Phosphorylation site independent single R-domain mutations affect CFTR channel activity

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Abstract We investigated CFTR channel activity of mature R-domain mutants showing single alterations at sites other than the predicted phosphorylation sites. All mutations were found in cystic fibrosis (CF) patients (H620Q, E822K and E826K). The macroscopic CFTR chloride conductance induced by phosphorylation was significantly enhanced in *Xenopus* oocytes injected with mRNA of H620Q but reduced in the E822K and E826K mutants compared to wild type CFTR. The anion permeability sequence for all three mutants was the same as that of wild type CFTR. Cell attached single channel studies in COS cells revealed that both open channel probability and/or the number of functional channels were either higher (H620Q) or lower (E822K and E826K) than in wild type CFTR. Single channel conductances were unchanged in all mutants. Our results suggest that additional sites in the R-domain other than phosphorylation sites influence gating of CFTR channels.

Key words: Cystic fibrosis transmembrane conductance regulator; R-domain mutation; *Xenopus* oocyte; COS cell; Green fluorescent protein

1. Introduction

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease that is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a 169 kDa protein composed of two transmembrane domains (TMD1, TMD2), two nucleotide binding domains (NBD1, NBD2) and a unique regulatory R-domain [1]. Although there is little doubt that CFTR itself is a cAMP activated chloride channel [2], the precise gating mechanism of the CFTR channel is unclear.

The phosphorylation on the R-domain has been shown to be one of most important mechanisms for CFTR channel regulation [3–5]. More than nine phosphorylation sites on the R-domain have been identified as functional sites for phosphorylation by cAMP dependent protein kinase (PKA) and protein kinase C (PKC) [4,6,7]. It has been reported that after mutating some of the serines to alanine in predicted phosphorylation sites in the R-domain, CFTR can still be phosphorylated by PKA to yield an activated Cl^- channel with a substantially reduced open probability [4,7]. The complete deletion of the R-domain (residues 708–835) would not induce the CFTR channel to close but leave it activated at a relatively low level even in the absence of phosphorylation [6]. Up to now the role of the R-domain in channel regulation has still not been resolved, but it seems that the negative charges introduced by phosphorylation might be important for channel gating [6]. The importance of electric charges on the properties of CFTR has been studied for TMD1, and it was found that mutations of the lysines at positions 95 or 335 to acidic amino acids converted the selectivity sequence to $I^- > Br^- > Cl^-$ [8]. These changes of the halide permeability and conductance might imply that changing positively charged amino acids to negatively charged amino acids in TMD1 and R-domains, or vice versa, could alter ionic channel properties of CFTR. Phosphorylation of the R-domain, however, may also influence the conformation of NBD1 since it enhances its affinity for ATP [9]. More recent studies show that an alteration in the conformation of the R-domain other than a consensus phosphorylation site may also affect the CFTR channel [10].

During the studies of R-domain mutants from patients, we found that some mutant CFTR with a single mutation in the R-domain at other sites than its predicted phosphorylation sites could significantly change the CFTR channel activity. These data may suggest that a conformational change alone or combined with changes of electric charge in conserved R-domains region other than the predicted phosphorylation sites can also have a significant effect on CFTR channel regulation.

2. Materials and methods

2.1. Cells and CFTR expression system

A *Xho*I-*Kpn*I fragment containing the CFTR coding region was isolated from the prokaryotic vector pTG5960 (Transgene S.A.) and inserted in the eukaryotic expression vector pcDNA3 (Invitrogen). Three different mutations, t1992g (= H620Q), g2596a (= E822K) and g2608a (= E826K), were introduced using the Transformer Site-Directed Mutagenesis kit (Clontech). The complete CFTR coding region of the wild type and the three mutant constructs was characterized by dideoxy sequencing. The different constructs contained only the introduced mutations.

For single channel measurements, the different mutant and wild type CFTR cDNAs were transferred to the bicistronic green fluorescent protein (GFP) expression vector pCINeo/IRES-GFP [11].

2.2. Expression, pulse chase and immunoprecipitation of CFTR

20 μg of plasmid DNA was electroporated (Bio-Rad Gene Pulser, Bio-Rad Laboratories, Hercules, CA) into $1.5–3 \times 10^7$ COS cells. The transfected cells were cultured for 48–72 h at 37°C in DMEM F12 (Life Technologies, Inchinnan, UK) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and subsequently selected with G418 disulfate (480 mg/l; Duchefa, Haarlem, The Netherlands). After 2 weeks selection, the cells were starved for 30 min in RPMI 1640 medium (Life Technologies, Inchinnan, UK) without methionine and cysteine, labelled during a 30 min pulse in RPMI 1640 supplemented with 100 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (ICN

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Pharmaceuticals, Costa Mesa, CA) and finally chased for different time periods in DMEM F12 supplemented with 10% fetal bovine serum. Cells were scraped and subsequently lysed by sonication in ice cold IPPA buffer (20 mM Tris, 150 mM NaCl, 1% Na-desoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) supplemented with protease inhibitors (20 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, 1 mg/ml antipain, 1 mg/ml chymostatin and 1 mM phenylmethylsulfonyl fluoride). The lysate was precleared with protein A Sepharose CL-4B beads (Pharmacia Biotech, Uppsala, Sweden) and CFTR proteins were affinity purified by incubation for 1 h with an anti-CFTR monoclonal antibody directed against the C-terminal part of CFTR (Genzyme Diagnostics, Cambridge, MA). The immunocomplexes were purified on protein A Sepharose CL-4B beads and after addition of loading buffer (1% β-mercaptoethanol, 16 mM Tris-HCl pH 6.8, 4% SDS, bromophenol blue and 10% glycerol), loaded on a 4–12% SDS gel (Novex, San Diego, CA). After drying, the gel was exposed at −70°C to a light sensitive film for 3–4 h.

2.3. RNA transcription

The different CFTR constructs were in vitro transcribed using the T7 RiboMAX Large Scale RNA Production System (Promega, Madison, WI), according to the protocol of the manufacturer. The quality of the RNA was determined by means of formaldehyde-agarose (1%) gel electrophoresis.

2.4. Cells

Oocytes were dispersed and their follicle cell layers were removed by blunt dissection after a 1 h incubation in a Ca^{2+} free ND96 solution (see solution below) containing 2 mg/ml collagenase A (Boehringer Mannheim Biochemicals, Indianapolis, IN). After 2–4 h incubation in normal ND96 solution, the defolliculated oocytes were injected with ~50 nl of 5 mg/ml cRNA using a microinjector (WPI, Sarasota, FL). Injected oocytes were incubated in ND96 solution supplemented with gentamicin (50 mg/l) at 18°C for 3 days prior to experimentation.

Cultured COS cells were grown in DMEM medium containing 10% fetal calf serum at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

2.5. Electrophysiology

2.5.1. Whole cell current measurements. 72 h after injection, oocytes were perfused with ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.5 with NaOH). Each oocyte was voltage clamped using a two microelectrode voltage clamp as previously described [12]. Microelectrodes filled with 3 M KCl had a resistance of about 1 MΩ. pCLAMP software (version 5.5.1, Axon Instruments) was used to generate voltage pulse protocols and to digitize the signals into the microcomputer. The cAMP mediated CFTR Cl^- current was activated by applying 10 μM forskolin and 1 mM IBMX (Sigma, St. Louis, MO) to the bath solution. All experiments were performed at room temperature and data were analyzed by the software WinASCD 3.0 (G. Droogmans, K.U. Leuven). The conductances were calculated from I-V curves according to Ohm's law.

The permeability of iodide or bromide relative to that for Cl^- was determined from the shift in reversal potential in anion substitution experiments and calculated from the equation

$$\frac{P_X}{P_{Cl}} = \frac{[Cl^-]_e \cdot \exp(-\Delta E_{rev} \cdot F/RT) - [Cl^-]_{res}}{[X^-]_e}$$

in which $[X^-]_e$ represents the extracellular iodide or bromide concentration, $[Cl^-]_e$ and $[Cl^-]_{res}$ the extracellular (before substitution) and 'residual' extracellular Cl^- concentrations (present in $CaCl_2$ and $MgCl_2$, during substitution), ΔE_{rev} is the measured shift in reversal potential.

2.5.2. Single channel measurements. The cell attached patch clamp configuration was carried out on COS cells co-expressing CFTR and GFP. A List EPC-7 amplifier (Darmstadt, Germany) was used for single channel measurements. A Zeiss Axiovert 100 microscope equipped with a xenon light source and epifluorescence optics (Zeiss, XBO75 and Zeiss-EPI unit fluorescence condenser) was used to excite GFP proteins (450–490 nm) and detect their light emission through a 520 nm long-pass filter. Only cells emitting green fluorescent light after excitation were used for single channel studies. The standard pipette and bath solutions were (in mM): 140 *N*-methyl-D-glucamine chloride (NMDG), 2 $MgCl_2$, 10 HEPES, 1 EGTA, 5 CsCl, 10 NaF (pH 7.4). Pipette resistance was about 10 MΩ when filled with the pipette solution. After giga seal formation, single channel currents were continuously recorded to a computer hard disk with AXO Tape software (version 2.02, Axon Instruments, Foster City, CA). Data were sampled at 1 kHz and filtered at 500 Hz. Since COS cells are more sensitive than *Xenopus* oocytes to stimulation with IBMX and forskolin, only 0.1 μM forskolin and 1 μM IBMX were applied to the chamber for activation of CFTR Cl^- channels.

2.6. Statistics

All data were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant. Data are reported as mean ± S.E.M.

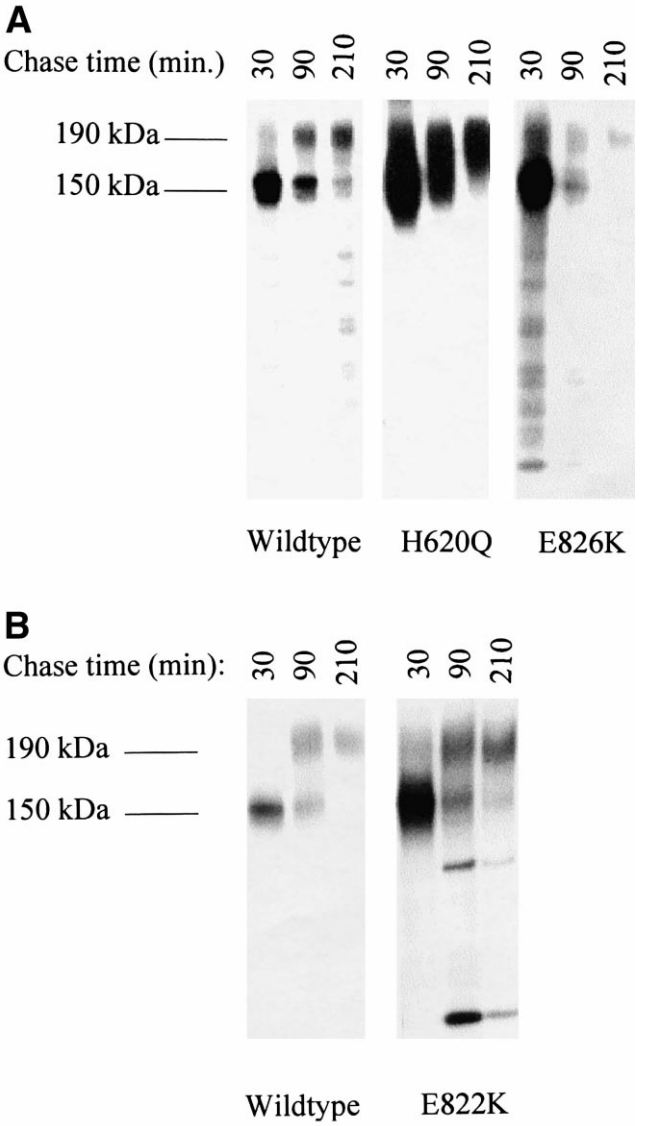

Fig. 1. Pulse chase and immunoprecipitation of CFTR. COS cells were transfected with wt CFTR, E822K CFTR (top), H620Q CFTR (bottom) and E826K CFTR (bottom) and selected for 2 weeks with G418. Proteins were subsequently labelled with translabel and chased for the indicated time periods. CFTR proteins were immunoprecipitated with a monoclonal antibody directed against the C-terminal part of CFTR (Genzyme). The isolated proteins were separated on a SDS gel. The different maturation forms are indicated: 150 kDa (core glycosylated protein) and 190 kDa (mature CFTR protein).

3. Results

3.1. Expression of mutant CFTR in COS cells

Normally, after translation (130 kDa), the wild type CFTR protein matures, through different glycosylation steps, from a core glycosylated protein (150 kDa) in the endoplasmic reticulum (ER) to a mature protein (190 kDa) in the trans-Golgi [13]. Only the mature form will reach the cell membrane to form a functional chloride channel [14]. It is known that some mutations located in the CFTR coding region give rise to a protein that only matures up to the core glycosylated form that is present in the ER [15–17]. These mutations seem to interfere with the folding pattern of the CFTR protein and aberrantly folded proteins are recognized and degraded by the control system that operates in this cell compartment [18]. Since the three maturation forms (130 kDa, 150 kDa and 190 kDa) can be separated on a SDS gel, the effect of a specific mutation on the maturation properties of the CFTR protein can be determined. In this study, the maturation pattern of three mutant R-domain proteins (H620Q-CFTR, E822K-CFTR and E826K-CFTR) has been characterized. Pulse chase experiments showed that all the mutants, like wild type CFTR, fully matured (Fig. 1). This implies that these mutations are not class II mutations and the change of CFTR channel activity is not due to misprocessing of the protein.

3.2. Whole cells currents of mutants in Xenopus oocytes

Whole cell membrane currents were recorded from *Xenopus* oocytes injected with RNA transcribed from either wild type or mutant (H620Q, E822K, E826K) constructs. After stabilization of the basal current in ND96 solution, oocytes were exposed to 1 mM IBMX and 10 μM forskolin (phosphorylation cocktail, phos-cock). As shown in Fig. 2A,B, application of phos-cock to the bath solution produced a substantial current in oocytes injected with wild type CFTR cRNA, but in oocytes injected with the vector only. Activation of the CFTR current usually occurred within 2 min and reached its maximal level after about 5 min. The I-V curves as shown in Fig. 2B are fairly linear, suggesting that the conductance is voltage independent. The mean size of the conductance amounted to 7.57 ± 0.65 μS ($n = 14$). For all three mutants we observed a similar activation time course as in wild type CFTR (not shown). The I-V relationships were also linear, although the macroscopic conductances were different. The two R-domain proteins E822K and E826K, in which a negatively charged glutamic acid was exchanged for a positively charged lysine, showed a significantly smaller phos-cock activated conductance. Oocytes expressing the mutant R-domain protein (H620Q), in which a predominantly positively charged histidine was substituted by a less charged glutamine (at pH 7.2), showed a much larger conductance activated by application of phos-cock. The pooled data for the whole cell conductance of the different R-domain mutants are compared to the wild type values in Fig. 2C. The conductance for E822K and E826K was 3.55 ± 0.44 μS ($n = 6$) and 4.24 ± 0.37 μS ($n = 6$), as compared to 7.57 ± 0.65 μS ($n = 14$) in the wild type. The conductance activated by phos-cock in H620Q expressed cells was 27.84 ± 5.7 μS ($n = 6$).

3.3. Anion permeation through R-domain mutants

It has been reported that mutations in the TMD1 that involve changes in electrical charge of the affected amino acids modify the anion permeability sequence [8]. It was therefore worthwhile to find out whether similar mutations in the R-domain would affect the anion permeability sequence of the CFTR channel. The anion permeability sequence of the cAMP activated current in wild type CFTR injected oocytes was $Br^- > Cl^- > I^-$ (Fig. 3A). These data were consistent

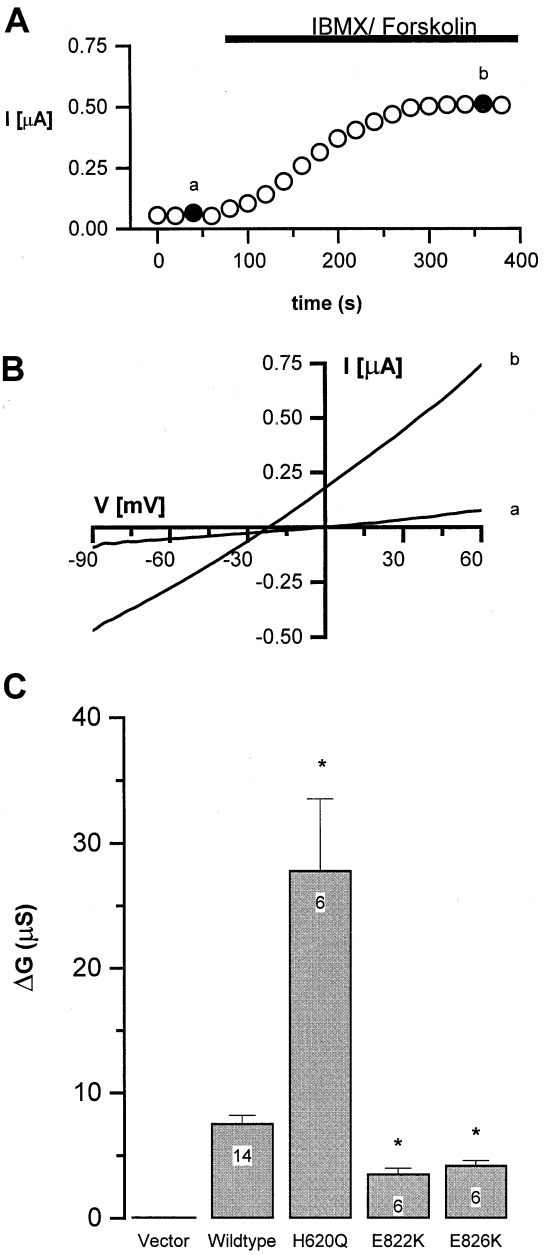

Fig. 2. Membrane current activated by phosphorylation cocktail in wild type CFTR injected *Xenopus* oocytes. A: Time course of membrane current in wild type CFTR injected *Xenopus* oocyte at +50 mV. B: Current-voltage relationships before and during the application of phos-cock at the time indicated in A with filled symbols. C: Summary of difference in conductance (ΔG) for stimulated wild type and mutant CFTR. ΔG was calculated from the difference of maximum conductance after phos-cock stimulation and conductance at control condition. The asterisk indicates a value different from wild type CFTR ($P < 0.05$, $n = 14$ for wild type, $n = 6$ for each mutant).

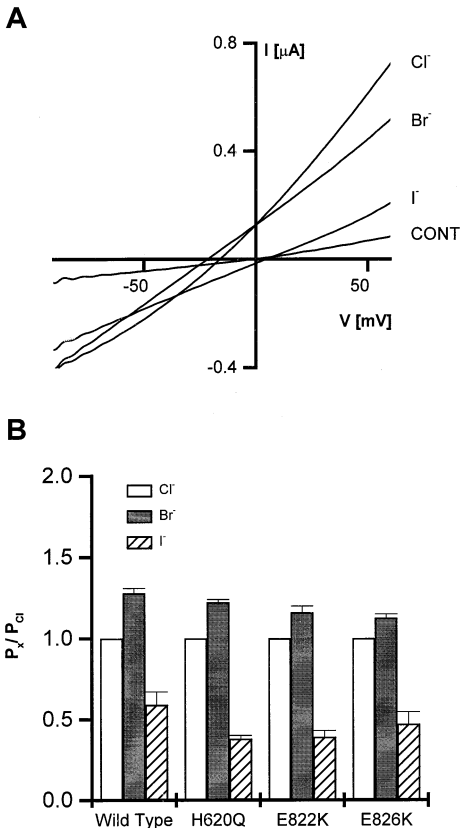

Fig. 3. Anion selectivity of cAMP activated CFTR Cl^- current in *Xenopus* oocytes. A: I-V relationships obtained from single wild type CFTR injected oocyte in ND-96 solution or anion substituted ND-96 solution after phos-cock stimulation. B: Comparison of the permeability ratios of wild type and mutant CFTR on anion substitution. Data are presented as mean ± S.E.M. ($n = 4$ for each group).

with results obtained for COS cells transiently transfected with wild type CFTR (data not shown) and are also in agreement with the permeability studies performed by other groups [8]. Fig. 3B summarizes the relative values of the permeability for Br^- and I^- for the wild type and the three mutants used in the present study. It is obvious that not only the permeability sequence is the same, but also the relative values are little affected by the mutations. These results imply that all these R-domain mutations do not affect the anion permeability sequence of CFTR.

3.4. Single channel properties of the R-domain mutants

A change in macroscopic conductance may reflect either a change in single channel conductance, or a change in channel open probability, or even a change in the number of functional channels. Whole cell current measurements therefore do not allow a discrimination between an effect of the mutations on open pore characteristics and gating properties of the CFTR channel. We have therefore performed single channel measurements of cAMP activated CFTR Cl^- channels in cell attached patches on transfected COS cells (Fig. 4). The rate of transient transfection of COS cells with CFTR was rather low (also on other cell lines we studied). We have therefore co-transfected them with GFP and CFTR which provides a reliable and easy method to identify CFTR transfected COS cells and increases the success rate of the single channel patch clamp experiments especially for the mutants with very low single channel open probability. Application of 0.1 μM forskolin and 1 μM IBMX to the bath solution can activate the CFTR Cl^- channels within 1 min in wild type CFTR and all three tested mutants. There were no significant differences between the time course of activation in wild type and mutant CFTR. Fig. 4A shows some representative traces and the corresponding amplitude histograms for the channel activity recorded in wild type and in the three mutants. Channel activation was completely reversible in all experiments and disappeared after wash-out of the phos-cock. Wild type CFTR channels expressed in COS cells were characterized by a small channel conductance of about 7 pS and a linear I-V relationship (Fig. 4B). This conductance was similar for all mutants as can be judged from the similar single channel current amplitudes recorded at −60 mV (Fig. 4C). This finding of an unchanged single channel conductance points to an effect on the gating properties of the mutant CFTR channels. We have also calculated the open probability p of the channel by $p = I/(n \cdot i)$ where I is the total averaged current over the sampling period, i the single channel current obtained from amplitude histograms and n the number of channels estimated from the maximal number of overlapping open events. We found that the open probability of the E822K and E826K mutants was significantly lower than that of wild type CFTR, whereas that of the H620Q mutant was strongly enhanced compared to wild type (not shown). We also noticed that these changes of open probability were also accompanied by changes in the number of activated channels. The average number of activated channels is 2.6 ± 0.306 ($n = 10$) for wild-type CFTR, 3.51 ± 0.428 ($n = 6$) for H620Q, 1.0 ± 0.000 ($n = 4$) for E822K and 1.66 ± 0.211 ($n = 6$) for E826K. The differences between wild type CFTR and E822K and E826K are significant ($P < 0.05$). These results suggest that these R-domain mutations affect CFTR channel activities at the single channel level.

4. Discussion

Our data show that some mutants of CFTR on single R-sites can affect the CFTR Cl^- channel activity. All the mutations studied here are located outside the phosphorylation sites, which may suggest that other regions in the R-domain, especially the highly conserved regions where E822K and E826K were l,ocated are important for the regulation of the CFTR Cl^- channel.

The R-domain of CFTR is a highly charged domain with nearly a third of the amino acids being charged residues. Many studies have shown that negative charges introduced by phosphorylation of the R-domain stimulate the CFTR Cl^- channel [4,6,19]. Substitution of multiple serines with negatively charged aspartates induced CFTR channel activity even without phosphorylation by PKA [6]. A comparison of the three R-domain mutants leads to a remarkable conclusion: both mutations, E822K and E826K, in which negatively charged glutamic acids were replaced by positively charged lysine had a significantly reduced CFTR channel activity, whereas the H620Q mutation, in which positively charged histidine was replaced by the more neutral amino acid glutamine, had a much higher channel activity. It therefore seems as if a decrease in the net negative charge in the R-domain reduces the CFTR activity and that the opposite change in-

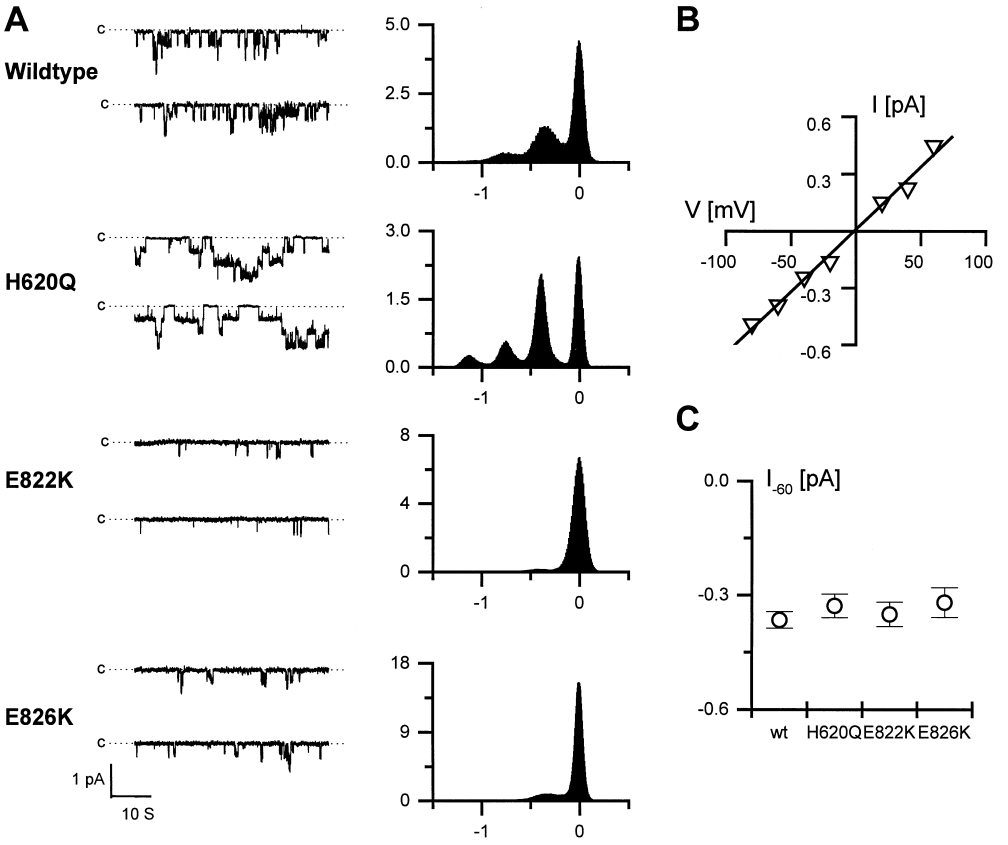

Fig. 4. Single channel current evoked by phos-cock in CFTR and GFP co-expressing COS cells. A: Single channel recordings of wild type and mutant CFTR with cell attached configuration at a holding potential of −60 mV. The dotted line indicates the closed level of single channel. Histograms alongside the original recording were obtained from the whole activation period. B: Single channel I-V relationship of wild type CFTR. C: Estimate of the single channel conductance by comparing of channel current amplitudes between wild type and mutant CFTR at −60 mV ($P > 0.05$, $n > 4$ for each group).

creases it. Since we only tested a few R-domain mutants obtained from patients, it is not clear whether this observation is coincidental or whether there is a correlation between channel activity and the presence of charged residues in these regions in the R-domain, but it might be worthwhile investigating this in more detail. It is also not clear how a change of net electric charge or charge distribution in the R-domain would affect channel activity. One possibility might be an effect either on phosphorylation or on binding of ATP to NBDs. However, it may be not enough for interpreting a mutant like E822K, in which only one positively charge mutation in the R-domain almost completely eliminates single channel activity. The effect on channel activity was more pronounced than that of the multiple mutations on serine residues [7], and seems to imply that the structure of this region (at the end of exon 13) has functional significance for channel regulation. It has recently been demonstrated [10] that covalent modification of the R-domain with a neutral, hydrophobic adduct at a site that is not phosphorylated can also stimulate CFTR, suggesting that an alteration in the conformation of the R-domain may be a key feature for channel regulation. Actually, negative charges introduced in the R-domain by phosphorylation are associated with structural changes [20,21]. From our experimental results, we cannot exclude that a mutation induced conformational change rather than changes in net electrical charges per se are responsible for the observed effects.

We also did not find any evidence for the role of the R-domain in the anion permeability sequence of CFTR at variance with other findings [8]. In this report, a marked change in channel selectivity by modifying charges on the side chains of two amino acids within TMD1 was demonstrated. It is therefore likely that changing electric charges in the R-domain will not affect open pore properties by electrostatic interactions within the protein. Similarly, these mutations also did not affect the single channel conductance. Taking together, these data indicate that the R-domain is not an essential part of the pore region, but merely acts as a CFTR Cl^- channel regulator.

In conclusion, our results suggest that the electrical charges in conserved R-domain regions other than the predicted phosphorylation sites might play an important role in the R-domain regulation of CFTR channel activity.

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