺ nor CuP produced any significant decrease of cGMP-activated current in the closed state. In the open state of the cysteine-free CNG channel, the current decrease for mutant channels G597C,

I600C, and M602C was 30 \pm 18%, 95 \pm 4%, and 30 \pm 12% after the application of Cd²⁺, and 87 \pm 10%, 92 \pm 8%, and 37 \pm 12% after the application of CuP.

Residues from Pro-587 to Met-592

Residues from Pro-587 to Met-592 displayed similar sensitivity to sulfhydryl reagents as those from Lys-603 to Leu-607. In the closed state, as shown in Fig. 4 A, Cd²⁺ strongly reduced the cGMP-gated current in mutant channels D588C, K590C, and G591C. Current reduction in mutant channels A589C and M592C was 20 \pm 4% and 50 \pm 5%, respectively. Perfusion with 1 μ M CuP for 2 min had a similar effect on the same mutant channels, as shown in Fig. 4 B. MTSET had a small effect on all of these mutant channels, reducing the current by 30% maximum, as shown

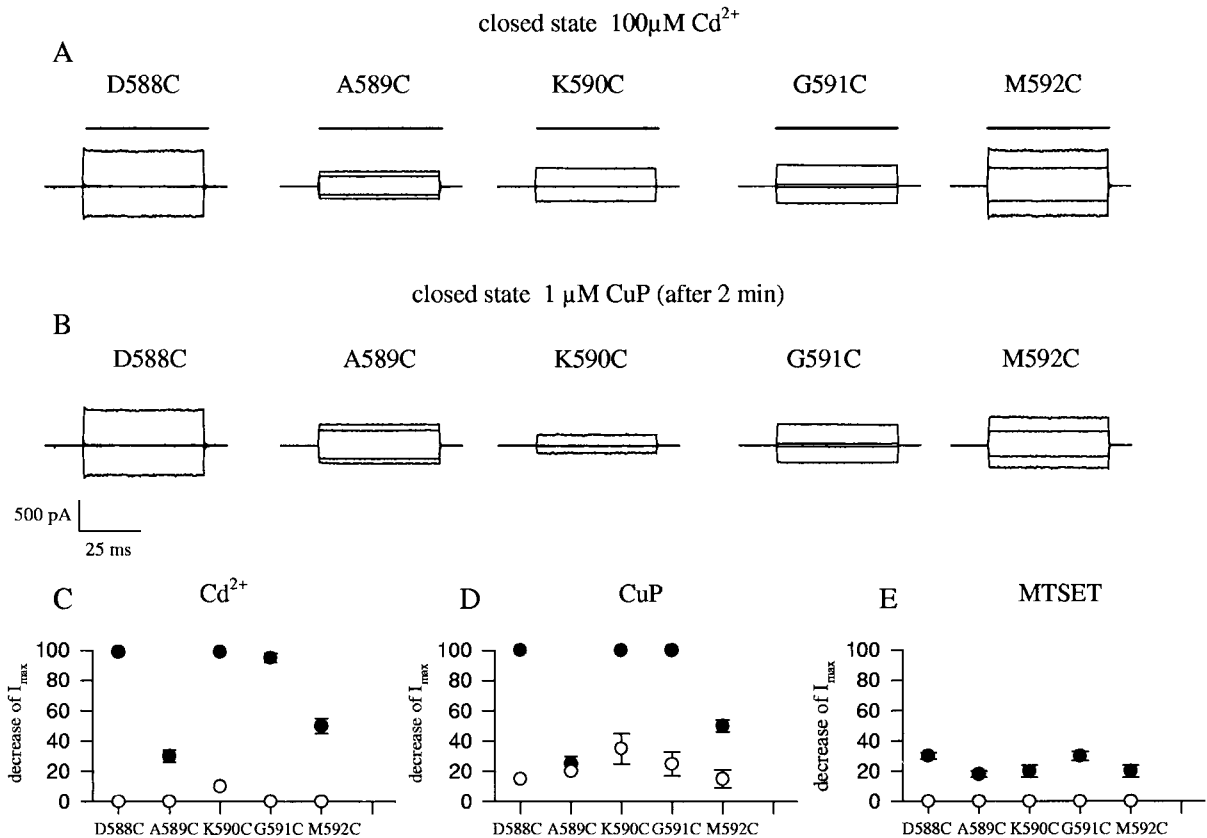


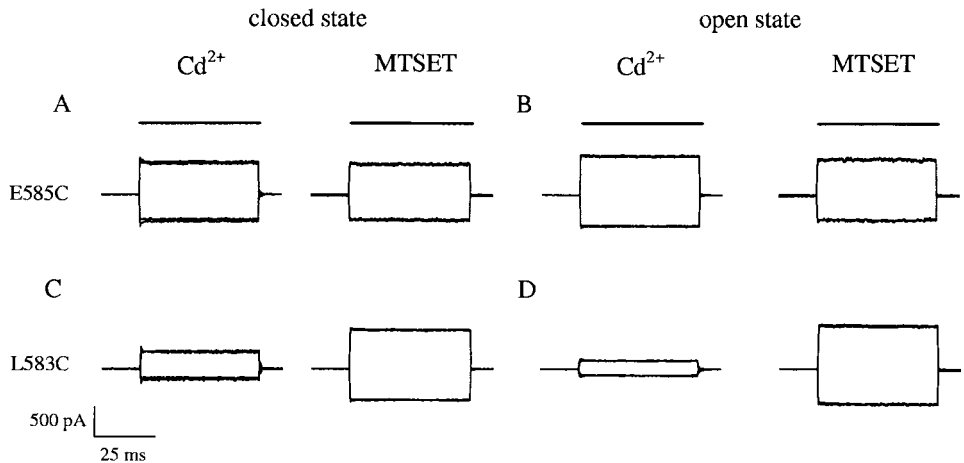
FIGURE 4 The effect of Cd²⁺ and CuP applied in the absence of cGMP on cysteine mutants from Asp-588 to Met-592. (A) Current traces before and after the addition of 100 μM Cd²⁺ for 5 min. From right to left: recordings from channel mutants M592C, G591C, K590C, and A589C and D588C. (B) As in A, but adding 1 μM CuP for 2 min. Voltage steps in A and B as in Fig. 1. (C) Percentage of 100 μM Cd²⁺ irreversible current reduction in the absence of cGMP (closed circles) and in the presence of 1 mM cGMP (open circles) for cysteine mutants Asp-588 to Met-592. (D) As in C, but for 1 μM CuP. (E) As in C, but for 2.5 mM MTSET.

in Fig. 4 E. In the open state, as shown in Fig. 4, C and D, current reduction by Cd²⁺ was at most 10% and by CuP was slightly higher, at a maximum of 30%. MTSET did not have any significant effect in the open state (Fig. 4 E).

Residues from Leu-583 to Tyr-586

A clear and evident cGMP-gated current was recorded from mutant channels E585C and L583C. No cGMP-gated cur-

FIGURE 5 The effect of Cd²⁺ and MTSET on mutant channels E585C and L583C in the presence and absence of cGMP. (A) Irreversible reduction of the cGMP-gated current caused by 100 μM Cd²⁺ (left) and 2.5 mM MTSET (right) added for 5 min in the absence of cGMP in mutant channel E585C. (B) As in A, but with sulfhydryl reagents added in the presence of 1 mM cGMP. (C) As in A, but for mutant channel L583C. (D) As in C, but in the presence of 1 mM cGMP. In A–D, voltage steps as in Fig. 1.



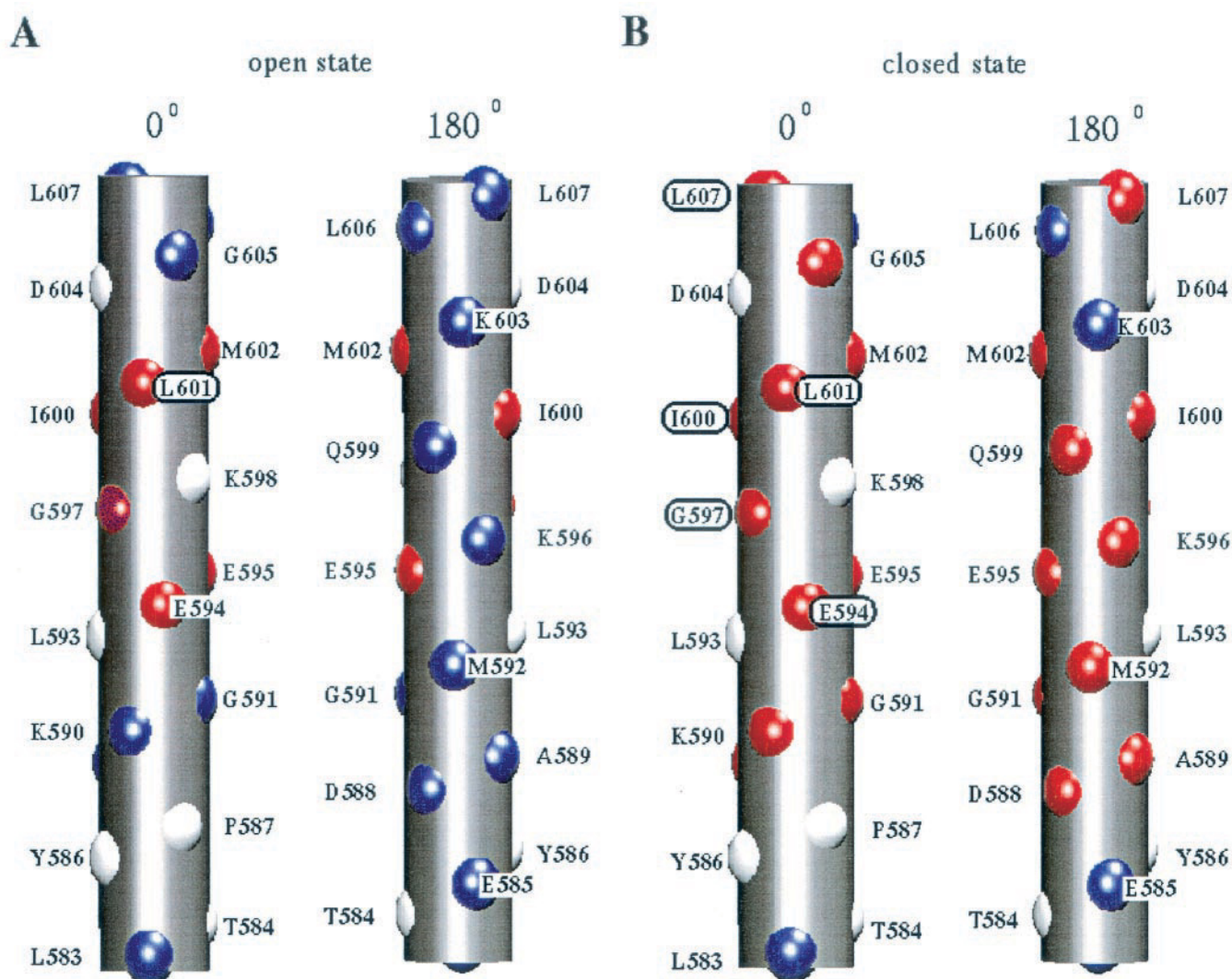


FIGURE 6 Map of Cd^{2+} blockage of cysteine mutants from Leu-583 to Leu-607. In each panel current reduction is shown in a color-coded map (red indicates reduction higher than 30%, blue between 0 and 30%, white indicates residues where the corresponding cysteine mutant did not produce functional channels with a cGMP-gated current) with residues located on an ideal α -helix. Two views of the α -helix, rotated by 180° around its symmetry axis, are shown. (A) Current reduction in the presence of 1 mM cGMP. (B) Current reduction in the absence of cGMP. The black circles indicate residues where the corresponding cysteine mutant was significantly (>40%) blocked by 2.5 mM MTSET. Gly-597 is colored blue and red because the current of mutant channel G597C is reduced by <30% by Cd^{2+} and ~70% by CuP.

rent was recorded from oocytes injected with the mRNA of mutant channels Y586C and L584C. As shown in Fig. 5, 100 μM Cd^{2+} did not produce any irreversible decrease of the cGMP-gated current in mutant channels E585C (A and B) and L583C (C and D) either in the open or in the closed state. Similarly, neither the addition of 1 μM nor 1 mM CuP to the bathing medium caused any permanent decrease of the cGMP-gated current. MTSET caused no current reduction when applied to the channels both in the closed and in the open states. Therefore, the sensitivity to sulfhydryl reagents of cysteine mutants from Tyr-586 to Leu-583 is similar to that observed in the wt channel.

DISCUSSION

The current reduction induced by Cd^{2+} and CuP illustrated in Figs. 1–5 is better summarized and rationalized when the residues responsible for the altered channel activity are mapped on an ideal α -helix. The degree of reduction of the cGMP-gated current in the corresponding cysteine mutant is shown in a color-coded scale, as shown in Fig. 6, A and B. Red indicates a significant reduction of the cGMP-activated current, (larger than 30%), blue indicates the absence of a significant reduction, i.e., lower than 30%, and white indicates residues where the corresponding cysteine mutant did not produce functional channels.

As clearly shown in Fig. 6 *A*, the current reduction of Cd^{2+} on cysteine mutants in the open state is well localized on one side of the helix, covering three turns of the helix from Glu-594 to Leu-601. The effect of CuP has a similar pattern, with the largest decrease coinciding with that of Cd^{2+} , the only exception being mutant channel G597C, where CuP caused a stronger current reduction ($67 \pm 7\%$) than Cd^{2+} ($23 \pm 4\%$), possibly because of a low accessibility of Gly-597 to Cd^{2+} . On the contrary, the reduction of the current caused by Cd^{2+} and CuP in the closed state is rather diffuse and it is significant for the majority of cysteine mutants from Asp-588 to Leu-607 (Fig. 6 *B*). Sulfhydryl reagents did not have any effect on cysteine mutants downstream from Pro-587. In the open state, MTSET caused a reduction of the current only in the Leu-601 cysteine mutant. In the closed state, a current decrease, larger than 40%, caused by MTSET, was observed only in cysteine mutants Leu-607, Leu-601, Iso-600, Gly-597, and Glu-594. Therefore, in the open state, the effect of Cd^{2+} and CuP among these mutants coincides almost completely with that of MTSET in the closed state, with the exception of Leu-607.

The different action of MTSET and Cd^{2+} and the similar action of Cd^{2+} and CuP can be rationalized by their different interactions with cysteine mutants. One molecule of MTSET forms a disulfide bond with the thiol group of a single cysteine molecule, whereas one Cd^{2+} atom is usually coordinated by two or more (up to four) cysteines and CuP enhances the formation of disulfide bonds between two cysteines. A Cd^{2+} ion has an approximate diameter of 1.82 Å (Glusker, 1991). Inspection of the 3D structure of metallothioneins deposited in the Protein Data Bank (Berman et al., 2000) indicates that the distance between a Cd^{2+} ion and the sulfur atom of a coordinating cysteine is ~ 2.5 Å, and that distances between the C_α of two cysteines coordinating the same Cd^{2+} ion ranges between 4 and 9 Å (Krovetz et al., 1997; Ermler et al., 1998; Maroney, 1999). The distance between the C_α of two cysteines forming a disulfide bond ranges between 4 and 6.5 Å (Srinivasan et al., 1989). Given the thermal fluctuations of the protein, the maximum distance between the C_α of two cysteines able to form a disulfide bond or to coordinate one Cd^{2+} ion (establishing bonds at the previously reported distances) can be estimated as being around 10 Å (Johnson and Zagotta, 2001; Careaga and Falke, 1992; Krovetz et al., 1997; Ermler et al., 1998; Maroney, 1999). The space occupied by a molecule of MTSET is approximately a cylinder with a diameter of 6 Å and a height of 10 Å (Akabas et al., 1992). These distances and the observed effects of the sulfhydryl reagents will be used to understand the experimental data and to develop a model of the relative location of two C-helices in the open state.

Current reduction in the open state

The clear action of Cd^{2+} and CuP in the open state, when residues are mapped on an ideal α -helix (see Fig. 6 *A*), has two major implications. First, it gives experimental evi-

dence that in the open state, residues from Asp-588 to Leu-607 have the secondary structure of an α -helix. Such a secondary structure was previously assumed, in analogy with the tertiary structure of CAP (Weber and Steitz, 1987; Passner et al., 2000). Second, it suggests that in the open state, C-helices from two subunits come in close contact, also like those in CAP. This last conclusion, however, depends on and is consistent with the notion that the C-helices are interacting as dimers. However, the experimental data do not rule out other physical mechanisms and different structural configurations.

In the open state, the action of Cd^{2+} and CuP strongly suggests that the distance between the C_α of residues Leu-601, Ile-600, Gly-597, and Glu-594 of two subunits is between 4 and 10 Å. Under these conditions, in the corresponding cysteine mutants, disulfide bonds can be formed and Cd^{2+} ions can find pairs of neighboring cysteines for an energetically favorable coordination. The formation of these bonds between pairs of neighboring cysteines leads to the disruption of the gating mechanism and the subsequent suppression of the cGMP-gated current. This last conclusion does not depend on the presumed structural homology with CAP.

CAP residues forming the C-helix and the hinge with the B-helix, from Arg-103 to Asn-133, are shown in Fig. 7 *A*, and are compared to the sequence of residues from Met-580 to Asn-610 of the BROD CNGA1 channel. Pro-110 and Asp-111 at the hinge of the B- and C-helix of CAP are conserved in the BROD CNGA1 channel and correspond to Pro-587 and Asp-588. Sequence similarity between CAP and BROD CNGA1 downstream of these two conserved residues is not high. Two views (rotated by 90° around the twofold symmetry axis of the template) of the 3D structure of the two C-helices of CAP and their hinge with B-helices are shown in Fig. 7, *B* and *C*. In CAP the two C-helices have their closest contact in correspondence of Leu-124, with the C_α at a distance of 5.3 Å. The angle between the two C-helices is $\sim 35^\circ$.

If the C-helices of the BROD CNGA1 channel have the same 3D structure as those in CAP using the sequence alignment of Fig. 7 *A*, the C_α of residues Glu-594, Gly-597, Leu-601, and Asp-604 will be at a distance of < 10 Å, in agreement with the Cd^{2+} and CuP action in the open state. This comparative model, however, predicts the C_α of Iso-600 of two neighboring subunits to be at 12 Å, a distance too high to be in agreement with the action of Cd^{2+} and CuP on mutant channel I600C. In addition, the comparative model also suggests that the C_α of Leu-607 of two neighboring subunits are at 8.5 Å, a distance compatible with a strong effect of Cd^{2+} and CuP on the cGMP-gated current, not experimentally observed (see Figs. 1 and 6).

A way to obtain a better relation between the experimental data and the comparative model of the CNB domain is to increase the crossing angle between the C-helices, as shown

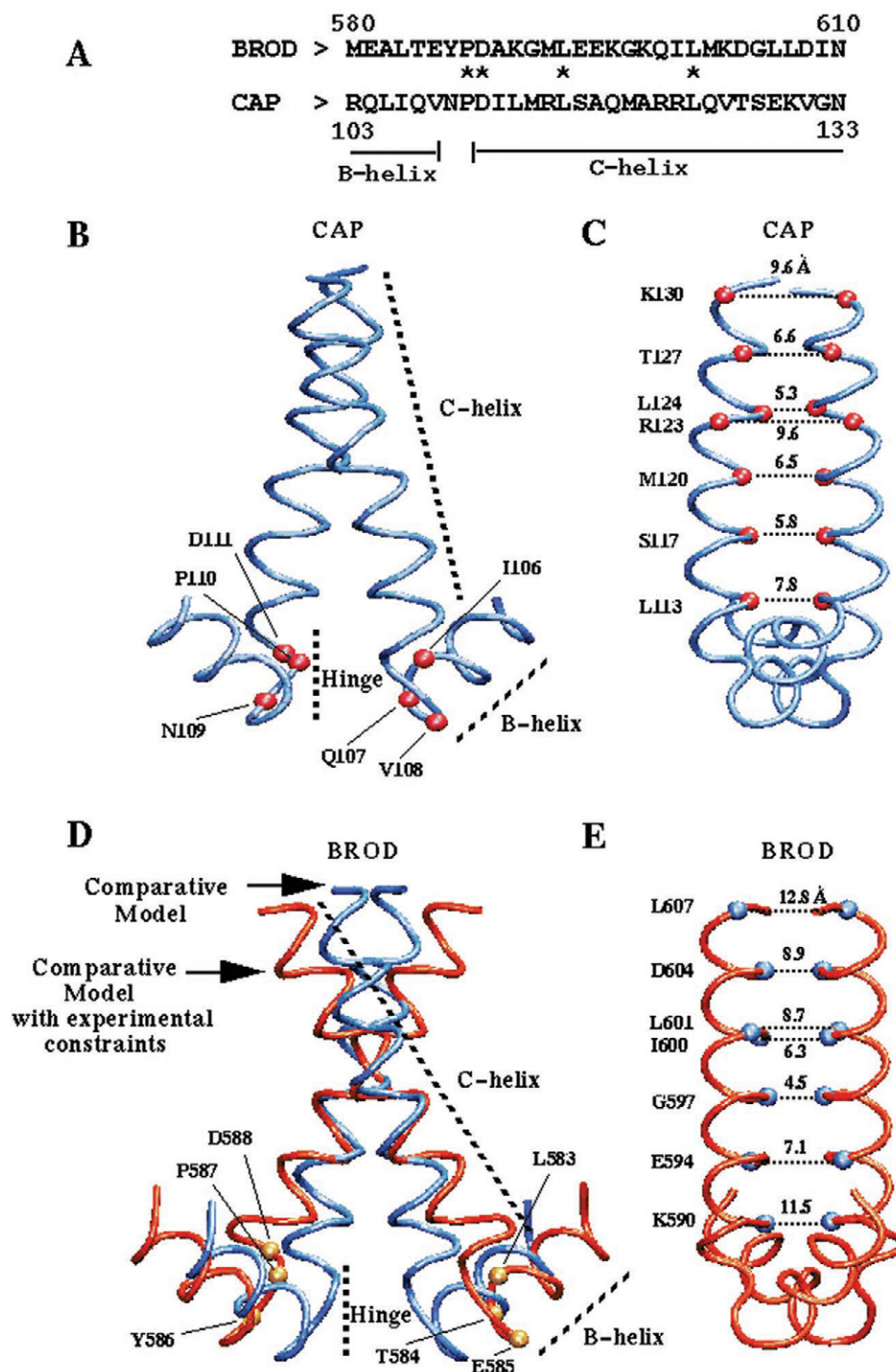


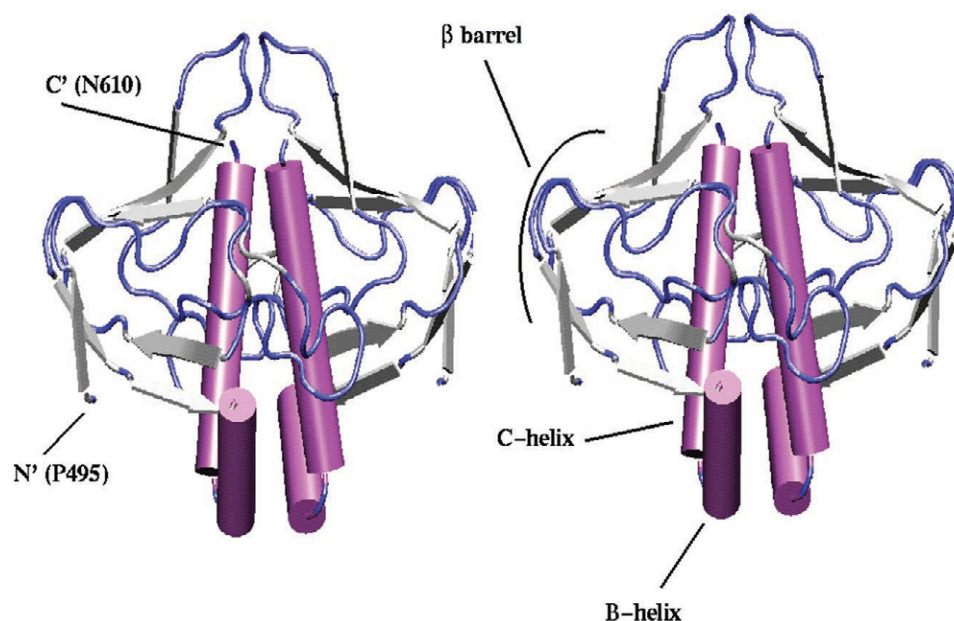
FIGURE 7 C-helices in CAP and in BROD CNGA1. (A) B- and C-helix alignment between BROD CNGA1 and CAP sequences. (B and C) Two different views of the 3D location of C-helices in CAP; C_{α} of the residues at the hinge between B- and C-helix (Asn-109, Pro-110, and Asp-111), at the end of the B-helix (Ile-106, Gln-107, and Val-108) (B) and at helix-helix interface (Leu-113, Ser-117, Met-120, Arg-123, Leu-124, Thr-127, and Lys-130) are shown (C); distances in C are in Angstroms (Å). (D and E) Two different views of the putative 3D location of C-helices in the open state of BROD CNGA1. In D, the crossing angle in the comparative model (blue) is compared to that one obtained considering the experimental constraints (red); C_{α} of the residues at the hinge between the B- and C-helix (Tyr-586, Pro-587, and Asp-588), at the end of the B-helix (Leu-583, Thr-584, and Glu-585) (D), and at helix-helix interface (Lys-590, Glu-594, Gly-597, Ile-600, Leu-601, Asp-604, and Leu-607) are shown (E); distances in E are in Angstroms (Å).

in Fig. 7 D, so that residues Leu-601, Ile-600, Gly-597, and Glu-594 of the two C-helices are in close contact. In this case the closest contact between the two C-helices is at Gly-597, with the C_{α} at a distance of 4.3 Å and with the angle between the two C-helices being 60°. With this geometry, the distance between the C_{α} of Leu-607 is 12.8 Å (Fig. 7 D), compatible with the absence of current reduction

by Cd^{2+} and CuP in the mutant channel L607C when the channel is in the open state.

Residues downstream from Pro-587, of two neighboring subunits, such as Glu-585 and Leu-583, are expected to be rather distant, as mutant channels G585C and L583C are neither affected by Cd^{2+} nor by CuP and they might form the B-helix of the BROD CNGA1 channel. Residues from

FIGURE 8 A putative model of the CNB domain in the open state composed of two dimers homologous to the CNB domain of CAP (Weber and Steitz, 1987).



Glu-585 to Asp-588 probably form the hinge between the C- and B-helices.

The 3D structure of the C-helices shown in Fig. 7, *D* and *E* provides an explanation also for the action of MTSET. In the open state, MTSET affects only the mutant channel L601C, implying that Leu-601 is accessible to the reagent. Leu-601 is indeed at the interface of the two C-helices with a C_{α} - C_{α} distance of 8.7 Å and, during its thermal fluctuations, can bind the large compound MTSET. Once bound, MTSET is likely to prevent the C-helix from reaching the correct position necessary for triggering the gating of the channel.

Current reduction in the closed state

As shown in Figs. 1–5, in the Results section, and summarized in Fig. 6 *B*, in the closed state of the channels, Cd^{2+} and CuP cause a decrease of the current in almost all of the cysteine mutants, from Asp-588 to Leu-607. A significantly lower decrease was observed only in cysteine mutants Leu-606, Lys-603 and, to some extent, in Met-602, Met-592, and Ala-589. All these residues, as shown in Fig. 6 *B*, are located on the same face of the α -helix but at the opposite side of those associated with the current reduction of Cd^{2+} and CuP in the open state. The effect of Cd^{2+} and CuP is likely to be mediated by the interaction among thiol groups of endogenous and exogenously introduced cysteines, transiently entering in close proximity.

In the closed state, when MTSET binds to the thiol of cysteine mutants, the gating mechanism is significantly impaired for mutants L607C, L601C, I600C, G597C, and E594C. In the open state, Leu-601, Ile-600, Gly-597, and Glu-594 are at the interface of the C-helices and, in the

closed state, when MTSET binds to the corresponding cysteine mutants, it blocks the motion of the C-helices toward the necessary position for opening the channel. Therefore, the cGMP-activated current in mutant channels L601C, I600C, G597C, and E594C is decreased by Cd^{2+} and CuP in the open state and by MTSET in the closed state.

In the closed state, MTSET potentiates mutant channels L606C, K603C, K596C, and E595C and protects G605C, M602C, I600C, and E595C from the effect of Cd^{2+} and CuP. Therefore, Leu-606, Gly-605, Lys-603, Met-602, Leu-601, Ile-600, Gly-597, Lys-596, and Glu-595 are accessible to MTSET. Glu-594 does not seem to be accessible to MTSET in the closed or in the open state, as the mutant channel E594C is neither potentiated by MTSET nor protected from Cd^{2+} current reduction by MTSET. The current reduction pattern of Cd^{2+} , CuP, and MTSET in the closed state suggests two conclusions: first, that in the closed state the great majority of residues from Asp-588 to Leu-607 are accessible to sulfhydryl reagents, and second, that these residues undergo significant rearrangements compared to their open state conformations.

CONCLUSIONS

The conclusions of the present manuscript can be summarized in the model shown in Fig. 8. In the presence of cGMP, i.e., in the open state, the CNB domain of the BROD CNGA1 channel is composed of two dimers, each of which is similar, but not identical to, the CNB domain of CAP, where the two C-helices cross and are in close contact. In the absence of cGMP, C-helices are free to move around their hinge. During these rearrangements C-helices may

kink and bend at variable angles and at different residue positions.

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