CFTR inhibition by glibenclamide requires a positive charge in cytoplasmic loop three

Patricia Melin [a,*], Eric Hosy [b], Michel Vivaudou [b], Frédéric Becq [a]

[a] *Institut de Physiologie et Biologie Cellulaires, Université de Poitiers, CNRS UMR 6187, 86022 Poitiers cedex, France*
[b] *CEA, DRDC, Biophysique Moléculaire et Cellulaire, UMR CNRS/UJF/CEA 5090, 17 Rue, des Martyrs, 38054, Grenoble, France*

Abstract

The sulfonylurea glibenclamide is widely used as an open-channel blocker of the CFTR chloride channel. Here, we used site-directed mutagenesis to identify glibenclamide site of interaction: a positively charged residue K978, located in the cytoplasmic loop 3. Charge-neutralizing mutations K978A, K978Q, K978S abolished the inhibition of forskolin-activated CFTR chloride current by glibenclamide but not by $CFTR_{inh}$-172. The charge-conservative mutation K978R did not alter glibenclamide sensitivity of CFTR current. Mutations of the neighbouring R975 (R975A, R975S, R975Q) did not affect electrophysiological and pharmacological properties of CFTR. No alteration of halide selectivity was observed with any of these CFTR mutant channels. This study identifies a novel potential inhibitor site within the CFTR molecule, and suggests a novel role of cytoplasmic loop three, within the second transmembrane domain of CFTR protein. This work is the first to report on the role of a residue in a cytoplasmic loop in the mechanism of action of the channel blocker glibenclamide.
© 2007 Elsevier B.V. All rights reserved.

1. Introduction

The ATP-binding cassette (ABC) genes represent a large family of transmembrane proteins widespread in archea, bacteria and eukaryotes. ABC transporters bind ATP and couple the energy released by ATP hydrolysis to transport of wide variety of substances into or out of cells or organelles [1–3]. The typical functional unit consists of a pair of ATP-binding domains (Nucleotide Binding Domains), providing necessary energy for solute transport, and two transmembrane domains (TMD) delimiting a transport pathway. Among the 49 human ABC transporters [4], only CFTR (ABCC7) is an ion channel: a chloride channel regulated by cyclic AMP and phosphorylation [5]. CFTR belongs to the ABCC subfamily,

the largest in the human ABC family, consisting in 12 full members and one unusual truncated ABCC13 [6]. Each TMD in ABC transporters is usually a bundle of six alpha-helices. The number of helices within TMD of ABC transporters is however variable. Six alpha-helices are generally found, but 10 helices are also frequently encountered. Moreover, in some transporters such as SUR1, additional transmembrane domains are found [1,2,4].

The sulfonylurea drug glibenclamide inhibits several ABC proteins: P-glycoprotein [7], CFTR [8], SUR1,2 [9,10], MRP1–5 [11–14]. Therefore, glibenclamide appears to be a general inhibitor of ABC transporters, suggesting an interaction with some conserved motif [7]. ABCC8 (SUR1) and ABCC9 (SUR2) are high-affinity receptors for sulfonylureas [9] that, upon binding to multiple sites, promote the inhibition of the associated K^+ channel, Kir6.1 or Kir6.2 [15,16]. Although structurally not yet well defined, the glibenclamide binding site of SUR1 appears to comprise cytoplasmic loop 3 (CL3), between TM5 and TM6, and CL8, between TM15 and TM16 [17–20].

Open channel blockers prevent chloride flow through the channel by occluding the CFTR pore [21]. The organic

* Corresponding author.
E-mail address: patricia.melin@univ-poitiers.fr (P. Melin).

anion glibenclamide inhibits CFTR with a low affinity by a complex mechanism involving multiple binding sites [22–24]. Glibenclamide and chloride ions are presumed to compete for common sites located within a large intracellular vestibule that is part of the CFTR pore [23]. The molecular determinants of the CFTR channel pore are not fully known but TM1 and TM6 are proposed to be part of the pore region [25,26]. The positively charged residues K95 and R303 attract large anionic molecules (glibenclamide, DIDS, NPPB) into the wide inner vestibule where they bind to block chloride permeation [27,28]. To our knowledge, residues involved in the mechanism of action of glibenclamide are all located into the pore of the CFTR molecule.

In the CFTR protein, the CL3, between TM8 and TM9, is speculated to be close to the intracellular mouth of the CFTR pore [29]. Within CL3, we have now identified an octapeptide sequence $I_{972}LNRFSKD_{979}$ relatively well conserved among ABCC subfamily proteins (Supplemental Fig. 1), in which 8 proteins are glibenclamide sensitive (Fig. 1B). Could the positively charged residues R975 and K978 be involved in the glibenclamide sensitivity of CFTR? The present data indicate that the charge of the side chain of K978, but not R975, is

required for CFTR inhibition by glibenclamide. The analogue mutation in SUR1 was also studied (SUR1–S1127A). Despite the sequence conservation, this mechanism could not be extended to SUR1 protein.

2. Experimental procedures

2.1. Plasmid constructs

Mutations were introduced by PCR using oligonucleotide-directed mutagenesis system (Quick Change[XL] Site-Directed Mutagenesis Kit, Stratagene) into (1) the pEGFP-CFTRwt (human cDNA) [30], (2) pcDNA3-SUR1 (hamster cDNA) [31]. The presence of mutations was confirmed by dideoxynucleotide sequencing on both strands. Other details appear elsewhere [32].

2.2. Functional analysis of ABCC proteins

For CFTR chloride channels, experiments were carried out on HEK-293 cells transiently transfected with wild-type or mutant EGFP-CFTR, at room temperature (20–25 °C). Experiments using broken-patch whole cell mode were performed as previously described [33]. The holding potential was −40 mV. Current/voltage (*I/V*) relationships were built by clamping the membrane potential to −40 mV and by pulses from −100 mV to +100 mV in 20 mV increments. The intrapipette solution contained (in mM): 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 MgCl$_2$, 1 EGTA, 3 MgATP *(ex-temporane)*,
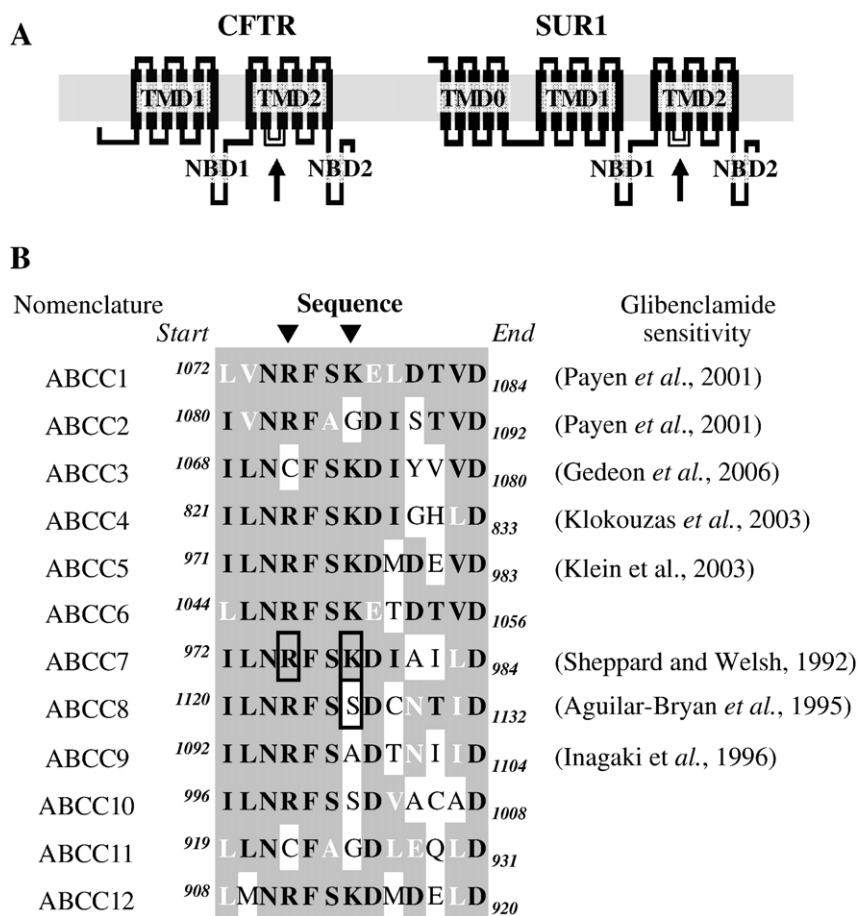

Fig. 1. Lysine 978 of CFTR is located in a conserved sequence among ABC proteins subfamily C. (A) Predicted topologies of CFTR and SUR1. Conserved sequence is positioned in the cytoplasmic loop (CL3 in CFTR) indicated by arrows. (B) Alignment of the cytoplasmic loop sequences of all functional members of the human ABCC proteins. Classification of proteins is given. Starting and ending residue numbers are specified. Key positions are indicated by arrows and mutated residues in the present work are boxed. Note that residue S1126 of human SUR1 corresponds to residue S1127 of hamster SUR1 experimentally tested.

10 TES, titrated with CsOH to pH 7.2, 285±5 mOsmol. The external bath solution contained (in mM): 145 NaCl, 4 CsCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 TES, titrated with NaOH to pH 7.4, 315±5 mOsmol. Following membrane rupture, CFTR chloride channels were activated by exposure extracellular side to Fsk 10 μM. After stable activation, CFTR inhibitors were added to the patch-clamp chamber from solutions made up fresh every 3 h in normal extracellular solution (pH checked). To prevent molecules photodegradation, experiments were performed in the dark. To quantify current inhibition, I/I_o ratios were calculated, I being the current density in the presence of blocker (glibenclamide or CFTR$_{inh}$-172) and I_o the unblocked current density (with Fsk). To compare the relative effect of glibenclamide on wild type or mutants CFTR channels, the Kd was determined according to the equation Kd=$[B]/((1/(I-I_0))-1)$, with $[B]$, concentration of blocker [27]. To measure the reversal potential of chloride currents, equivalent amount of NaCl was replaced by NaBr or NaI in the extracellular solution. Reversal potential was measured after stable activation of channels by Fsk 10 μM. The ABCC8 KATP channel activity was characterized by the patch-clamp technique in the excised inside-out configuration on mRNA-injected *Xenopus laevis* oocytes as previously described [34].

2.3. Statistics

Results are expressed as means±S.E.M. of n experiments. Sets of data were compared with a Student's *t* test. Differences were considered statistically significant when $P < 0.05$. ns: non significant difference, *$P < 0.05$, **$P < 0.01$,

***$P < 0.001$. All statistical tests were performed using GraphPad Prism 4.0 for Windows (Graphpad Software).

2.4. Chemicals

All chemicals were from Sigma Chemicals except forskolin (PKC Pharmaceuticals) and CFTR$_{inh}$-172 (Calbiochem). Stock solutions of glibenclamide and forskolin: 100 mM, CFTR$_{inh}$-172: 10 mM, were prepared in DMSO.

3. Results

3.1. Effects of mutagenesis of K978 on the glibenclamide inhibition of CFTR

Positive amino acids of the TM1 of CFTR play an important role in attracting both permeant and blocking anions into the CFTR channel pore [27]. We investigated the role of the charged residue K978 in the sequence ILNRFSKD of CL3 (Fig. 1B) described as a region physically close to the pore [29] and examined the interaction of glibenclamide with mutated EGFP-CFTR channels: K978A, K978Q, K978R,
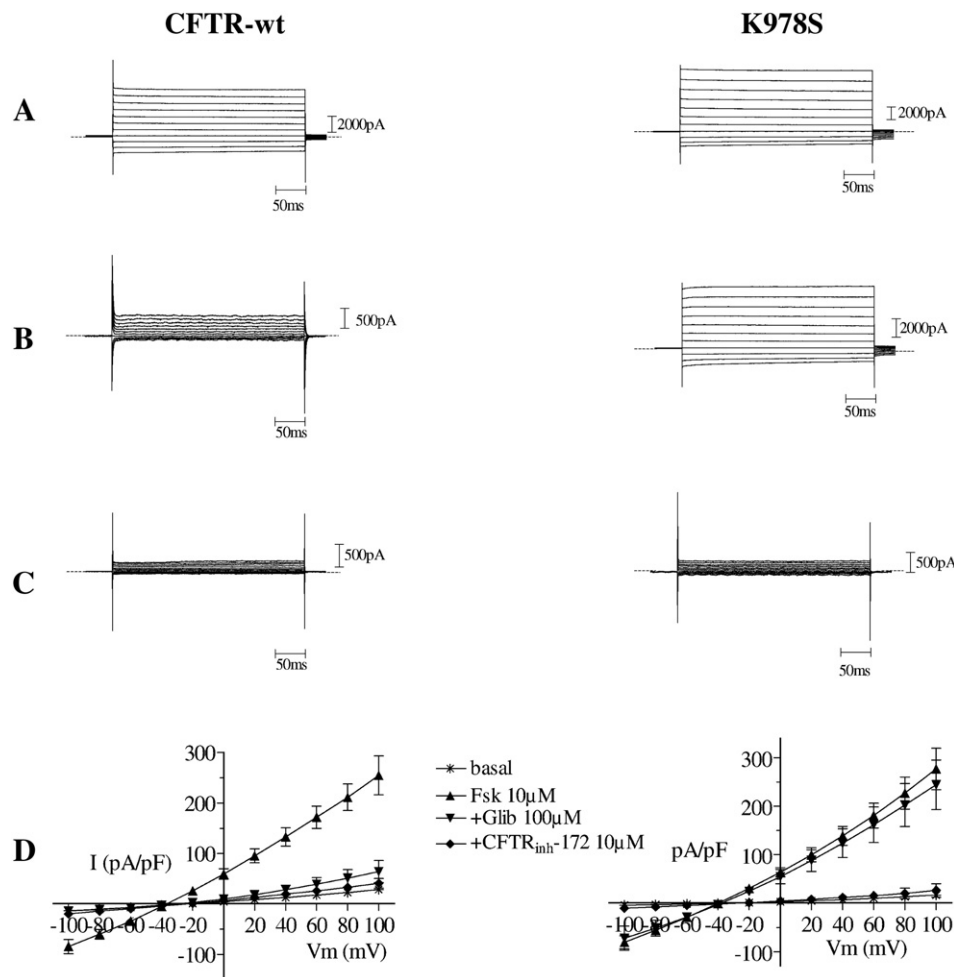

Fig. 2. Effect of K978S mutation on the whole cell EGFP–CFTR chloride currents. Representative traces of global currents recorded on HEK-293 transfected with EGFP–CFTR channels wild-type (left) and charge neutralizing mutant K978S (right) are shown in presence of Fsk 10 μM (A), after perfusion of glibenclamide 100 μM (B), and sub-sequential addition of CFTR$_{inh}$-172 10 μM (C). (D) Corresponding current–voltage relationships normalized by cell capacitance ($n=6$ for wt, $n=10$ for K978S).

K978S compared to CFTR-wt. EGF fusion to the N-terminus of CFTR does not alter CFTR chloride function or CFTR pharmacology [30]. With HEK-293 cells transiently expressing EGFP-CFTR-wt proteins, stimulated with Fsk 10 μM, the whole-cell chloride current increased linearly with voltage (Fig. 2A, D, left). Similar effects in the presence of Fsk were recorded with cells expressing K978A, K978Q, K978R and K978S mutants CFTR. Representative chloride currents recorded with K978S are shown in Fig. 2A (right). Consistent with previous works, extracellular perfusion with 100 μM glibenclamide produced a reduction in current with a slight outward rectification of the CFTR-wt I/V relationship (Fig. 2B left). Interestingly, perfusion of glibenclamide did not induce inhibition of forskolin-activated chloride currents in EGFP–CFTR–K978S expressing cells (Fig. 2B right). The difference between the two I/V curves in the presence of Fsk versus Fsk+Glib, at all voltages for K978S channels, was not statistically different ($P>0.05$) using analysis of variance followed by a Bonferroni post hoc test (Fig. 2D right). Because voltage might alter the gating of CFTR chloride channel [35] and CFTR glibenclamide inhibition is voltage dependent [8], inhibition was evaluated at hyperpolarization (−100 mV) and depolarization (+40 mV) of HEK transfected membrane cells. The current amplitude recorded at +40 mV revealed a >85% block in presence of Fsk+Glib for CFTR-wt, >98% block at −100 mV (Fig. 3A, left). Further addition of CFTR$_{inh}$-172 had no supplementary inhibitor action

($P>0.05$). In contrast, K978S activity, after glibenclamide application, was fully inhibited by CFTR$_{inh}$-172 (Fig. 3A, right). From continuous whole cell recordings with voltage steps between −40 mV and +40 mV, we estimated that full inhibition of CFTR-wt current by glibenclamide was achieved in ~250 s (Fig. 3B left) whereas K978S activity remained remarkably stable as long as the sulfonylurea was present (Fig. 3B right). The inhibition by CFTR$_{inh}$-172 of CFTR–K978S was, on the contrary, almost immediate and very rapid as illustrated at the end of the recording, Fig. 3B (right). Thus, the charge neutralizing mutant K978S is resistant to glibenclamide block, but remains sensitive to the blocker CFTR$_{inh}$-172. To investigate the role of the charge of the side chain of K978 in glibenclamide resistance, different amino acid substitutions were examined: K978A, K978Q, K978R. Because glibenclamide inhibits CFTR channels in a voltage-dependent manner [8], more pronounced inhibition occurs at negative voltages. Fig. 4A presents the ratio Iglib/Ifsk, recorded at −100 mV, with 100 μM glibenclamide, for K978A, K978Q, K978R, K978S channels compared to CFTR-wt. These results strongly suggest that the side chain charge at position 978 is important for glibenclamide inhibition of CFTR. Indeed, the ratio at −100 mV for the charge conservative mutant K978R (0.17 ± 0.04, $n=4$) was not significantly different from that of CFTR-wt. However, for K978S channels, the ratio at this voltage was significantly ($P<0.001$) increased to 0.81 ± 0.04 ($n=4$). Other charge
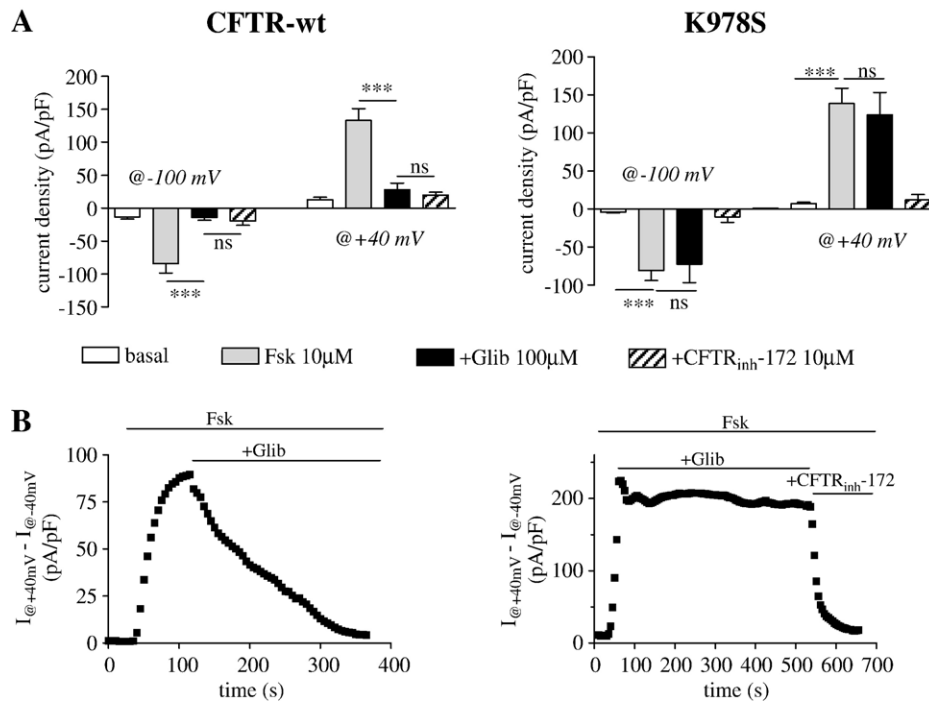

Fig. 3. Mutagenesis of positively charged K978 prevents blockage of CFTR channels by glibenclamide but not by CFTRinh-172. (A) Summary of mean current densities (pA/pF), recorded at −100 mV and +40 mV, in various conditions (indicated on graph) for HEK cells transfected with EGFP–CFTR-wt (left) or EGFP–CFTR–K978S (right). Data are mean±S.E.M. of n experiments ($n=7$ for wt, $n=10$ for K978S). (B) Representative currents amplitude time course recorded between +40 mV and −40 mV, normalized by cell capacitance, in presence of various agonists (Fsk 10 μM, Fsk+Glib 100 μM, Fsk+ CFTR$_{inh}$-172 10 μM). Note that EGFP–CFTR–K978S channels were inhibited by CFTR$_{inh}$-172 but not by glibenclamide.
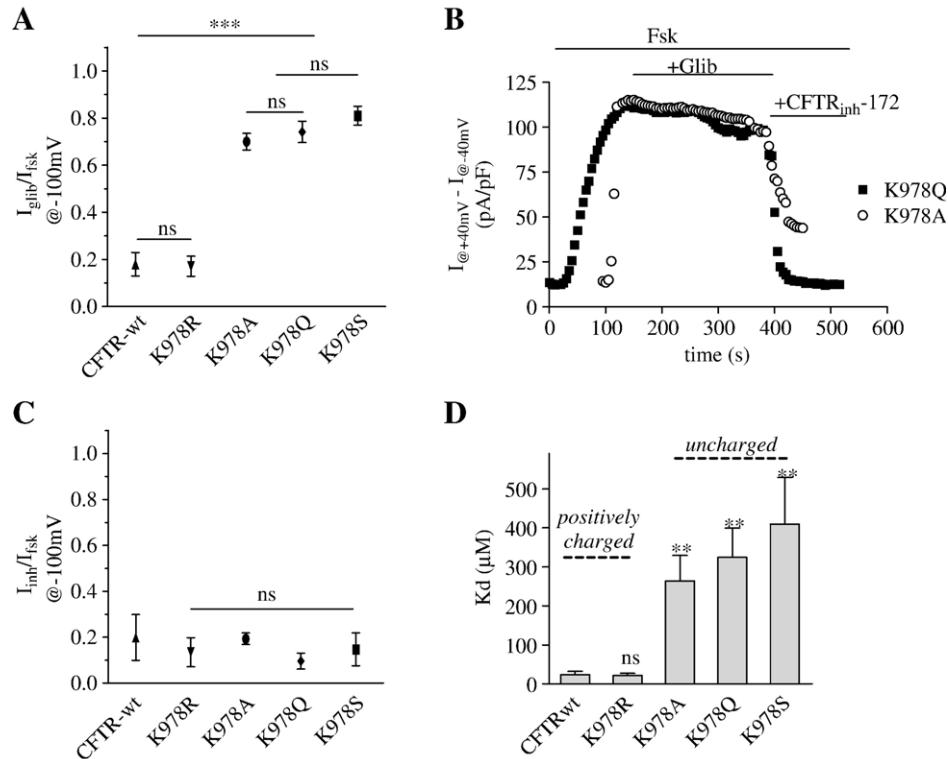
Fig. 4. The charge of the side chain of the amino acid 978 is involved in glibenclamide inhibition of CFTR channels. (A), Mean ratio (±S.E.M.) of currents recorded with glibenclamide (I, Fsk 10 µM + Glib 100 µM) on unblocked currents (I_o, Fsk), at a membrane potential of −100 mV, from different EGFP–CFTR mutants of K978. (B) Examples of time course of current densities (between +40 mV and −40 mV) from the charge neutralizing mutants CFTR–K978Q (■) and CFTR–K978A (○) in presence of Fsk 10 µM, and Fsk + Glib 100 µM, and Fsk + CFTR$_{inh}$-172 10 µM. (C), Mean ratio ± S.E.M. of currents recorded, at −100 mV, with blocker CFTR$_{inh}$-172 (Fsk 10 µM + CFTR$_{inh}$-172 10 µM) on unblocked currents (Fsk), at −100 mV, from different mutants of K978. (D) Mean Kd ± S.E.M. (in µM) of CFTR current block by glibenclamide with different mutants of K978 vs. wt-CFTR.

neutralizing mutations (K978A, K978Q) rendered channels insensitive to inhibition by glibenclamide as demonstrated by the time course of current recorded at +40 mV (Fig. 4B). By contrast, CFTR$_{inh}$-172 fully inhibited mutant channels with the same efficiency as CFTR-wt (Fig. 4C). Sensitivity to glibenclamide depends on charge of the side chain of the residue 978 (Fig. 4D). The apparent Kd at −100 mV was not affected by the charge conservative mutation (<25 µM), but was significantly increased in charge neutralizing mutation (>250 µM). Thus, the charge of the side chain of residue 978 is crucial for CFTR sensitivity to glibenclamide but has no incidence on the inhibition by CFTR$_{inh}$-172.

3.2. Effects of mutagenesis of arginine 975 on the glibenclamide inhibition of CFTR

We studied the effect of substitutions of R975, another positively charged residue located in the conserved sequence ILN**R**FSKD (Fig. 1B). After expression in HEK-293 cells, the whole-cell chloride currents recorded with charge neutralizing mutants CFTR–R975A, R975Q and R975S (Fig. 5) showed similar electrophysiological properties as CFTR-wt: linear activation with Fsk, inhibition by glibenclamide and by CFTR$_{inh}$-172. Thus, R975 of CL3 has no apparent role in pharmacological inhibition of CFTR.

3.3. Effects of mutagenesis of K978 and R975 on the halide selectivity of CFTR

Several sites of electrostatic interaction with chloride ions have been identified, among them positively charged lysine and arginine located in the TMs of CFTR [36]. However, CL3 is apparently not an integral part of the CFTR pore [29]. Nevertheless, the halide selectivity sequence was examined for the CFTR mutants and compared to CFTR-wt. For K978S channels, refractory to glibenclamide, the experimental reversal potentials were −43±1.5 mV (n=4) for bromide, −42.5± 1.5 mV (n=4) for chloride, and −29±5 mV (n=3) for iodide. The selectivity sequence of CFTR currents was unaffected by the replacement of the K978 or R975 (data not shown). So, the charge of K978 and R975 of CL3 has no influence on the anion permeation sequence.

3.4. Role of serine 1127 in the glibenclamide sensitivity of SUR1

SUR1 and SUR2 bind glibenclamide with widely different affinities, ∼0.6 nM and ∼300 nM, respectively [37]. In SUR1, the residue aligned to the critical K978 of CFTR is a serine (S1127) while it is an alanine in SUR2 (Fig. 1B). We reasoned that if this residue also controls
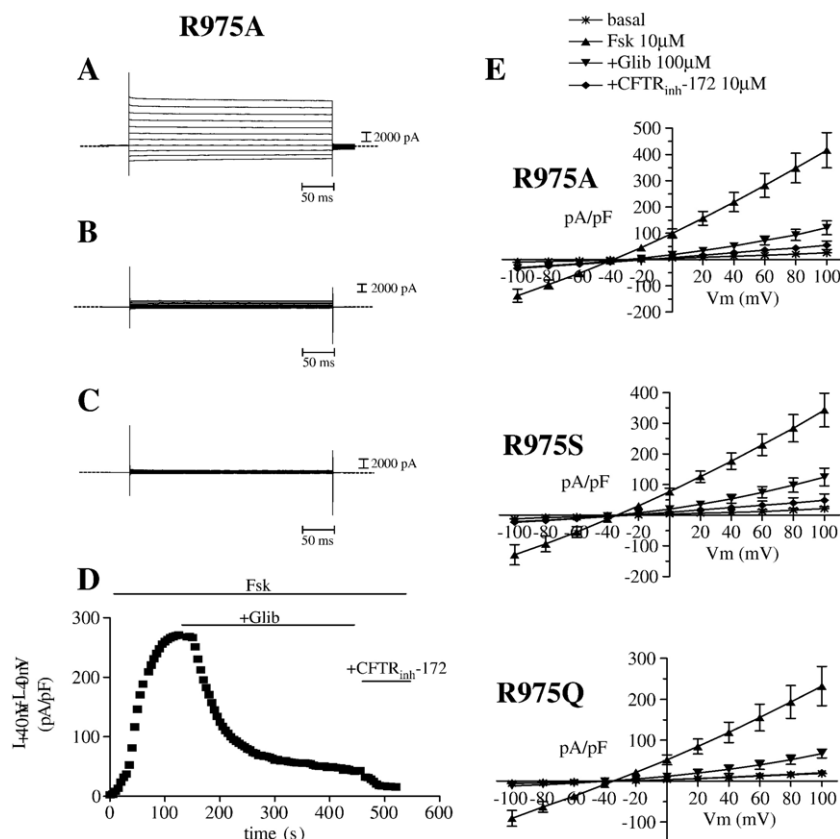
Fig. 5. Mutagenesis of R975 does not affect CFTR chloride channels properties. Original currents recorded on HEK-293 transfected with EGFP–CFTR–R975A with Fsk 10 μ M (A), Fsk+Glib 100 μM (B), and Fsk+CFTR$_{inh}$-172 10 μM (C). (D) Representative time course of current densities from the charge neutralizing mutant EGFP–CFTR–R975A in presence of Fsk 10 μM, Fsk+Glib 100 μM, Fsk+CFTR$_{inh}$-172 10 μM. (E) Current–voltage relationships normalized by cell capacitance recorded from R975A ($n=11$), R975S ($n=7$) and R975Q ($n=4$) EGFP–CFTR channels in presence of agonists. Data are mean±S.E.M. of n experiments.

glibenclamide sensitivity in SUR1, its mutation to the alanine found in SUR2 might reduce glibenclamide sensitivity. The effect the mutation SUR1–S1127A was evaluated by comparing glibenclamide block of wild-type and mutated KATP channels after expression in *Xenopus* oocytes. However, application of 100 nM glibenclamide blocked both the wild-type ($85\pm13\%$, $n=11$) and the mutated channel ($95\pm4\%$, $n=7$) (Fig. 6). The half inhibition times of wild-type and S1127A were not statistically different (4.7 ± 2.2 s, $n=7$ and 6.4 ± 4 s, $n=10$, respectively). Thus, mutation on the SUR1 subunit of the ILNRFSSD sequence into ILNRFSAD within the CL7, between TM13 and TM14, does not alter the sensitivity to glibenclamide of KATP channels.

4. Discussion

The present report showed that several substitutions of lysine 978 of CFTR alter the inhibitory response of CFTR channels to the anionic glibenclamide but not to the uncharged inhibitor CFTR$_{inh}$-172. This was the case for the mutations that neutralized the charge of the side chain of the residue 978 (K978A, K978Q, K978S). Charge neutralizing mutations decreased CFTR channel sensitivity to glibenclamide while they did not affect sensitivity to CFTR$_{inh}$-172. In

contrast, substitution of lysine 978 with another positively charged residue (K978R) had no incidence on glibenclamide sensitivity of CFTR channels. This charge conservative mutant shared the same properties of inhibition as CFTR-wt. We conclude that the nature of the residue 978 in CFTR is not crucial, but the positive charge of the side chain at this location is involved into glibenclamide inhibition. These results would be consistent with electrostatically attractive interaction between the positive charge of K978 and the negative charge of glibenclamide. Therefore our study suggests a novel role for the CL3 of the CFTR protein implicated as a potential pharmacological inhibitory site. As K978 is involved in the site of action of the open channel blocker glibenclamide, the CL3 is presumed to be close to the pore. This is in agreement with the recent elucidation of the crystal structure of a bacterial ABC transporter (Sav1866) from *Staphylococcus aureus* [38]. In this context, it is also important to note that the structure of the bacterial Sav1866 transporter is made of two transmembrane domains with six helices, like CFTR [38].

A current view of the CFTR pore (for review [36]) involves the first and the sixth transmembrane regions of the protein [26]. Some positively charged amino acids act by attracting chloride ions into the pore structure. For example, K95 in TM1 [27] and R334 in TM6 [39,40] create
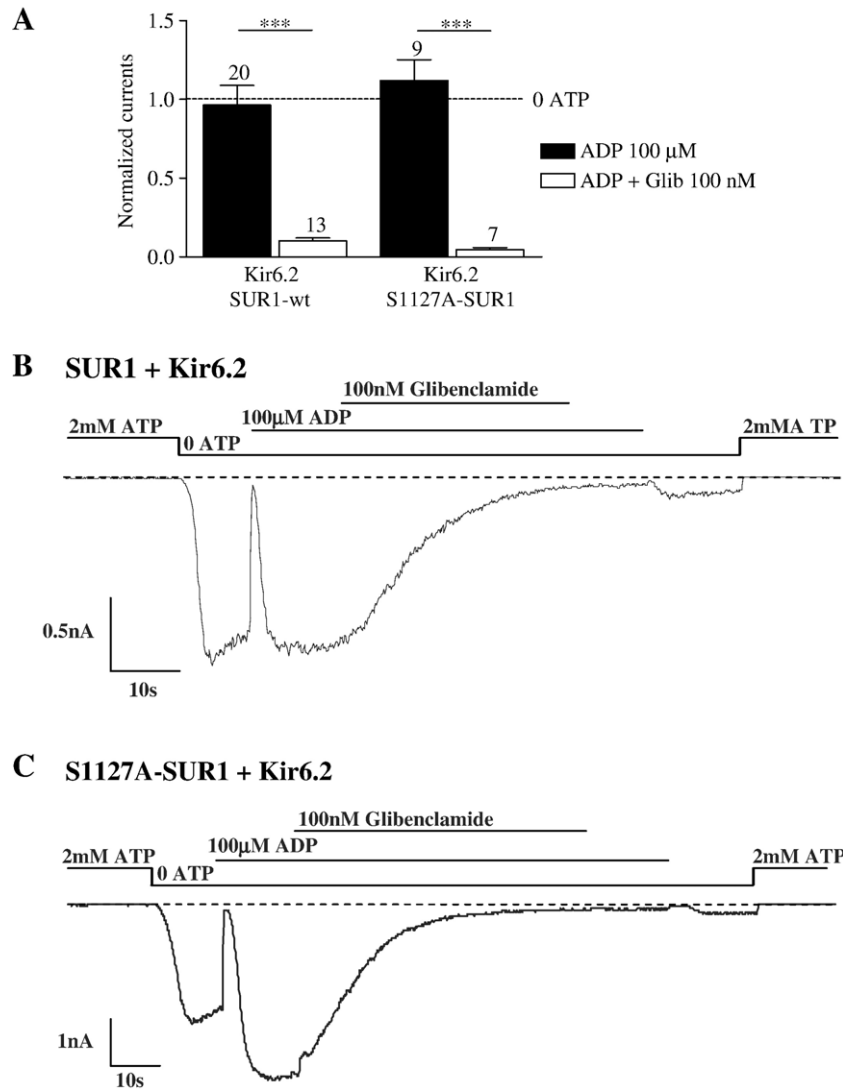
Fig. 6. Mutation S1127A on SUR1 subunit does not alter the glibenclamide sensitivity of KATP channels. (A) Average currents for SUR1+Kir6.2 and SUR1 S1127A+Kir6.2 measured in 100 μM ADP before (dark bars) or after (white bars) application of Glibenclamide (100 nM). Currents were normalized to the current measured in 0 ATP before application of nucleotides. Error bars represent S.E.M. and numbers of repetition are noted above the bars. (B) Representative currents recorded in inside-out patches from *Xenopus* oocytes expressing SUR1+Kir6.2 and S1127A–SUR1+Kir6.2 (C). Glibenclamide (100 nM) was applied in the presence of 100 μM ADP.

electrostatic potential influencing anion conduction. A recent study shows that the positively charged R303 and R352, located near the intracellular ends of the TM5 and TM6 regions, regulate anion conduction through the CFTR pore [25]. K95 and R303 also attract large blocker molecules, such as glibenclamide, into the CFTR pore [27,28]. In the CL3 region, we showed that R975 and K978, on the contrary, do not influence the chloride permeation. Thus, our results highlight the role of lysine 978 only in glibenclamide interaction with CFTR protein.

SUR1, another ABCC protein, has considerable sequence similarity with CFTR and it binds glibenclamide, though with a much greater affinity. It is interesting to note that the binding affinity for SUR of glibenclamide is favoured by the anionic group of hypoglycaemic sulfonylureas and acidic analogues [41]. S1127 of SUR1 matches K978 of CFTR and

a single serine (S1237) was identified as molecular site involved in the binding of tolbutamide and glibenclamide [17]. We tested the involvement of this residue in glibenclamide efficiency by mutating it to the aligned residue of SUR2, a SUR isoform with a lower affinity for glibenclamide than SUR1. In absence of ADP, blocking affinity in inside-out patches $EC_{50}=4.2$ nM for SUR1 and $EC_{50}=27$ nM for SUR2A [42]. However, they show that with 100 μM ADP, 100 nM of glibenclamide blocks ∼90% SUR1/Kir6.2 and >∼10% SUR2A/Kir6.2. We have also observed similar differences in our model. However, the mutation S1127A did not reduce glibenclamide block of KATP channels, implying a lesser role of this residue than in CFTR. The glibenclamide binding site of SUR1 is not yet well defined. It appears to be made up of multiple regions including the TM5–TM6 and TM15–TM16 cytoplasmic

loops [19,20,43], equivalent to the N-terminal loop and CL4 of CFTR.

In conclusion, we propose a model in which the positive charge of lysine 978 within the cytoplasmic loop 3 of CFTR could act as a first bait to drive glibenclamide (possibly through electrostatic interactions) into the pore. Glibenclamide could then be stabilized by additional positively charged amino-acids (like K95 and R303) located in the depth of the channel pore [27,28]. Our study also suggests a position close to the CFTR pore for cytoplasmic loop 3 and especially for the side chain of residue 978. Finally, whether the inhibition by glibenclamide of other ABCC members, such as MRP1–5 (Fig. 1), is also dependent on the corresponding sequence ILNRFSKD is not known but will be the subject of future studies.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.05.013.

References

[1] M. Dean, R. Allikmets, Evolution of ATP-binding cassette transporter genes, Curr. Opin. Genet. Dev. 5 (1995) 779–785.

[2] C.F. Higgins, ABC transporters: from microorganisms to man, Annu. Rev. Cell Biol. 8 (1992) 67–113.

[3] S. Childs, V. Ling, The MDR superfamily of genes and its biological implications, Important Adv. Oncol. (1994) 21–36.

[4] M. Dean, T. Annilo, Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates, Annu. Rev. Genomics. Hum. Genet. 6 (2005) 123–142.

[5] D.C. Gadsby, A.C. Nairn, Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis, Physiol. Rev. 79 (1999) S77–S107.

[6] H. Yabuuchi, S. Takayanagi, K. Yoshinaga, N. Taniguchi, H. Aburatani, T. Ishikawa, ABCC13, an unusual truncated ABC transporter, is highly expressed in fetal human liver, Biochem. Biophys. Res. Commun. 299 (2002) 410–417.

[7] P.E. Golstein, A. Boom, J. van Geffel, P. Jacobs, B. Masereel, R. Beauwens, P-glycoprotein inhibition by glibenclamide and related compounds, Pflugers Arch. 437 (1999) 652–660.

[8] D.N. Sheppard, M.J. Welsh, Effect of ATP-sensitive K+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents, J. Gen. Physiol. 100 (1992) 573–591.

[9] L. Aguilar-Bryan, C.G. Nichols, S.W. Wechsler, J.P. Clement, A.E. Boyd III, G. Gonzalez, H. Herrera-Sosa, K. Nguy, J. Bryan, D.A. Nelson, Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion, Science 268 (1995) 423–426.

[10] N. Inagaki, T. Gonoi, J.P. Clement, C.Z. Wang, L. Aguilar-Bryan, J. Bryan, S. Seino, A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K+ channels, Neuron 16 (1996) 1011–1017.

[11] M. Klein, L. Perfus-Barbeoch, A. Frelet, N. Gaedeke, D. Reinhardt, B. Mueller-Roeber, E. Martinoia, C. Forestier, The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use, Plant J. 33 (2003) 119–129.

[12] A. Klokouzas, C.P. Wu, H.W. van Veen, M.A. Barrand, S.B. Hladky, cGMP and glutathione-conjugate transport in human erythrocytes, Eur. J. Biochem. 270 (2003) 3696–3708.

[13] C. Gedeon, J. Behravan, G. Koren, M. Piquette-Miller, Transport of glyburide by placental ABC transporters: implications in fetal drug exposure, Placenta 27 (2006) 1096–1102.

[14] L. Payen, L. Delugin, A. Courtois, Y. Trinquart, A. Guillouzo, O. Fardel, The sulphonylurea glibenclamide inhibits multidrug resistance protein (MRP1) activity in human lung cancer cells, Br. J. Pharmacol. 132 (2001) 778–784.

[15] F.M. Gribble, R. Ashfield, C. Ammala, F.M. Ashcroft, Properties of cloned ATP-sensitive K+ currents expressed in *Xenopus* oocytes, J. Physiol. 498 (Pt 1) (1997) 87–98.

[16] L. Gros, A. Virsolvy, G. Salazar, D. Bataille, P. Blache, Characterization of low-affinity binding sites for glibenclamide on the Kir6.2 subunit of the beta-cell KATP channel, Biochem. Biophys. Res. Commun. 257 (1999) 766–770.

[17] R. Ashfield, F.M. Gribble, S.J. Ashcroft, F.M. Ashcroft, Identification of the high-affinity tolbutamide site on the SUR1 subunit of the K(ATP) channel, Diabetes 48 (1999) 1341–1347.

[18] M.V. Mikhailov, S.J. Ashcroft, Interactions of the sulfonylurea receptor 1 subunit in the molecular assembly of beta-cell K(ATP) channels, J. Biol. Chem. 275 (2000) 3360–3364.

[19] M.V. Mikhailov, E.A. Mikhailova, S.J. Ashcroft, Molecular structure of the glibenclamide binding site of the beta-cell K(ATP) channel, FEBS Lett. 499 (2001) 154–160.

[20] M.V. Mikhailov, E.A. Mikhailova, S.J. Ashcroft, Structure–function relationships in the beta-cell K(ATP) channel, Biochem. Soc. Trans. 30 (2002) 323–327.

[21] T.C. Hwang, D.N. Sheppard, Molecular pharmacology of the CFTR Cl-channel, Trends Pharmacol. Sci. 20 (1999) 448–453.

[22] Z.R. Zhang, S. Zeltwanger, N.A. McCarty, Steady-state interactions of glibenclamide with CFTR: evidence for multiple sites in the pore, J. Membr. Biol. 199 (2004) 15–28.

[23] D.N. Sheppard, K.A. Robinson, Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl-channels expressed in a murine cell line, J. Physiol. 503 (Pt 2) (1997) 333–346.

[24] B.D. Schultz, A.D. DeRoos, C.J. Venglarik, A.K. Singh, R.A. Frizzell, R.J. Bridges, Glibenclamide blockade of CFTR chloride channels, Am. J. Physiol. 271 (1996) L192–L200.

[25] C.N. Aubin, P. Linsdell, Positive charges at the intracellular mouth of the pore regulate anion conduction in the CFTR chloride channel, J. Gen. Physiol. 128 (2006) 535–545.

[26] N. Ge, C.N. Muise, X. Gong, P. Linsdell, Direct comparison of the functional roles played by different transmembrane regions in the cystic fibrosis transmembrane conductance regulator chloride channel pore, J. Biol. Chem. 279 (2004) 55283–55289.

[27] P. Linsdell, Location of a common inhibitor binding site in the cytoplasmic vestibule of the cystic fibrosis transmembrane conductance regulator chloride channel pore, J. Biol. Chem. 280 (2005) 8945–8950.

[28] C.N. St. Aubin, J.J. Zhou, P. Linsdell, Identification of a second blocker binding site at the cytoplasmic mouth of the CFTR chloride channel pore, Mol. Pharmacol. 71 (2007) 1360–1368.

[29] F.S. Seibert, P. Linsdell, T.W. Loo, J.W. Hanrahan, J.R. Riordan, D.M. Clarke, Cytoplasmic loop three of cystic fibrosis transmembrane conductance regulator contributes to regulation of chloride channel activity, J. Biol. Chem. 271 (1996) 27493–27499.

[30] B.D. Moyer, J. Loffing, E.M. Schwiebert, D. Loffing-Cueni, P.A. Halpin, K.H. Karlson, Ismailov II, W.B. Guggino, G.M. Langford, B.A. Stanton, Membrane trafficking of the cystic fibrosis gene product, cystic fibrosis transmembrane conductance regulator, tagged with green fluorescent protein in Madin–Darby canine kidney cells, J. Biol. Chem. 273 (1998) 21759–21768.

[31] E.R. Liman, J. Tytgat, P. Hess, Subunit stoichiometry of a mammalian K+ channel determined by construction of multimeric cDNAs, Neuron 9 (1992) 861–871.

[32] P. Melin, V. Thoreau, C. Norez, F. Bilan, A. Kitzis, F. Becq, The cystic fibrosis mutation G1349D within the signature motif LSHGH of NBD2 abolishes the activation of CFTR chloride channels by genistein, Biochem. Pharmacol. 67 (2004) 2187–2196.

[33] P. Melin, C. Norez, I. Callebaut, F. Becq, The glycine residues G551 and G1349 within the ATP-binding cassette signature motifs play critical roles in the activation and inhibition of cystic fibrosis transmembrane conductance regulator channels by phloxine B, J. Membr. Biol. 208 (2005) 203–212.

[34] A.L. Prost, A. Bloc, N. Hussy, R. Derand, M. Vivaudou, Zinc is both an intracellular and extracellular regulator of KATP channel function, J. Physiol. 559 (2004) 157–167.

[35] Z. Cai, T.S. Scott-Ward, D.N. Sheppard, Voltage-dependent gating of the cystic fibrosis transmembrane conductance regulator Cl-channel, J. Gen. Physiol. 122 (2003) 605–620.

[36] P. Linsdell, Mechanism of chloride permeation in the cystic fibrosis transmembrane conductance regulator chloride channel, Exp. Physiol. 91 (2006) 123–129.

[37] H. Dorschner, E. Brekardin, I. Uhde, C. Schwanstecher, M. Schwan-

[38] R.J. Dawson, K.P. Locher, Structure of a bacterial multidrug ABC transporter, Nature 443 (2006) 180–185.

[39] X. Gong, P. Linsdell, Molecular determinants and role of an anion binding site in the external mouth of the CFTR chloride channel pore, J. Physiol. 549 (2003) 387–397.

[40] X. Liu, S.S. Smith, D.C. Dawson, CFTR: what's it like inside the pore? J. Exp. Zool., A Comp. Exp. Biol. 300 (2003) 69–75.

[41] M. Meyer, F. Chudziak, C. Schwanstecher, M. Schwanstecher, U. Panten, Structural requirements of sulphonylureas and analogues for interaction with sulphonylurea receptor subtypes, Br. J. Pharmacol. 128 (1999) 27–34.

[42] F.M. Gribble, S.J. Tucker, S. Seino, F.M. Ashcroft, Tissue specificity of sulfonylureas: studies on cloned cardiac and beta-cell K(ATP) channels, Diabetes 47 (1998) 1412–1418.

[43] J. Bryan, A. Crane, W.H. Vila-Carriles, A.P. Babenko, L. Aguilar-Bryan, Insulin secretagogues, sulfonylurea receptors and K(ATP) channels, Curr. Pharm. Des. 11 (2005) 2699–2716.

stecher, Stoichiometry of sulfonylurea-induced ATP-sensitive potassium channel closure, Mol. Pharmacol. 55 (1999) 1060–1066.