J Membrane Biol (2009) 230:133–141
DOI 10.1007/s00232-009-9193-1

Role of the C-Terminal Part of the Extracellular Domain of the α-ENaC in Activation by Sulfonylurea Glibenclamide

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Received: 22 April 2009 / Accepted: 28 July 2009 / Published online: 21 August 2009

Abstract The epithelial sodium channel (ENaC) is regulated by hormones and by other intracellular or extracellular factors. It is activated by the sulfonylurea drug glibenclamide. The activator effect of glibenclamide is fast and reversible and was observed in *Xenopus* oocytes coexpressing the α subunit from human, *Xenopus*, or guinea pig (but not rat) with $\beta\gamma$-rat ENaC subunits. The mechanism of this effect is not yet well understood. We hypothesize that the extracellular loop of ENaC plays a major role in this activation. Mutants and chimeras of α subunits harboring different parts of the rat and guinea pig α-subunit extracellular loops were generated and coexpressed with $\beta\gamma$-rat subunits in *Xenopus* oocytes. The effect of glibenclamide on ENaC activity was measured using two-electrode voltage-clamp technique. The α-rat ENaC chimera containing the C-terminal part of the extracellular loop of the α-guinea pig ENaC was significantly stimulated by glibenclamide (1.26-fold), whereas the rat-α combination was not activated by this sulfonylurea. Mutagenesis of specific residues on the rat α subunit did not generate channels sensitive to glibenclamide, suggesting that the overall structure of the extracellular loop is required for activation of the channel by this drug. These results support the hypothesis of the existence of a role played by the last 100 amino acids of the extracellular loop and confirm that the ENaC behaves as a ligand-gated channel similar to several other members of the ENaC/degenerin family.

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Keywords Epithelial sodium channel · Amiloride · Glibenclamide · Extracellular activation · *Xenopus* oocyte

Abbreviations

ENaC	Epithelial sodium channel
ASIC	Acid-sensing ion channel
SUR	Sulfonylurea receptor
ABC	ATP-binding cassette family protein member
CFTR	Cystic fibrosis transmembrane regulator
TEVC	Two-electrode voltage clamp technique

The epithelial sodium channel (ENaC) is the limiting step for sodium reabsorption across epithelia such as the distal nephron (Rossier et al. 1994), airways (Hummler et al. 1996), and distal colon (Epple et al. 2000). ENaC comprises three homologous subunits (Canessa et al. 1994) and belongs to the degenerin (DEG)/ENaC. Each subunit is characterized by two transmembrane spanning domains, cytoplasmic N- and C-termini, and a large (70% of the size) extracellular loop. The stoichiometry is still under debate but several studies suggest a tetrameric (2α, 1β, 1γ) (Anantharam A 2007; Firsov et al. 1998) or nonameric (3α, 3β, 3γ) (Snyder et al. 1998) structure of the channel. Recently, a trimeric stoichiometry (1α, 1β, 1γ) (Stockand et al. 2008) has been proposed based on the crystal structure of the acid-sensing ion channel (ASIC) (Jasti et al. 2007), another member of the DEG/ENaC family (Garty and Palmer 1997).

ATP-binding cassette (ABC) family protein members including the cystic fibrosis transmembrane regulator (CFTR), and K_{ATP} channels have been suggested to modulate the ENaC by down-regulating apical sodium

reabsorption. Konstas and collaborators (2001) have shown that sulfonylurea receptors (SURs) inhibit the ENaC by reducing cell surface expression in *Xenopus* oocytes. In airway epithelia, inhibition of K_{ATP} channels decreased sodium and water reabsorption to a similar level as seen with amiloride, an ENaC-specific pore blocker (Leroy et al. 2006). However, it has been reported that glibenclamide, a high-affinity blocker of SURs of the K_{ATP} channel, directly stimulates ENaC activity in *Xenopus* oocytes (Chraibi and Horisberger 1999). This observation is supported by patch-clamp experiments in an outside-out configuration where the N.Po (the product of N channels and open probability P_o) of the channel was doubled after glibenclamide perfusion (Chraibi and Horisberger 1999). Interestingly, ENaC channels composed of the α subunit of guinea pig (αgp) combined with the β and γ subunits of rat (βr and γr) coexpressed in *Xenopus* oocytes are sensitive to glibenclamide, while αr and $\beta r\gamma r$ ENaC are resistant (Schnizler et al. 2003), indicating that the α subunit carries a major determinant of the sensitivity to glibenclamide. Many extracellular factors, such as cpt-cAMP (Bhalla et al. 2006; Chraibi et al. 2001; Schnizler et al. 2000), serine proteases (Chraibi et al. 1998; Hughey et al. 2003, 2007; Rossier 2004; Vallet et al. 1997, 2002), capsazepine, a competitive antagonist for transient receptor potential vanilloid subfamily 1 (Yamamura et al. 2004), and S3969, a small organic molecule (Lu et al. 2008), are known to stimulate the ENaC through direct interaction with the extracellular loop of the channel. These extracellular factors are known to activate the ENaC by increasing the N.Po of the channel, and for several of them it is probably the open probability that is modified (Horisberger and Chraibi 2004). Serine proteases like trypsin and endogenous channel-activating proteases 1 (CAP1) increased ENaC activity by proteolysis of the channel subunits. Recently, we demonstrated that Ile-481 of the αgp extracellular domain plays a crucial role in the sensitivity of ENaC to cpt-cAMP (Renauld et al. 2008). Capsazepine activates human $\delta\beta\gamma$-ENaC in a concentration-dependent manner, with an EC_{50} value of 8 μM. S3969 enhances hENaC activity through interactions requiring the extracellular part of the β subunit. This stimulation did not necessitate cleavage by the furin protease, indicating that nonproteolyzed channels can be opened.

The molecular basis of the activation of the channel by these different factors is unknown and their interaction site remains to be discovered. Identification of specific sites involved in ligand-induced ENaC regulation is crucial to understand the structure-function relationship of the extracellular moiety of the channel. Even if glibenclamide is not a physiological regulator of ENaC activity, understanding the molecular mechanism of its effect will help to clarify the mechanism of action of other factors such as S3969 and cpt-cAMP, which also stimulate ENaC activity through interaction with its extracellular loop. Knowledge in this research area would be helpful in pathology like pseudohypoaldosteronism type 1 (PHA-1), characterized by a loss of ENaC function.

The specific purpose of the present study was to determine the region of αgp involved in activation of the sodium channel by glibenclamide. To do so, first, we made four chimeras of the α- subunit harboring different parts of the αr and αgp extracellular loop sequences. Second, we performed amino acid substitutions on αr with the corresponding residues from the αgp sequence. These chimeras and mutants were coexpressed in *Xenopus* laevis oocytes, and amiloride-sensitive currents were measured using two-electrode voltage-clamp technique (TEVC). Our results indicate that the C-terminal region of the extracellular loop plays a major role in the activation of ENaC by glibenclamide. We also performed amiloride concentration-response experiments to address the possibility of a shift of the inhibition constant (K_i) for amiloride induced by glibenclamide. In our experiments, glibenclamide did not alter K_i for amiloride in oocytes expressing chimeras or wild-type α subunits together with $\beta r\gamma r$, suggesting that the sulfonylurea drug did not induce a conformational change of the amiloride binding site.

Materials and Methods

Construction of Chimeric α Subunits and Site-Directed Mutagenesis

We constructed a set of chimeric channels with parts of αgpENaC exchanged with the corresponding parts of αrENaC. For each chimeric construct, two restriction sites were introduced into αgp cDNA at homologous positions as into αr cDNA by PCR. Each cDNA chimeric construct was inserted into the pSD5 vector (Invitrogen, Burlington, ON, Canada) as previously described (Renauld et al. 2008). As shown in Fig. 2a, chimera I consists of a replacement of the αr12-268 sequence (which contained the first transmembrane domain together with the N-terminal part of extracellular loop) by its homologue in αgp. In chimera II, the part (268–456) of the extracellular loop of αr was exchanged for its homologue in αgp. Chimera III consists of a replacement of αr12-456 by its homologue in αgp, whereas chimera IV contains the C-terminal part of the extracellular loop and the second transmembrane domain jointly with the intracellular C-terminus of αgp. Site-directed mutagenesis was performed using the pfu turbo DNA polymerase kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Thirty nucleotide sense and antisense primers contained mutations

at the center of the sequence. After a 30-cycle PCR reaction, parental cDNA was degraded using *Dpn*I methylated specific restriction enzyme (Promega, San Luis Obispo, CA, USA). All mutations and chimeric constructions were confirmed by sequencing (University Core DNA Services, Calgary, AB, Canada).

Expression of ENaC in *Xenopus laevis* Oocytes

In vitro transcribed cRNA of the wild type or mutant αr subunit and chimeric constructs were coinjected with cRNA of βrγr ENaC into stage V/VI *Xenopus* oocytes (2 ng of cRNA of each subunit in 50 nl total) as described earlier (Chraibi et al. 1998). Injected oocytes were incubated at 18.5°C in a low-temperature incubator (Fisher Scientific, Ottawa, ON, Canada) in a low-Na^+ modified Barth's solution that contained (mM) 10 NaCl, 2 KCl, 0.7 $CaCl_2$, 0.8 $MgCl_2$, 0.33 $Ca(NO_3)_2$, 80 *N*-methyl-D-glucamine (NMDG)-Cl, and 10.0 NMDG-HEPES, pH 7.4. The low-Na^+ modified Barth's was chosen to prevent excessive Na^+ loading during the time needed for ENaC expression. Electrophysiological experiments were performed 1 or 2 days after cRNA injection.

Electrophysiological Measurements in Whole Oocytes

Amiloride-sensitive currents were measured using TEVC with a Dagan TEV voltage-clamp apparatus (Dagan, Minneapolis, MN, USA), at room temperature and a holding potential of −60 mV in a solution containing (mM) 100 Na-gluconate, 0.4 $CaCl_2$, 0.8 $MgCl_2$, 5 $BaCl_2$, 10 tetraethyl-ammonium chloride (TEA-Cl), and 10 NMDG-HEPES (pH 7.4). A low Cl^-concentration and K^+ channel blockers (Barium and TEA; Sigma-Aldrich Oakville, ON, Canada) were used to reduce the background membrane conductance. Extracellular solutions flowed under gravity at a flow rate of 6–8 ml/min. Current signals were filtered at 20 Hz using the internal filter of the Dagan apparatus and continuously recorded and sampled at 1,000 Hz with pCLAMP 10 Software (Axon Instruments, Sunnyvale, CA, USA).

The relationship between amiloride concentrations and current responses were fitted using the following equation after normalization: $Y = \min + (\max–\min)/(1 + ([A]/K_i)^n)$, where min is the component resistant to amiloride, K_i is the apparent inhibition constant of amiloride, [A] is the amiloride concentration, and n is the Hill coefficient.

Statistics

Statistical analysis was performed with Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). All results are expressed as mean ± SE, and statistical significance was evaluated by paired Student *t*-test as indicated in the text.

Chemicals and Drugs

Amiloride and glibenclamide were obtained from Sigma-Aldrich, Oakville, ON, Canada. A stock solution of 0.2 M glibenclamide in dimethyl sulfoxide (DMSO) was used. In all experiments, appropriate amounts of DMSO were added to the solutions to obtain the same DMSO concentration (0.1%) in control (i.e., without glibenclamide) and glibenclamide-containing solutions. Under these experimental conditions, no effect of DMSO alone could be detected in oocytes expressing rat, guinea pig, or chimera channels [$(Iam_{\text{after perfusion of DMSO}}/Iam_{\text{before perfusion DMSO}}) = 0.99 \pm 0.02$ (n = 11), 1.01 ± 0.02 (n = 8), or 1.01 ± 0.01 (n = 9), respectively].

Results

Effect of Glibenclamide on the Sodium Amiloride-Sensitive Current

Original current recordings using TEVC on oocytes expressing αgp or αr together with βrγr are shown in Fig. 1. Sodium amiloride-sensitive currents (I_{am}) were measured before and after 30 s to 1 min of exposure to 100 μM glibenclamide at a holding potential of −60 mV. As observed earlier with oocytes expressing the *Xenopus* ENaC (Chraibi and Horisberger 1999), a rapid increase of inward sodium current was observed in oocytes expressing αgpβrγr following glibenclamide perfusion (Fig. 1a). However, glibenclamide did not affect the sodium current when αr was expressed (Fig. 1b). The mean changes in sodium amiloride-sensitive current induced by glibenclamide for αgpβrγr and αrβrγr channels expressed in *Xenopus* oocytes are shown in Fig. 1c. This effect is also consistent with a previous report in which oocytes expressing αgpENaC showed sensitivity to glibenclamide (Schnizler et al. 2003) and support the hypothesis of the role of the extracellular moiety in ENaC activation in our model.

Determination of the Region of the Extracellular Domain of αgp Involved in Activation of ENaC by Glibenclamide

Previously we have shown, in patch-clamp experiments, that glibenclamide did not activate the ENaC through stimulation of an intracellular pathway, suggesting a direct interaction with the channel (Chraibi and Horisberger 1999). We thus focused on the extracellular domain of αgp to determine the region of interest of this regulation. To do

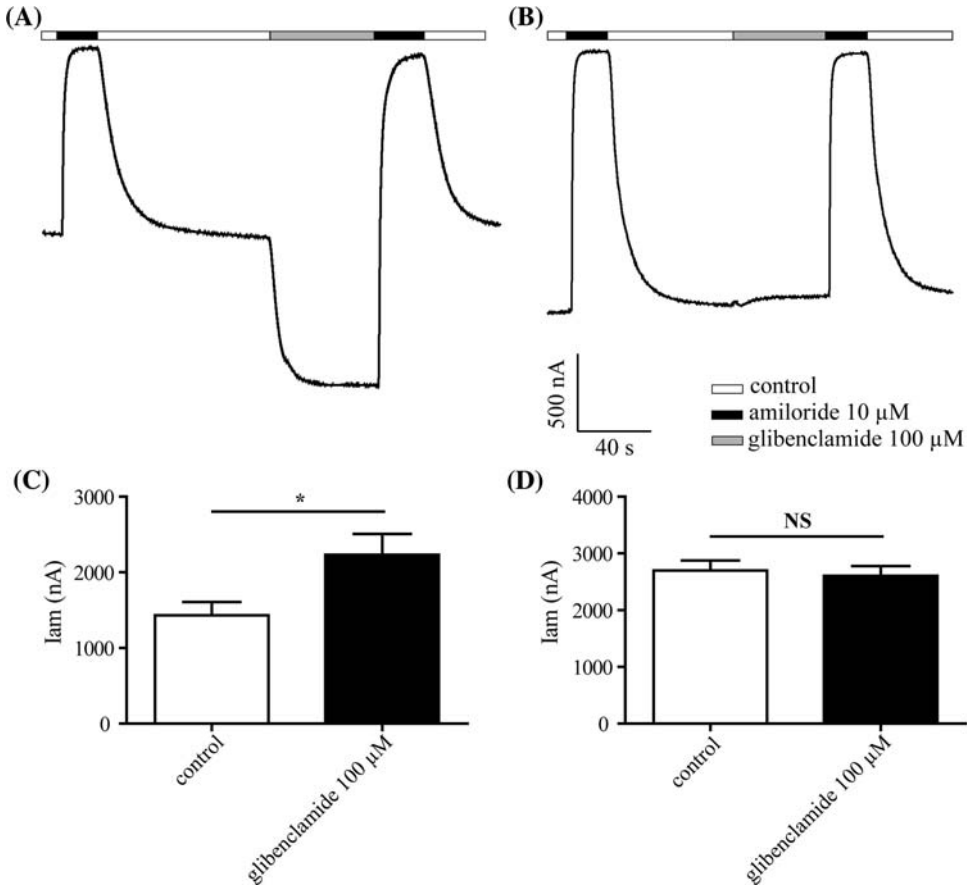

Fig. 1 Effect of glibenclamide on amiloride-sensitive currents in oocytes expressing αgp or αr with βrγr. Original inward Na^+ current recordings in oocytes expressing αgpβrγr (**a**) and αrβrγr (**b**) measured before and after 100 µM glibenclamide perfusion. **c** Mean I_{am} recorded in αgpβrγr-expessing oocytes before and after glibencalmide perfusion. I_{am} increased from 1431.60 ± 176.22 to 2233.58 ± 274.93 nA (n = 66) after glibenclamide perfusion. **d** Mean I_{am} recorded in αrβrγr-expressing oocytes. I_{am} was 2698.77 ± 177.53 and 2604.28 ± 174.18 nA before and after glibenclamide treatment, respectively (n = 65). *$P < 0.001$ versus control, paired t-test, NS, non-significant

so, we generated four chimeras of the α subunit in which we replaced different parts of the αr sequence with αgp at homologous positions (Fig. 2a and Materials and Methods). Each chimera was coexpressed with βrγr in *Xenopus* oocytes and the amiloride-sensitive sodium current was measured using TEVC, before and after a 30-s to 1-min glibenclamide perfusion (100 µM). As shown in Fig. 2b, glibenclamide induced a significant increase, 1.63 ± 0.09-fold, in the sodium amiloride-sensitive current generated by αgpβrγr channels (n = 70; $P < 0.001$ compared to control, paired t-test, i.e., before glibenclamide perfusion in the same oocyte). Oocytes expressing αr are not sensitive to glibenclamide (n = 78). When chimera I (n = 12), II (n = 11), or III (n = 25) was coexpressed in oocytes with βrγr, glibenclamide did not induce an increase in I_{am}, suggesting that the first transmembrane domain and approximately the first two-thirds of the extracellular loop of αgp (residues 12 to 456; see Fig. 2a and Materials and Methods) are not critical for this regulation. Interestingly, for chimera IV, which contained the C-terminal part of the extracellular loop and the second transmembrane domain together with the intracellular C-terminus of αgp, the sodium amiloride-sensitive current was increased by glibenclamide. Thus, a significant stimulation of 1.26 ± 0.04-fold ($P < 0.001$; n = 27) was observed, suggesting that this region of αgp (residues 456 to 623) plays a crucial role in activation of the ENaC by glibenclamide. Interestingly, it was reported that the activity of chimeric channels containing the αr with both intracellular termini originating from the αgp were not affected by glibenclamide (Schnizler et al. 2003). Altogether, these results suggest that essential regions involved in activation of the ENaC by glibenclamide reside in the extracellular loop and/or the second transmembrane domain. On the basis of those data, we focused on the region from residue 456 to residue 570 to determine specific amino acids involved in this regulation (see Figs. 2a, 3a).

Determination of Specific Amino Acids Involved in the Interaction of αgp with Glibenclamide

Sequence alignment analysis of the extracellular domain of αgp and αr between residues 456 and 539 revealed 11 mismatches (Fig. 3a). To determine specific amino acids potentially involved in activation of ENaC by glibenclamide, we generated the latter 11 swapping mutants on αr. These mutants were coexpressed with βrγr and we used TEVC to evaluate their impact on glibenclamide-induced activation. The effect of glibenclamide was reported as described above. As shown in Fig. 3b, glibenclamide did not induce a significantly different increase in I_{am} (paired

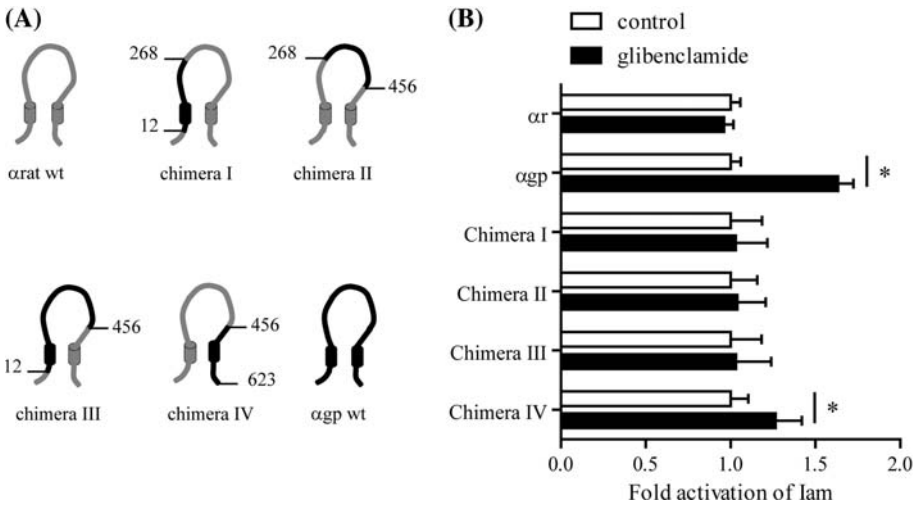

Fig. 2 Determination of the extracellular region of αgp involved in activation of the ENaC by glibenclamide. **a** Schematic representation of wild-type (wt) αgp (black), αr (gray), and chimeras of α subunits. Numbers on chimeras indicate residues at corresponding positions on αgp sequence. **b** Mean effect of glibenclamide on inward Na^+ current generated by αgp, αr, and chimeras coexpressed with βrγr in *Xenopus* oocytes. Glibenclamide increased the amiloride-sensitive current in αgp- and chimera IV-expressing oocytes by 1.63 ± 0.09-fold ($n = 70$) and 1.26 ± 0.04-fold ($n = 27$), respectively. Glibenclamide did not significantly increase the inward current when chimera I, II, or III was expressed in *Xenopus* oocytes compared to αr. *$P < 0.001$ versus control, paired *t*-test

t-test vs. control; $n = 12$ to 27). According to this result, we did not identify one or several major residues in αgp involved in ENaC stimulation by glibenclamide. Next, we compared the properties of these 11 amino acids to determine a combination of residues potentially involved. We noted six residues on αgp differing from αr in their polarity properties. Because glibenclamide is a nonpolar molecule, we evaluated the potential role played by these residues. We generated a swapping mutant of αr containing those six αgp residues at homologous positions: αr L464S, I480T, N481I, K495T, E502Q, and K521N. As shown in Fig. 3b, the coexpression of this mutant (αr VI) with βrγr did not generate channels sensitive to glibenclamide (paired *t*-test vs. control; $n = 14$).

The Second Transmembrane Domain of α-ENaC Is Not Responsible for Glibenclamide Activation

We have established the major role of the C-terminal part of αgp in this activation (Fig. 2b), but our results do not exclude an involvement of the second transmembrane domain (TM2). Canessa and colleagues first established the location of TM2 from Val 540 to Arg 585 (Fig. 3a; gray line) according to the hydrophobicity properties of amino acids in this segment (Canessa et al. 1993). The recent model of the human ENaC based on the ASIC-1 crystal structure (Stockand et al. 2008) harbors a TM2 shorter than previously described (see black line in Fig. 3a). Considering both the Canessa et al. and the Stockand et al. point of view, sequence alignment analysis of αgp and αr revealed three mismatches in TM2. We examined whether these residues could be involved in this regulation pathway. We thus generated substitution of the Asp-Val-Ile motif from αr TM2 with Glu-Phe-Met from αgp at the corresponding position. The activity of channels containing αr with Glu-Phe-Met from αgp (αr M2), coexpressed with βrγr, was not affected by glibenclamide perfusion (Fig. 3b). These results suggest that activation of the ENaC by glibenclamide requires the overall structure of the C-terminal part of the extracellular domain of αgp. However, we do not exclude that the mechanisms of activation may be exerted when certain conformational changes of the protein occur.

Effect of Glibenclamide on the Inhibition Constant (K_i) for Amiloride

A mutagenesis screen of the amino acid residue identified S583 (in α rat ENaC) and its homologues in β and γENaC (βG525, γG537), each of which, when mutated, drastically reduced the apparent channel affinity for amiloride (Schild et al. 1997). These residues are located in pre-M2, the hydrophobic domain directly preceding TM2 (Kellenberger and Schild 2002).

We have shown that the C-terminal part of the extracellular domain of αgp plays a major role in regulation of the ENaC by glibenclamide. This region is near pre-M2, which harbors the serine residue involved in amiloride block (αgp-S556, 583 in α rat; see Fig. 3a). Schnizler and colleagues have shown that cpt-cAMP slightly shifted the

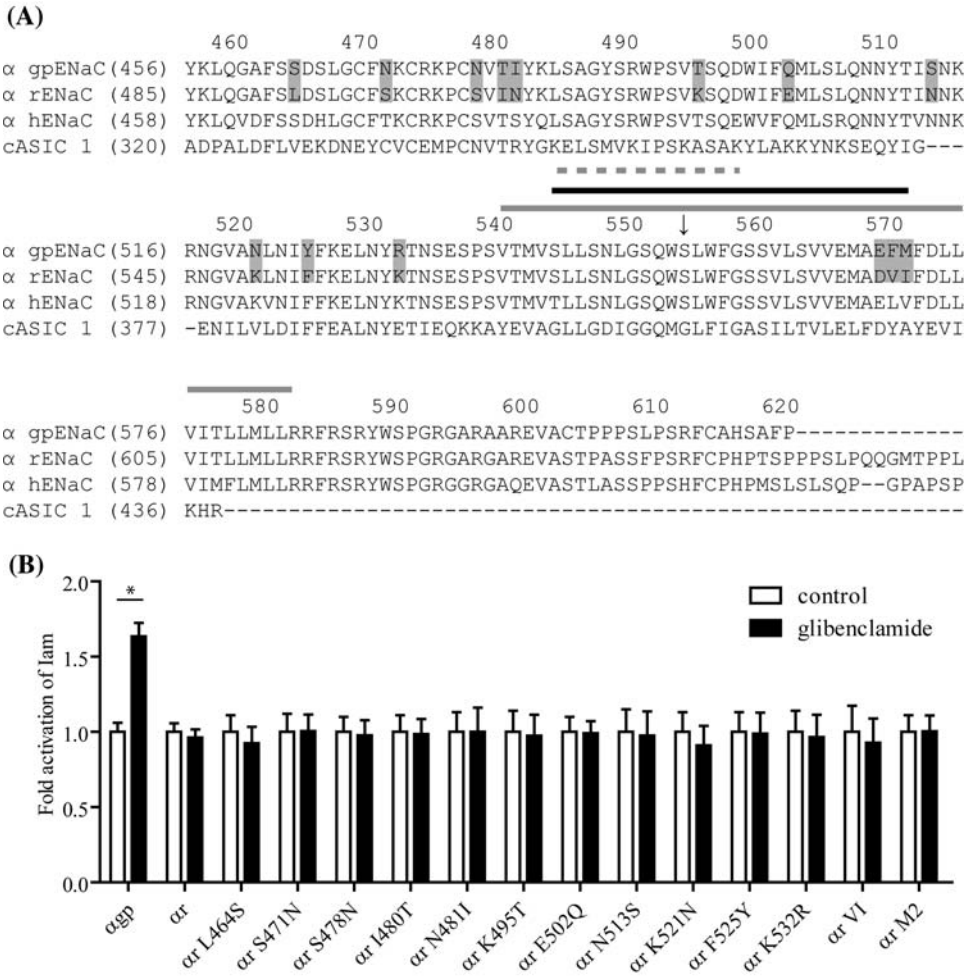

Fig. 3 Determination of specific residues of αgp involved in activation of the ENaC by glibenclamide. **a** Sequence alignment of the C-terminal region of αgp (GenBank accession number AJ249296), αr (GenBank accession number NM_031548), αh (GenBank accession number NM 001038), and cASIC1 (PDB number 2QTS). Sequence analysis showed mismatches on 11 residues (shaded gray) between αgp and αr in the extracellular loop and 3 residues in the second transmembrane domain (TM2). The gray and black lines represent the TM2 domain according to Canessa et al. (1993) and Stockand et al. (2008). respectively. The pre-M2 domain is indicated by the dotted line (according to Kellenberger and Schild 2002). The arrow indicates the location of the amiloride-binding site. **b** Mean effect of glibenclamide on the inward Na^+ current generated by α wild type (wt) and mutants of αr coexpressed with βrγr in *Xenopus* oocytes. Single and multiple amino acid substitutions on αr did not increase the sensitivity of the ENaC to glibenclamide. $^*P < 0.001$ versus control, paired *t*-test

K_i for amiloride, suggesting that cpt-cAMP could interact with the amiloride binding site (Schnizler et al. 2000). Hence, to test whether glibenclamide could interfere with αgp-S556, we performed amiloride concentration-response recordings using TEVC on oocytes expressing αgp, αr, or chimera IV together with βrγr. Each oocyte was exposed to increasing amiloride concentrations, from 10^{-4} to 2×10^2 μM, with or without 100 μM glibenclamide in the perfusion solution. K_i values for amiloride were determined by fits of curves obtained from three experiments after normalization (four to six oocytes per experiment). Original recordings performed on the same oocyte expressing αgpβrγr are shown in Fig. 4a. In αgpβrγr- and αrβrγr-expressing oocytes, K_i values for amiloride were 0.35 ± 0.06 and 0.27 ± 0.03 μM, respectively, in the absence of glibenclamide (Fig. 4b). Since the serine residue S556 is unchanged in αgp and chimera IV, we were expecting a similar K_i for amiloride. Interestingly, the K_i for amiloride in chimera IVβrγr ENaC channels was 2.04 ± 0.14 μM, corresponding to an apparent affinity for inhibition six to seven times lower than that of αgp and αrβrγr ENaC. Glibenclamide only slightly shifted the K_i for amiloride in αgpβrγr (0.32 ± 0.08 μM), αrβrγr (0.34 ± 0.08 μM), and IVβrγr (2.07 ± 0.15 μM), but there was no significant difference in amiloride response without versus with glibenclamide ($n = 12$–18; paired *t*-test). Thus, ENaC activation by glibenclamide did not interfere with the pharmacological properties of amiloride.

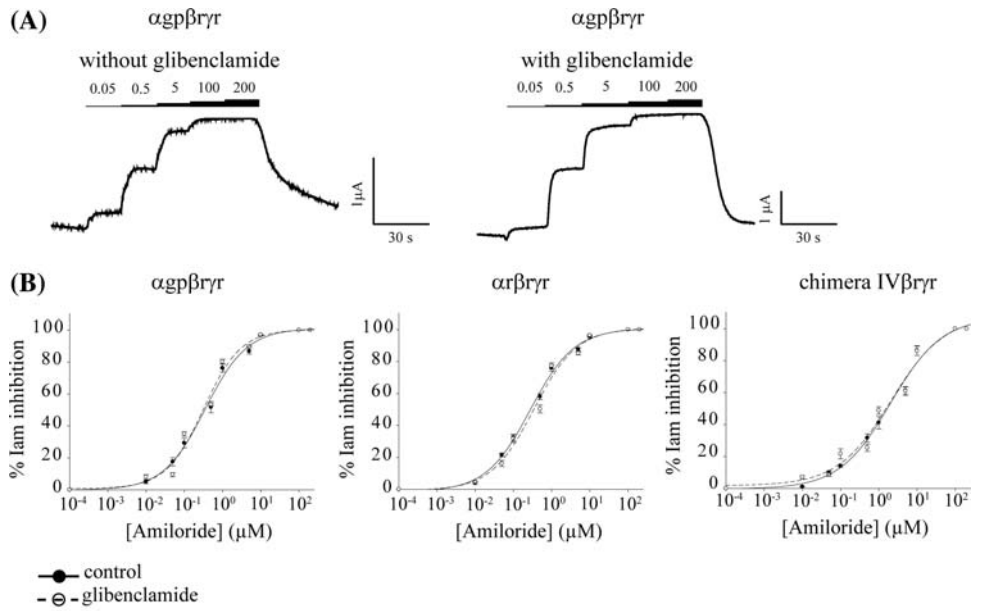

Fig. 4 Effect of glibenclamide on the K_i of amiloride. The K_i for amiloride was measured in each oocyte without and with 100 μM glibenclamide in perfusion solutions. **a** Original recordings of amiloride dose-response on αgp*β*r*γ*r ENaC without (left) and with (right) 100 μM glibenclamide performed in the same oocyte with TEVC at −60 mV. Values above each recording indicate the amiloride concentration (μM). Amiloride at 200 μM fully inhibits the inward sodium current. **b** Mean effect of glibenclamide on the inhibition constant (K_i) for amiloride of αgp*β*r*γ*r, αr*β*r*γ*r, and chimera IV *β*r*γ*r ENaC channels. Under all conditions, inhibition was normalized to the maximal effect recorded with 200 μM amiloride. The K_i for amiloride was 0.35 ± 0.06, 0.27 ± 0.03, and 2.04 ± 0.14 μM for αgp*β*r*γ*r, αr*β*r*γ*r, and chimera IV *β*r*γ*r, respectively, and was not affected by glibenclamide (paired *t*-test). K_i values were determined by fits of curves obtained from three experiments after normalization (four to six oocytes per experiment)

Discussion

Several hormonal and nonhormonal factors regulate the ENaC but the mechanisms of this regulation are not yet completely understood. Diverse pathways including regulation of channel expression (synthesis, degradation, and trafficking) and single-channel activity have been reported (Bhalla and Hallows 2008). It also has been proposed that the function of the ENaC may be modulated by several proteins belonging to the family of ATP-binding cassette transporter proteins, such as SUR, CFTR, and K_{ATP} (Berdiev et al. 2009; Konstas et al. 2001; Leroy et al. 2006). Several mechanisms have been proposed to explain these regulations. In particular, using coexpression in *Xenopus* oocytes, protein-protein interaction between the ENaC and the CFTR has been suggested. Furthermore, it has been demonstrated that coexpression of the SUR reduces the sodium-amiloride sensitive current (Konstas et al. 2001). This inhibitory effect, caused by a decrease in expression of the ENaC at the plasma membrane, does not depend on an interaction between the SUR and the C-termini of the α, *β*, and *γ* ENaC subunits. Also, it has been demonstrated that the SUR does not confer the sensitivity of the ENaC to glibenclamide, a drug known for its high-affinity inhibitor of the SUR. These results are consistent with our studies. Indeed, we and others have demonstrated that glibenclamide activated ENaC channels expressed in *Xenopus* oocytes. This up-regulation requires a high concentration (1000-fold higher than that used to inhibit the SUR) and it is species dependent. It requires expression of the *Xenopus* or guinea pig α subunit, while the α subunit of rat is not able to generate glibenclamide-sensitive ENaC channels (Chraibi and Horisberger 1999; Konstas et al. 2001; Schnizler et al. 2003). In our previous work, we showed that this stimulation did not depend on intracellular pathways but probably was a result of a direct interaction with the extracellular domain of the channel (Chraibi and Horisberger 1999). This finding is also compatible with data reported by Schnizler and colleagues showing that exchange of both of the intracellular αgp termini for the respective termini from the rat did not prevent stimulation of the amiloride-sensitive sodium current by glibenclamide. At the molecular level, glibenclamide produced an increase in the N.Po of the sodium channel measured in an outside-out configuration of the patch-clamp, reinforcing the hypothesis of a direct interaction with the extracellular domain of the channel (Chraibi and Horisberger 1999). However, our data do not allow exclusion of the possibility that glibenclamide binds to an ABC protein as yet unidentified in the *Xenopus* oocyte membrane.

In the present study, we generated four chimeras of the α subunit harboring different regions of αgp and αr. ENaC

channels made of chimeras containing the first transmembrane domain and approximately the first two-thirds of the extracellular loop of αgp were not stimulated by glibenclamide. However, the chimera enclosing the αgp C-terminal part of the extracellular loop was the only one able to generate ENaC channels sensitive to glibenclamide, although the activation induced by this ligand was significantly lower from the oocyte expressing αgpβrγr. Taken together, our data suggest that residues 456 to 539 of the guinea pig α subunit play a major role in glibenclamide-induced activation of the ENaC expressed in *Xenopus* oocytes. However, they do not exclude a potential function of other parts of the ENaC subunits since αgpβrγr showed a stronger activation than chimera IV (see Fig. 2b). Sequence alignment analysis between αgp and αr in the extracellular domain of this region revealed 11 mismatches. Site-directed mutagenesis on the αr subunit did not allow us to identify specific amino acids potentially involved in this up-regulation. Thus, our results suggest that the overall structure of the extracellular domain is required for full activation of the ENaC by glibenclamide.

In a recent study, Stockand and coworkers (2008) established a model of ENaC structure based on the crystal structure of ASIC1 (Jasti et al. 2007). The authors described the extracellular domain as a large structure forming cavities probably involved in protein or ligand interacting sites located in the extracellular domain. According to this model and our results, we propose that the glibenclamide-dependent activating domain is located throughout the extracellular domain.

We also investigated the potential role of the second transmembrane domain in the activation of ENaC by glibenclamide. Mutation of this domain from the αr sequence did not generate a channel sensitive to the compound. In this mutant, Asp-Val-Ile was replaced by Glu-Phe-Met from αgp, which have similar properties to their homologous residues (negative charge of Asp-Glu and polarity of Val-Phe and Ile-Met).

Glibenclamide was not able to shift the K_i for amiloride in oocytes expressing αgp, αr, or chimera IV together with βrγr. This observation suggests that glibenclamide did not induce a conformational change of the amiloride binding site. Interestingly, the K_i for amiloride in chimera IV-expressing oocytes was six- to sevenfold higher than that for αgpβrγr. The amiloride binding site is located in the extracellular loop close to the channel pore (Garty and Palmer 1997). Since the amiloride binding-site sequences in αgp and chimera IV are identical, we could expect a similar K_i for amiloride in oocytes expressing αgp and chimera IV. McNicholas and Canessa (1997) suggested that the guanidinium portion of amiloride interacts with the pore of the channel, while the pyrazine ring of the molecule is stabilized by another region of the extracellular loop. A second amiloride-binding region in the N-terminal region (residues 278–283) of the αr ENaC subunit stabilizing amiloride has been identified and characterized (Ismailov et al. 1997; Kelly et al. 2003). Mutations in this domain (W278A or H282R) increased the K_i for amiloride to a value similar to that measured in chimera IV. These mutations could generate conformational changes within the extracellular loop and alter the block by amiloride. The K_i value for amiloride measured in chimera IV could mean that the amiloride-binding domain in the N-terminal region is not able to stabilize amiloride. Further experiments are necessary to determine whether interaction and stabilization of amiloride occur in different ways in channels composed of αgp and chimera IV.

Several extracellular factors involved in the modulation of ENaC activity, such as S3969 (Lu et al. 2008), capsazepine (Yamamura et al. 2004), and serine proteases (Chraibi et al. 1998; Hughey et al. 2003, 2007; Rossier 2004; Vallet et al. 1997, 2002), were identified. However, the region of the extracellular domain responsible for the interaction with these factors remains to be determined. Previously, we have demonstrated that the C-terminal region of the extracellular loop of αgp plays a major role in the interaction with cpt-cAMP (Renauld et al. 2008). In this paper, we propose that this domain of αgp is also important for the up-regulation of the sodium channel by glibenclamide. Taken together, these data provide evidence that the extracellular loop of the ENaC moiety harbors activation domains potentially accessible to small molecules and indicate that this sodium channel appears to function like a ligand-gated ion channel. However, they do not exclude that the mechanisms of this stimulation may be exerted when certain conformational changes of the protein take place. Among these molecules, S3969 stimulates the function of a PHA-1 variant by increasing its open probability (Lu et al. 2008); a similar mechanism was also observed in activation of the ENaC by glibenclamide (Chraibi and Horisberger 1999). Thus, knowledge in this research area would be helpful in pathologies characterized by a loss of ENaC function such as pseudohypoaldosteronism type 1.

Acknowledgments We thank Dr. Karine Tremblay for critically reading the manuscript. This work was supported by the Canadian Institutes of Health Research, Kidney Foundation of Canada, and Canada Foundation for Innovation. Dr. A. Chraïbi holds a Chercheur Boursier Junior-2 Award from the Fonds de la Recherche en Santé du Québec. We thank Dr. Wolfgang Clauss for αgpENaC cDNA and Drs. Bernard Rossier and Cecilia Canessa for the rENaC clone.

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