Intracellular regulation of human ClC-5 by adenine nucleotides

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ClC-5, an endosomal Cl⁻/H⁺ antiporter that is mutated in Dent disease, is essential for endosomal acidification and re-uptake of small molecular weight proteins in the renal proximal tubule. Eukaryotic chloride channels (CLCs) contain two cytoplasmic CBS domains, motifs present in different proteins, the function of which is still poorly understood. Structural studies have shown that ClC-5 can bind to ATP at the interface between the CBS domains, but so far the potential functional consequences of nucleotide binding to ClC-5 have not been investigated. Here, we show that the direct application of ATP, ADP and AMP in inside-out patch experiments potentiates the current mediated by ClC-5 with similar affinities. The nucleotides increase the probability of ClC-5 to be in an active, transporting state. The residues Tyr 617 and Asp 727, but not Ser 618, are crucial for the potentiation. These results provide a mechanistic and structural framework for the interpretation of nucleotide regulation of a CLC transporter.

Keywords: chloride channel; Cl⁻/H⁺ antiporter; CBS domains; nucleotides; transport

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INTRODUCTION

The CLC protein family is crucial for many physiological processes that range from cell volume regulation and transepithelial transport to electrical excitability (Zifarelli & Pusch, 2007; Jentsch, 2008). It is unique in that it comprises, in a similar structural scaffold, both ion channels in which Cl⁻ ions diffuse passively down their electrochemical gradient and antiporters for which anion transport is thermodynamically coupled to the transport of protons in the opposite direction (Accardi & Miller, 2004; Picollo & Pusch, 2005; Scheel *et al*, 2005; De Angeli *et al*, 2006; Graves *et al*, 2008; Zifarelli & Pusch, 2009).

ClC-5 is a Cl⁻/H⁺ antiporter (Picollo & Pusch, 2005; Scheel *et al*, 2005) that has a crucial role in the acidification of early endosomes in the proximal tubule, a segment of the nephron responsible for the re-uptake of small molecular weight proteins

(Günther *et al*, 1998, 2003; Piwon *et al*, 2000; Wang *et al*, 2000; Mohammad-Panah *et al*, 2003). In fact, ClC-5 co-localizes with the V-type ATPase and other endosomal markers; knockout mice show that impaired endosomal acidification and loss-of-function mutations in ClC-5 lead to Dent disease (for reviews, see Jentsch, 2005, 2008; Zifarelli & Pusch, 2007), a disorder characterized by defective endocytosis leading to kidney stones and renal failure (Lloyd *et al*, 1996).

CLC proteins are homodimers with each subunit containing an independent ion translocation pathway (Ludewig *et al*, 1996; Middleton *et al*, 1996; Weinreich & Jentsch, 2001; Dutzler *et al*, 2002). Similar to all eukaryotic CLCs, each ClC-5 monomer has a cytoplasmic region comprising two CBS domains (Estévez *et al*, 2004), motifs present in many different proteins in all organisms (Bateman, 1997; Ponting, 1997). Mutations in CBS domains are causative in several hereditary diseases in humans (Scott *et al*, 2004), but their function is only poorly understood.

It has been suggested that CBS domains might constitute binding modules for nucleotides that regulate the activity of several CLC proteins. In fact, ATP binds to the isolated C-terminal region of ClC-2 (Scott *et al*, 2004) and slightly alters the gating kinetics of ClC-2 (Niemeyer *et al*, 2004). Conflicting results have been reported about the regulatory role of nucleotides on ClC-1 (Bennetts *et al*, 2005, 2007; Tseng *et al*, 2007; Zhang *et al*, 2008; Zifarelli & Pusch, 2008); however, C-terminal fragments of ClC-0 and ClC-Ka did not show ATP binding (Meyer & Dutzler, 2006; Markovic & Dutzler, 2007).

For ClC-5, biochemical studies have shown the binding of adenine nucleotides to the isolated C-terminus (Wellhauser *et al*, 2006; Meyer *et al*, 2007). By using X-ray crystallography, Meyer *et al* (2007) found that ATP and ADP bind to the C-terminus in a cleft formed at the interface between the two CBS domains in each monomer, with crucial interactions provided by the side chains of Tyr 617 and Asp 727 (Meyer *et al*, 2007). This breakthrough was the first direct evidence that nucleotides bind to a CLC protein and immediately suggest a potential functional role of nucleotides for ClC-5. However, evidence for a regulation of ClC-5 function by nucleotides is lacking.

Here, we used the inside-out configuration of the patch-clamp technique to study directly the regulation of ClC-5 by intracellular nucleotides. We found that ClC-5 is potentiated by intracellular AMP, ADP and ATP with similar affinities, whereas other adenine-containing molecules had little or no effect. The ATP

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dose–response and noise analysis suggest that the current potentiation is due to an increased probability for CIC-5 to be in an active, transporting state. Mutational analysis confirms that Tyr617 and Asp727 are essential for mediating the nucleotide-induced potentiation.

RESULTS AND DISCUSSION

We applied ATP to the intracellular side of inside-out patches expressing CIC-5. At concentrations of 1 and 10 mM, respectively, ATP increases currents by a factor approximately 1.5 and approximately 2 (Fig 1A). ATP potentiation is not accompanied by an alteration of the rectification (Fig 1B), showing that its effect is voltage independent and does not involve a major modification of the transport mechanism.

The concentration–response relationship (Fig 1C) shows a small but significant increase in current at 1 μ M with saturation at around 10 mM. The line in Fig 1C represents a fit to the data using equation (1) described in the Methods section. From the fit, we obtain for the apparent dissociation constant for the inactive state, $\tilde{K}_i$, and for the active state, K_A , values of 0.9 and 0.4 mM, respectively, which are in reasonable agreement with biochemical studies on isolated C-terminal fragments (Wellhauser *et al*, 2006; Meyer *et al*, 2007). The increase in current is explained by the fact that $K_A < \tilde{K}_i$ —that is, ATP stabilizes the active state. Half-maximal potentiation is obtained at about 1 mM, a value that is close to the ‘physiological’ intracellular ATP concentration.

The above interpretation is in accordance with our recent proposal that CIC-5 activity occurs in bursts, so that the spectral noise of the current is dominated by the transition between the active and inactive state (Hilgemann, 1996; Zdebik *et al*, 2008; Zifarelli & Pusch, 2009). In this situation, noise analysis can be used to determine the transport turnover rate. By using this technique, we find that the single transporter conductance is not affected by ATP (conductance values were 0.34 ± 0.08 pS and 0.32 ± 0.08 pS without ATP and with 1 mM ATP, respectively, $n=4$), confirming the conclusion that ATP increases the probability of CIC-5 being in an active, transporting mode.

Interestingly, the pH of the intracellular solution has a profound impact on the effect produced by ATP (Fig 2). Although application of 10 mM ATP increases currents by a factor of approximately 2 at pH 7.3 and by a factor of approximately 2.5 at pH 8.3, at pH 6 it does not produce any significant increase in current (Fig 2A,B). These results imply that ATP- and pH-mediated effects are not independent of each other.

The potentiation mediated by ADP and AMP (Fig 3A) is quantitatively almost the same as that produced by ATP, suggesting that the apparent affinity and efficacy of the three nucleotides is similar (Fig 3B). Experiments in which 1 mM ATP was first applied alone and then simultaneously with 1 mM ADP, show that the effect of these nucleotides is additive, confirming the similar apparent affinity of ATP and ADP (supplementary Fig S1 online). Our results are consistent with the similar binding affinities for AMP, ADP and ATP to the C-terminal fragment of CIC-5 reported by Meyer *et al* (2007) using equilibrium dialysis, but are in contrast to the work of Wellhauser *et al* (2006), who reported, using radio-ligand binding, that AMP could compete with ATP binding with μ M affinity. A potential source for this discrepancy is the different approach used (electrophysiological versus biochemical); however, our results are in agreement with

Fig 1 | ATP potentiates wild-type chloride proton antiporter 5.

(A) Representative recordings from an inside-out patch a perfused with a control solution (no ATP added) and with solutions containing 1 and 10 mM ATP. (B) Mean values of the currents obtained from experiments similar to that described in (A) in the presence of 1 mM ATP ($n=14$; open circles) or in control condition ($n=14$; filled squares). The currents were normalized using the value of the current in the control condition at 160 mV. As ATP potentiates CIC-5 currents, to have a more direct comparison of the rectification properties in the absence and in the presence of ATP, the normalized currents in the control solution were multiplied by the scaling factor 1.55, obtained from the minimization of the difference in current magnitude in the absence and in the presence of ATP. (C) Plot of normalized currents at 160 mV for ATP at 0.001 mM ($n=7$), 0.01 mM ($n=5$), 0.1 mM ($n=6$), 1 mM ($n=15$), 5 mM ($n=6$) and 10 mM ($n=6$). Normalization was carried out with current in a control solution. The solid line represents a fit with the equation described in the Methods section with $K_A = 0.43$ mM and $K_i = 0.89$ mM. Error bars indicate s.e.m. CIC-5, chloride proton antiporter 5; WT, wild type.

the biochemical results of Meyer *et al*. Furthermore, our results are consistent with the structural results obtained in the same study, showing that the adenosine moiety contributes predominantly to the binding affinity. This conclusion is supported further by mutational analysis and the different effects of the adenine-containing compounds included in this study (see below). There are, therefore, several lines of evidence arguing against significantly different affinities of ATP and AMP.

Interestingly, nicotinamide adenine dinucleotide (NAD), a molecule that contains an adenine and a nicotinamide nucleotide moiety, also increases CIC-5 currents (Fig 3B; supplementary Fig S2 online). However, the potentiation at 10 mM is significantly smaller compared with the other nucleotides tested, suggesting a lower efficacy of NAD. The potential physiological implications of NAD-mediated regulation of CIC-5 activity require further investigation.

The application of 10 mM adenosine produced only a marginal potentiation, whereas 3 mM adenine (close to the maximal concentration that we were able to dissolve in our solutions)

Fig 2 | Interdependence of the effect of intracellular ATP and pH. (A) Representative recordings from an inside-out patch perfused with a control solution (pH 7.3, no ATP), with a solution at pH 7.3 with 10 mM ATP, and with a solution at pH 6.0 without and with 10 mM ATP. For clarity, data were filtered at 3 kHz. (B) Mean values of the normalized currents obtained from experiments similar to that described in (A) with the addition of experiments with solution at pH 8.3 with 10 mM ATP ($n \geq 4$) and experiments with solutions at pH 8.3 without ATP that were published in a previous study by our group (Zifarelli & Pusch, 2009). All values are normalized to the currents measured at pH 7.3 without ATP. Note that the effective MgATP^{2-} concentration at pH 6.0 is only slightly reduced (by about 30%) compared with pH 7.3 (Storer & Cornish-Bowden, 1976). Thus, the lack of ATP effect at pH 6.0 is unlikely to be caused indirectly by a pH-dependent change of the unprotonated ATP concentration.

had no effect (Fig 3B). Furthermore, the addition of 3 mM adenine did not alter the effect of 1 mM ATP, indicating that adenine is not able to bind to CIC-5 (supplementary Fig S3 online). These results show that the minimal requirement for binding is the presence, in the ligand, of both the adenine and ribose moiety. Furthermore, the potentiating effect of AMP, ADP and ATP, but only marginal effect of adenosine, indicates that the presence of the α -phosphate group is necessary to produce a robust current potentiation.

There have been conflicting results about the possible role of ATP in regulating the activity of the muscle channel CIC-1. In contrast to what we have shown here for CIC-5, it has been proposed that ATP favours the closed state of CIC-1 (Bennetts *et al*, 2005) and that an acidic pH potentiates the effect of ATP (Bennetts *et al*, 2007; Tseng *et al*, 2007). In particular, Zhang *et al* (2008) suggested that the oxidation that follows patch excision could

Fig 3 | Effect of various adenine-containing ligands. (A) Representative recordings from an inside-out patch perfused with a control solution (no nucleotide added) and with solutions containing 10 mM ADP and AMP. (B) Mean values of the normalized currents at 160 mV obtained in the presence of the indicated ligand at the indicated concentration. Normalization was carried out using the current in the control condition at 160 mV ($n \geq 4$). ATP, ADP, AMP and NAD had no significant effect on patches from uninjected oocytes (data not shown, $n \geq 3$).

reduce or abrogate the effect of ATP, potentially explaining the lack of ATP effect reported by our group (Zifarelli & Pusch, 2008). To test whether this parameter could also modulate the effect of ATP on CIC-5, we carried out experiments in the presence of the reducing agent dithiothreitol (DTT). An exposure to 1 mM DTT, before or during the application of ATP, did not affect the potentiation mediated by 1 mM ATP or modified the behaviour of CIC-5 in the control condition (supplementary Fig S4 online).

These results suggest a fundamentally different mechanism underlying the ATP effect on CIC-5 compared with CIC-1, which is also consistent with the relatively poor conservation of the C-terminal region of the two proteins.

Meyer *et al* (2007) showed that Tyr 617 and Asp 727 are crucial residues for the binding of nucleotides, but could not detect any functional difference between the wild type and either of the

mutants Y617A and D727A. However, this test was carried out in voltage clamp experiments and not by the direct application of ATP in to the intracellular side of CIC-5. However, when the same mutations were introduced in to the background of the mutant E211A, they were reported to markedly change the rectification of the currents, rendering the E211A–Y617A mutant more similar to wild type CIC-5 than to the single mutant E211A. In contrast to these findings, in our experiments, the double mutant E211A–Y617A is similar to E211A, except for a decreased current magnitude (supplementary Fig S6 online). Similar results were obtained for the double-mutant E211A–D727A (data not shown, $n=7$). The origin of the discrepancy regarding the rectification of the double mutants is at present unclear. Possibly, the divergent results are related to the rather small currents of the double mutants compared with E211A. In this respect, endogenous oocyte currents are outwardly rectifying, resembling those carried by wild type CIC-5, which might explain the conclusion of Meyer *et al* (2007). By contrast, the rectification of E211A and of the double mutants observed in our study is characteristic and differs from typical leak currents.

The single mutants Y617A and D727A preserve the strong rectification of currents at negative voltages, typical of the wild type (supplementary Fig S5A online), and they are able to transport protons (supplementary Fig S5B online). However, they produce a decrease in current magnitude, which is more pronounced for the D727A mutant (supplementary Fig S5C online). More importantly, both mutations abrogate the potentiating effect of ATP (Fig 4). To rule out the possibility that this result originates from an impaired access of the perfusing solutions to the intracellular side of the patches, we always tested the patch accessibility by application of control solutions at pH 8.3 (Fig 4A,B) or pH 6.3 (data not shown), which were shown to inhibit and potentiate respectively, wild type CIC-5 currents in a reversible manner (Zifarelli & Pusch, 2009). Such control experiments directly prove that the lack of effect of ATP is specifically due to the introduced mutations. Furthermore, they show that the mutations Y617A and D727A have a similar response to the pH_{int} compared with wild type.

Conversely, mutation of Ser 618, which interacts with both the α - and γ -phosphate groups of ATP (Meyer *et al*, 2007), into alanine does not affect ATP-induced potentiation (Fig 4C), indicating that this residue is not critical for ATP binding, and does not participate in the conformational changes that follow nucleotide binding and determine the current increase. Thus, both our mutational analysis and previous structural findings indicate that the interactions of Tyr617 and Asp727 with the adenine and ribose moieties are most essential for binding, suggesting that the α -phosphate is involved in conformational changes that follow ATP binding. By contrast, mutation of Ser618, which interacts with the α -phosphate (Meyer *et al*, 2007), into alanine does not have any effect on the response to ATP compared with wild type, further supporting the idea that this interaction is not important for the binding and also indicating that this residue is not crucial in determining the current potentiation that follows ATP binding. Further studies are therefore required to identify the molecular mechanism coupling ATP binding and current potentiation.

The indiscriminate current potentiation caused by AMP, ADP, ATP and NAD casts doubts on the physiological relevance of these

Fig 4 | ATP effect on mutants Y617A, D727A and S618A. (A) Representative recordings from an inside-out patch on the mutant Y617A perfused with a control solution, with a solution at pH 8.3 and with 1 mM ATP. (B) Representative recordings from experiments as in panel (A) on the mutant D727A. (C) Mean of normalized currents obtained for the indicated mutant at 1 and 10 mM ATP ($n \geq 4$).

molecules for CIC-5 function. However, we still do not know whether the properties of heterologously expressed CIC-5 recapitulate those of the endosomal environment and therefore we cannot completely exclude the possibility that nucleotides do have a regulatory function in a physiological setting.

In summary, we show for the first time, to our knowledge, that CIC-5 is functionally regulated by intracellular nucleotides through binding to the cytoplasmic CBS domains. Our results provide a mechanistic and structural framework for assessing the physiological role of intracellular nucleotide regulation of CLC transporters.

METHODS

Detailed methods for oocyte expression, voltage clamp, non-stationary noise analysis and extracellular pH measurements are provided in the supplementary information online.

For inside-out patch clamp measurements, pipettes were pulled from aluminosilicate capillaries (Hilgenberg, Malsfeld, Germany), coated with Sylgard (Dow Corning Corporation, MI, USA) and fire-polished to a resistance of ~ 0.5 – $1.0 \text{ M}\Omega$. The extracellular (pipette) solution contained 100 mM *N*-methyl-D-glucamine chloride, 10 mM HEPES and 5 mM MgCl_2 (pH 7.3). The intracellular solution contained 100 mM *N*-methyl-D-glucamine chloride, 10 mM HEPES,

2 mM MgCl₂ and 1 mM EGTA (pH 7.3). Nucleotide- and adenosine-containing solutions were freshly prepared before experiments from stock solutions stored at −20 °C. Adenine and DTT were dissolved directly in the measuring solution. The pH was adjusted using NaOH or H₂SO₄ to the desired values. For pH < 7.3, MES buffer, and for pH 8.3, bis-tris-propane buffer were used instead of HEPES. All reagents were purchased from Sigma (Milan, Italy). Solutions were changed by inserting the patch pipette into the opening of ~0.5 mm diameter capillaries.

The voltage protocol consisted of steps of 30 or 50 ms from 160 to −80 mV in decrements of 20 mV. The holding potential was 0 mV. Filter frequency, unless otherwise stated, was 10 kHz. If not otherwise stated, error bars are s.d. Statistical differences were assessed using Student's *t*-test (*P* < 0.05).

The stimulating effect of ATP was described by the following allosteric model:

according to which the CIC-5 transporter can be in an inactive state (*I*) and an active, transporting state (*A*). The binding of one molecule of ATP is possible in both states with respective dissociation constants *K_I* and *K_A*. The parameter $r_0 = \frac{P_A}{P_I}$ is the ratio of the probabilities of the transporter to be in the active state and that to be in the inactive state in the absence of ATP. Similarly, $r_1 = \frac{P_{A_{ATP}}}{P_{I_{ATP}}}$ refers to the equivalent ratio in the presence of ATP.

According to the principle of microscopic reversibility, only three of the four parameters depicted in Scheme 1 are independent. Defining the 'apparent affinity of the inactive state', $\tilde{K}_I$, as

$$\tilde{K}_I = K_I \frac{1 + r_0}{1 + r_1}$$

the normalized current, *I_{norm}*, as a function of the concentration of ATP can be expressed by

$$\frac{I(\text{ATP})}{I(0)} = \frac{1 + \text{ATP}/K_A}{1 + \text{ATP}/\tilde{K}_I} \quad (1)$$

This equation was used to fit the data shown in Fig 1C. On the basis of noise analysis (Zdebik *et al*, 2008; Zifarelli & Pusch, 2009) it is reasonable to assume that $r_0 \ll 1$ that is the probability of the transporter to be in an active state is much smaller than unity. As nucleotides increase currents maximally by about twofold, even $r_1 \ll 1$. With these assumptions, the apparent dissociation constant of the inactive state is close to the true dissociation constant that is $\tilde{K}_I \approx K_I$.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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