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Mutations in the putative pore-forming domain of CFTR do not change anion selectivity of the cAMP activated Cl^- conductance

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Abstract **Cystic fibrosis transmembrane conductance regulator (CFTR) apparently forms Cl^- channels in apical membranes of secretory epithelial cells. A detailed model describes molecular structure and biophysical properties of CFTR and the impact of various mutations as they occur in cystic fibrosis. In the present report mutations were introduced into the putative 6th α-helical transmembrane pore forming domain of CFTR. The mutants were subsequently expressed in *Xenopus* oocytes by injection of the respective cRNAs. Whole cell (wc) conductances could be reversibly activated by IBMX (1 mmol/l) only in oocytes injected with wild-type (wt) or mutant CFTR but not in oocytes injected with water or antisense CFTR. The activated conductance was partially inhibited by (each 100 μmol/l) DIDS (27%) and glibenclamide (77%), but not by 10 μmol/l NPPB. The following mutations were examined: K335E, R347E, R334E, K335H, R347H, R334H. They did not measurably change the wt-CFTR anion permeability (P) and wc conductance (G) sequence of: $P_{Cl^-} > P_{Br^-} > P_{I^-}$ and $G_{Cl^-} > G_{Br^-} > G_{I^-}$, respectively. Moreover, anomalous mole fraction behavior for the cAMP activated current could not be detected: neither in wt-CFTR nor in R347E-CFTR. Various mutants for which positively charged amino acids were replaced by histidines (K335H, R347H, R334H) did not show pH sensitivity of the IBMX activated wc conductance. We, therefore, cannot confirm previous results. CFTR might have a different molecular structure than previously suggested or it might act as a regulator of ion conductances.**

Key words: Cystic fibrosis; CFTR; Cl^- conductance; Permeability; *Xenopus* oocyte; Site-directed mutagenesis

1. Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ABC family of proteins and is mutated in cystic fibrosis (CF) [6]. Several lines of evidence indicate that CFTR is responsible for cAMP-dependent Cl^- currents in epithelial cells. (i) Mutations of CFTR as they occur in CF result in impaired cAMP activated Cl^- conductance. (ii) Overexpression of exogenous CFTR in a variety of expression systems results in a cAMP-activated Cl^- conductance or Cl^- channels with an anion permeability and conductance sequence, respectively, of $Cl^- \approx Br^- > I^-$ [5]. (iii) Hydrophobicity plots of CFTR suggest 12 transmembrane α-helical domains forming an anion selective pore along with a regulatory subunit and two nucleotide binding domains [6]. (iv) Reconstitution of purified CFTR or fusion of CFTR containing liposomes with black lipid membranes induced Cl^- channels with a single channel conductance of about 7 pS [2,8]. (v) Mutations in the apparent 6th α-helical transmembrane domain of CFTR (K335E, R347E) resulted in changes in the halide selectivity of CFTR [1]. (vi) Furthermore, anomalous mole fraction behavior of wt-CFTR was abolished in one mutant of CFTR (R347D) [7]. These changes of the halide permeability and conductance sequence, respectively, strongly support the model of the anion conducting pore. We felt that these important observations had to be confirmed and expanded by additional studies using another CFTR expression system.

In the present study we demonstrate that CFTR, when mutated in this region and expressed in oocytes of *Xenopus laevis*, has the same halide selectivity as wt-CFTR. The data presented here, thus, do not confirm those of previous reports [1,7].

2. Materials and methods

2.1. CFTR-cRNA and site directed mutagenesis

A 4.7 kb fragment encoding CFTR was subcloned into p-Bluescript vector (Stratagene) via *Kpn*I and *Not*I and amplified in *E. coli* (TOP-10, Invitrogen). For in vitro transcription of cRNA the plasmid was linearized via *Kpn*I and cRNA was synthesized using the T3 promotor and a 5′ cap (mCAP RNA, Stratagene). For mutagenesis, a 2.1 kb *Eco*RI CFTR fragment, comprising the first six transmembrane domains and the first nucleotide binding fold (NBF1), was subcloned into p-alter vector (Altered Sites in vitro Mutagenesis System, Promega, Heidelberg, Germany) and single-stranded cDNA was obtained by helper phage R408. All mutations were performed in the cDNA sequence apparently encoding the 6th transmembrane domain of CFTR. Synthesis of mutated CFTR-cDNA was induced by annealing of ampicillin repair oligonucleotide and oligonucleotide primers carrying the respective mutation changing positively charged to negatively charged amino acids (R334E, R347E, K335E) or replacing R and K at these positions by histidines (R334H, R347H, K335H). After subcloning of the mutated fragment into p-Bluescript, correct mutations were confirmed by sequencing the entire subcloned CFTR fragment including ligation sites using DIG-Taq sequencing kit (Boehringer) and cycle sequencing kit (PRISM, Perkin Elmer) with an automated sequencer (Pharmacia, Germany).

2.2. Microinjection and voltage clamping of oocytes

Isolation and voltage clamping of oocytes have been described in previous reports [3]. In brief, adult *Xenopus laevis* female frogs were obtained from H. Kähler (Bedarf für Entwicklungsbiologie, Hamburg, Germany). After isolation, oocytes were dispersed and defolliculated by 1 h treatment with collagenase (type A, Boehringer) [3]. Subsequently, oocytes were rinsed 10 times and kept in a Na^+-HEPES buffer (pH 7.55), supplemented with pyruvate (2.5 mM), theophylline (0.5 mM) and gentamycin (50 mg/l) at 14–18°C. Oocytes of identical batches were injected each with 10–50 ng of cRNA dissolved in about 50 nl double-distilled H_2O (controls) (PV830 pneumatic pico-pump, WPI, Germany). 2–3 days after injection oocytes were impaled with two electrodes (Clark Instruments) which had input resistances of ≤ 1 MΩ when filled with 3 mol/l KCl. A flowing (3 mol/l) KCl electrode served as a bath reference. The membrane currents were measured by voltage clamping of the oocytes (OOC-1, WPI, Germany) from −80 to +80 mV in steps of 10–20 mV and conductances were calculated from current–voltage relations (of the outward current, i.e. anion entry). Permeability

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sequences were obtained from zero-current voltages using the Goldman-Hodgkin-Katz equation.

All used compounds were of highest available grade of purity. They were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). Agonists were dissolved from a stock in standard bath solution and prepared freshly every day.

All data were presented as original recordings or as mean values ± S.E.M. (n = number of observations). Statistical analysis was performed according to Students t-test. P values < 0.05 were accepted to indicate statistical significance.

3. Results

3.1. Cl^- currents activated in oocytes overexpressing CFTR

A time-independent whole cell (wc) current was activated by 1 mmol/l IBMX in oocytes which were injected with wild-type (wt)-CFTR (Fig. 1A,B). Wc conductances were significantly increased from 1.6 ± 0.2 to 12.6 ± 4.2 μS (n = 9) with 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate. When extracellular Cl^- was replaced by equimolar concentration of the impermeable anion gluconate, the reversal potential for wc currents (zero current voltage) of stimulated oocytes was shifted to more depolarized values by 33.9 ± 4.1 mV (n = 9), indicating that a wc Cl^- conductance was activated. Moreover, the activated wc current was inhibited significantly by 4,4′-dinitro-stilbene-2,2′-disulphonic acid (DIDS, 100 μmol/l) and glibenclamide [9] (100 μmol/l) by 26.6 ± 0.3% (n = 9) and 77.3 ± 8.2% (n = 4), respectively, but was insensitive towards 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB [9], 10 μmol/l (n = 7). No currents were activated in oocytes injected with either water or antisense CFTR-cRNA (Fig. 1B).

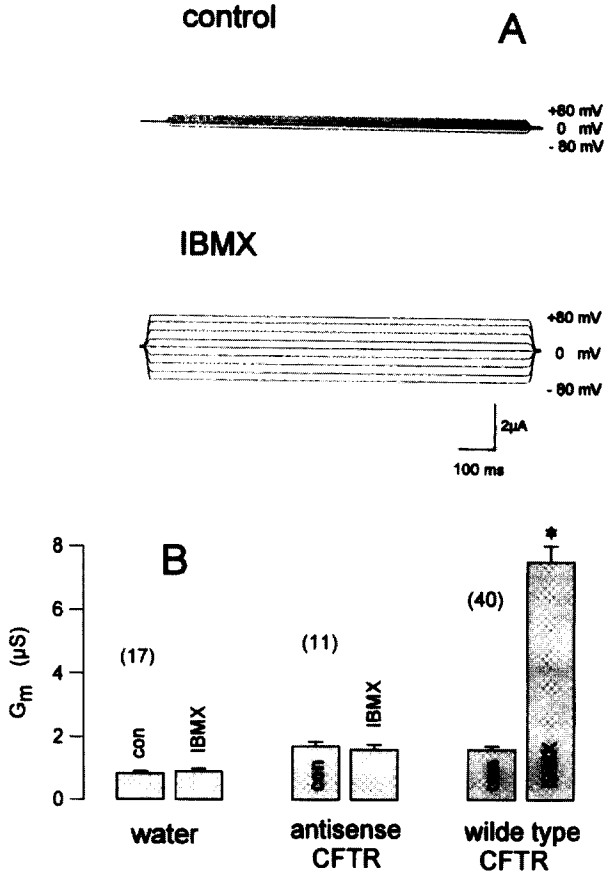

Fig. 1. (A) Recording of the wc currents obtained in a voltage clamp experiment with an oocyte from *Xenopus laevis* transfected with wild-type CFTR. The oocyte was voltage clamped from −80 to +80 mV in steps of 20 mV. Wc steady state currents were increased substantially by 1 mmol/l 3-isobutyl-1-methylxanthine (IBMX). (B) Summary of the wc conductances of *Xenopus* oocytes as measured in voltage clamp experiments. IBMX increased wc conductance in oocytes injected with wt CFTR-cRNA but not in water or antisense injected oocytes. Asterisks indicate significant differences (n = number of experiments).

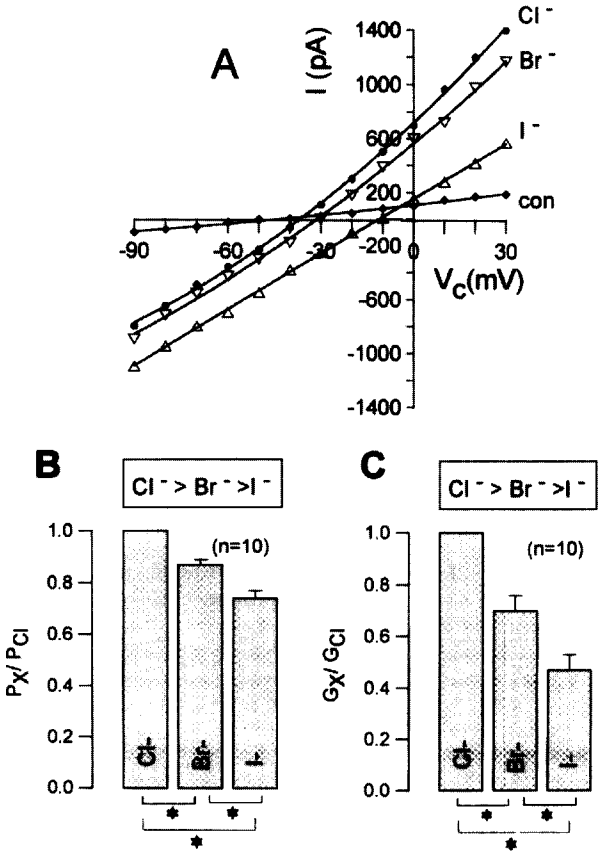

Fig. 2. Anion selectivity of the IBMX induced wc current in *Xenopus* oocytes. (A) *I/V* curves obtained under control conditions and after stimulation with IBMX. Note the shift and decreased slope of the *I/V* curves, when extracellular Cl^- was replaced by either I^- or Br^-. Summary of the permeability ratios (B) and conductance ratios (C) for the respective anions obtained from intercepts and slopes (outward current) of the respective *I/V* curves.

3.2. Anion selectivities of wt and mutant CFTRs are similar

In wt CFTR overexpressing oocytes, anion permeability and conductance sequences were examined by a complete replacement of extracellular Cl^- by I^- or Br^-. Fig. 2A demonstrates typical current–voltage (I/V) curves of an IBMX stimulated oocyte for either Cl^-, I^- or Br^- as the extracellular anion. The data of this set of experiments are summerized in Fig. 2B,C and indicate that anion permeability and conductance sequences of the wt CFTR for the examined anions were identical and were $Cl^- > Br^- > I^-$. This is the reverse anion sequence obtained for the Ca^{2+}-dependent Cl^- conductance endogenously present in oocytes, which was $I^- > Br^- > Cl^-$ for both permeability as well as conductance (n = 14).

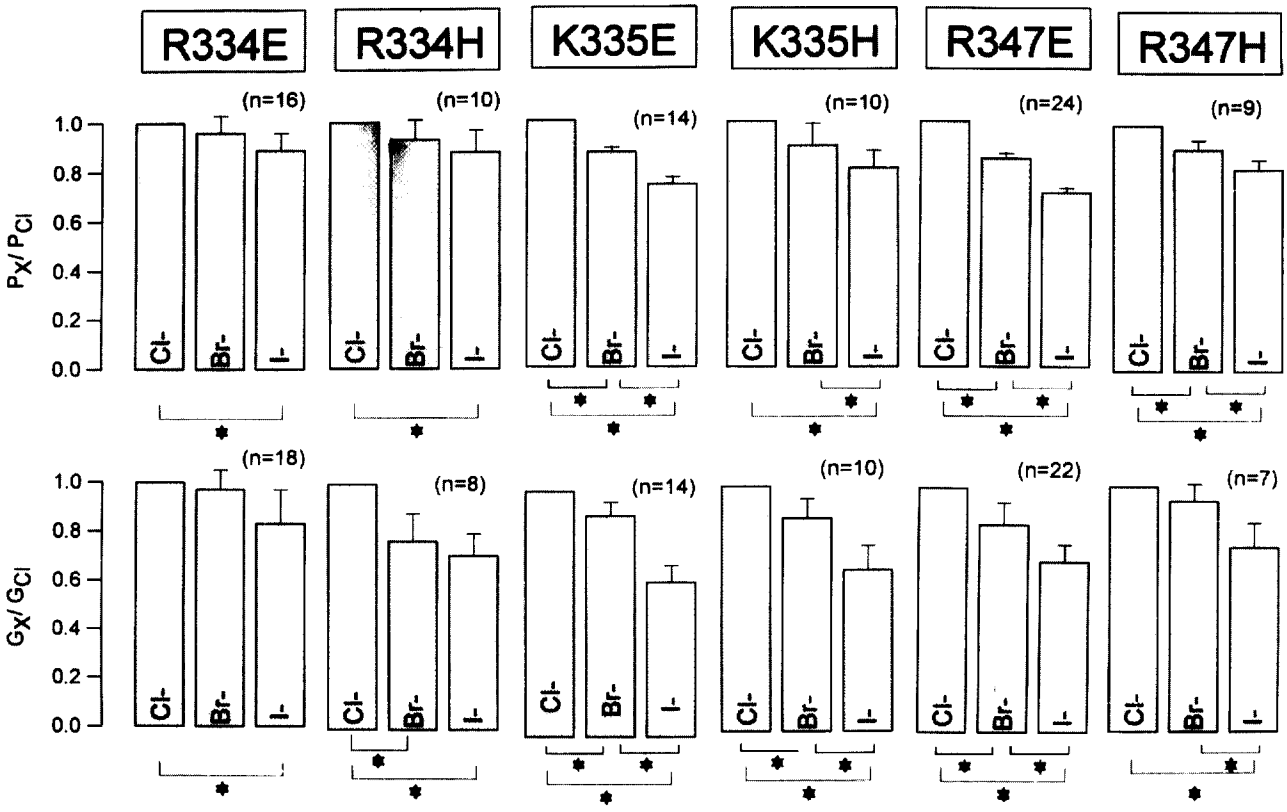

Fig. 3. Summary of the permeability and conductance ratios obtained in experiments with oocytes injected with cRNA of various CFTR mutants. The G values were determined as the ratio of the conductance measured in the presence of IBMX minus that under control conditions over that obtained for Cl^-. Note that similar permeability as well as conductance ratios were obtained for all mutants when compared to wild-type CFTR. Asterisks indicate statistical differences.

Next, positively charged amino acids R334, R347, K335 located in the putative 6th pore forming transmembrane α-helical domain of CFTR, were exchanged by histidines (R334H, R347H, K335H) or by the negatively charged glutamate (R334E, R347E, K335E). Wc conductances were activated significantly by IBMX in all 6 mutants but to variable degrees (ΔG in μS): 3.2 ± 0.6 (R334E, n = 20), 2.7 ± 0.6 (R334H, n = 13), 7.1 ± 0.9 (K335E, n = 20), 2.8 ± 0.7 (K335H, n = 10), 3.2 ± 0.04 (R347E, n = 32) and 1.8 ± 0.3 (R347H, n = 10). This indicates that some of these mutations led to reduced expression or function. None of the mutations exposed an altered halide permeability or conductance sequence, respectively, of the IBMX activated wc Cl^- conductance. This is summarized in Fig. 3.

3.3. SCN^- conductance in wt and R347E CFTR and pH insensitivity of histidine mutants

Extracellular Cl^- (101 mmol/l) was partially (7 mmol/l) or almost completely (96 mmol/l) replaced by equal concentrations of SCN^- and wc currents were measured in IBMX stimulated oocytes. For both wt CFTR and R347E-CFTR we found reduced wc conductance when Cl^- was replaced by SCN^-. Anomalous mole fraction behavior could neither be detected for wt-CFTR nor for R347E mutants (Fig. 4). Interestingly, the calculated permeability ratio (P_{SCN}/P_{Cl}) was significantly larger than the conductance ratio. It was 2.2 ± 0.3 (wt CFTR, n = 17) and 2.0 ± 0.2 (R334E, n = 19), respectively.

Following previous experiments [7] wc Cl^- conductances were examined in mutants bearing a histidine mutation (K335H, R347H, R334H) at different extracellular pH values. However, unlike in the previous study in R347H [7] no significant changes of G could be detected when extracellular pH was

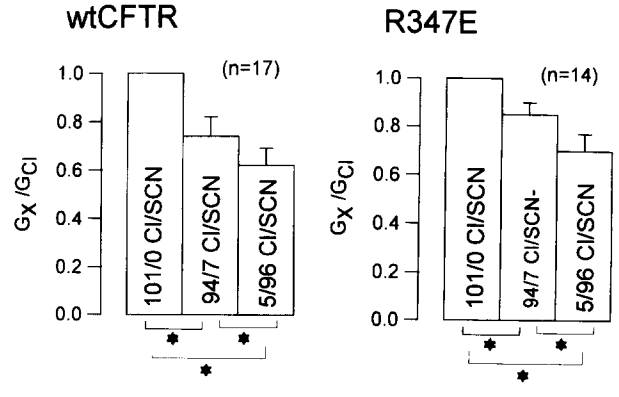

Fig. 4. Summary of the conductance ratios obtained in wt and R347E-CFTR transfected oocytes stimulated by IBMX when 101 mmol/l extracellular Cl^- was replaced by (mmol/l) 94 Cl^- and 7 SCN^- (94/7) and 5 Cl^- and 96 SCN^- (5/96), respectively. The G values were determined as the ratio of the conductance measured in the presence of IBMX minus that under control conditions over that obtained for Cl^-. Note that conductance decreased in both series when extracellular Cl^- was replaced by SCN^-.

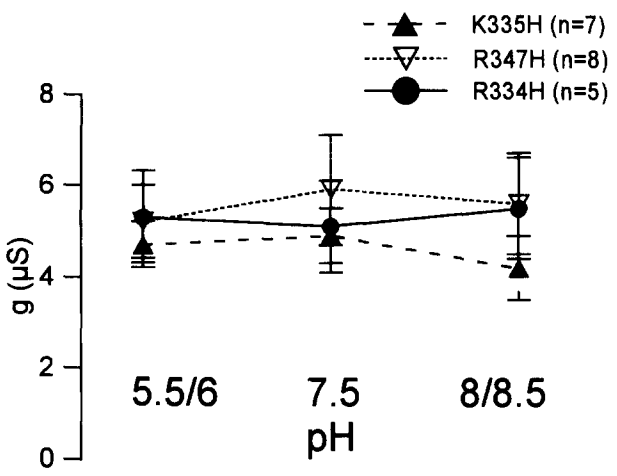

Fig. 5. Summary of the conductances obtained from IBMX stimulated oocytes at different extracellular pH values. Experiments were performed with oocytes overexpressing three different CFTR mutants: K335H, R347H, R334H. Note that for all three mutants wc conductances were not significantly changed when the extracellular solution was either acidified (pH 5.5 or 6) or alkalinized (pH 8 or 8.5).

alkalinized to 8 and 8.5 or was acidified to 6 or even lower to 5.5 (Fig. 5).

4. Discussion

The present experiments were performed in order to examine the applicability of the current molecular model of CFTR. While numerous reports suggest CFTR as a protein kinase activated Cl^- channel and present detailed concepts about channel regulation, kinetic properties and dysfunction in CF, a more direct evidence for the channel function of CFTR was deduced from the analysis of the conductive properties of site directed mutants. To this end, a number of mutations were introduced into the apparent 6th pore forming domain of CFTR. The 6th transmembrane stretch contains several positively charged amino acids and forms a α-helical secondary structure [6]. Positively charged amino acids should significantly determine the conductive properties of the channel pore [4,10]. Consequently, mutation of these positively charged amino acids presumably lining the conductive pore by negatively charged amino acids might considerably influence the anion selectivity of CFTR.

In fact, in a previous study lysine and arginine were replaced by negatively charged amino acids in the first and sixth transmembrane domain (K95D, K335E, R347E), respectively [1]. The authors found a reversed permeability and conductance ratios for chloride over iodide of the cAMP induced wc current in HeLa cells overexpressing these mutant CFTRs. From these results it was concluded that : (i) CFTR forms a Cl^- channel; and that (ii) the molecular model of CFTR as predicted from hydrophobicity plots is basically correct.

In the present study we repeated some of the published (K335E, R347E, R347H) and performed additional mutations (R334E, R334H, K335H) which are all located in the putative sixth transmembrane domain and overexpressed the respective CFTRs in oocytes. To our surprise, for none of the mutants a change of the permeability or conductance sequence of $Cl^- \geq Br^- > I^-$ for wt CFTR could be detected. However, the amount of wc Cl^- current activated by cAMP was significantly reduced for most of the mutants when compared to wt CFTR.

In another study anomalous mole fraction effects were reported for single CFTR channels recorded in Chinese hamster ovary (CHO) cells [7]. Comparable wc measurements were performed in the present study (K335E, R347E compared to R347D in [7]) with SCN^- and Cl^- present in the extracellular bath solution at different concentration ratios. Measurements of the wc conductance did not indicate anomalous mole fraction behavior for wt CFTR. Even more important, SCN^- conductance was not different for the R347E mutant. However, it is important to keep in mind that SCN^- belongs to the group of chaotropic ions. Therefore, data from experiments with SCN^- should a priori be interpreted with caution.

Additional mutations were constructed in which positively charged lysine and two arginines in the sixth transmembrane domain were replaced by pH-sensitive histidines (R334H, K335H, R347H). Histidine should be predominantly positively charged at low pH but uncharged at high pH. Consequently, Cl^- conductance properties should be different for different pH values. In the present study, unlike in the previous one ([7], R347H), we did not find significant differences for the cAMP activated wc conductances at different pH values in all three mutants. Moreover, permeability and conductance sequences for Cl^-, Br^- and I^- of the histidine mutants did not differ from that of wt CFTR (data not shown).

We have no ready explanation of why the present data are in contrast to previous reports [1,7]. Obviously, the expression systems used in previous (HeLa cells and CHO cells [1,7]) and in the present studies are different. In the first report [1] the number of observations was rather limited and the variability of the data was appreciable. Furthermore, and surprisingly, the *I/V* curves obtained with outside anion replacements showed marked conductance changes even for the inward currents (Cl^- flowing out of the cell). This was not observed here (Fig. 2). The second report [7] shows distinct differences in single channel properties caused for the mutants. However, only single channel characteristics but not the wc conductance have been reported.

In summary, the data presented here do not support the concept that the putative sixth transmembrane α-helical domain contributes to the anion conductive pore. The current model of CFTR probably requires reevaluation and it should be considered that CFTR might be a regulator of Cl^- channels intrinsically present in various cell types.

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