To ATP or Not To ATP: This Is the Question

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The ability to sense the metabolic state of a cell and tune its electrical activity through the direct or indirect modulation of ion transport systems by ATP is essential in a multitude of physiological processes in both excitable and nonexcitable cells. For example in pancreatic β cells an increase in the ATP levels induces closure of the K_{ATP} channels, leading to depolarization of the membrane, triggering insulin secretion (Ashcroft, 2006), and the energy derived from ATP hydrolysis is used to drive inward nutrient movement or outward xenobiotic transport (Davidson and Maloney, 2007). The structural motifs regulating ATP binding and hydrolysis have been elucidated in atomic detail by the resolution of numerous crystal structures, both of isolated domains and of complete proteins (Walker et al., 1982; Hollenstein et al., 2007).

Members of the CLC family of Cl^- channels and transporters have only been peripherally involved in ATP-sensing processes. The cytoplasmic domains of all eukaryotic CLCs contain two cystathionine-β-synthase (CBS) domains homologous to those found in a wide variety of other systems (Scott et al., 2004). In several cases it has been shown that these domains mediate direct binding of and regulate modulation of their host proteins by adenosine ligands, and it has been proposed that CLC function might also be regulated by ATP (Scott et al., 2004). However the reports offered indirect evidence: ATP and its derivatives could prevent rundown of CLC-4 currents (Vanoye and George, 2002), and disease-causing mutations in the cytoplasmic domains of CLC-1 and CLC-2 led to changes in the ATP-binding properties of their isolated CBS domains (Scott et al., 2004; Wellhauser et al., 2006). The pathophysiological relevance of these domains in CLC-mediated Cl^- transport is highlighted by the numerous mutations localizing within their boundaries that compromise protein function and cause a number of genetically inherited disorders such as myotonia, Bartter syndrome, osteopetrosis, Dent's disease, and epilepsy (Jentsch et al., 2005; Ignoul and Eggermont, 2005). Functionally these domains have been implicated in regulating channel gating (Estévez et al., 2004; Bykova et al., 2006), but as to how they are involved, we do not know. Channel opening in CLC-1 is regulated by two distinct and interdependent processes: a single-pore gating mode, where each pore gates independently, and a common-pore gate, which allows both pores to open simultaneously (Saviane et al., 1999; Accardi and Pusch, 2000). In CLC-1 both processes depend upon voltage in a Boltzmann-like fashion: at positive potentials the maximal open probability approaches unity; at negative voltages the gates deactivate incompletely, so that at no voltage the channel is completely shut (Rychkov et al., 1996; Accardi and Pusch, 2000). This incomplete closure is a physiologically fundamental feature because the flow of Cl^- ions through the open CLC-1 channels stabilizes the resting potential of the skeletal muscle fibers. Given the technical difficulties of studying CLC-mediated Cl^- transport in native systems, with many CLCs inconveniently localizing to subcellular compartments or residing in electrically inaccessible cells, most investigations have been limited to heterologous expression systems such as cultured cells or *Xenopus* oocytes.

In 2005, Bennetts and colleagues provided the first evidence that the CLCs can be directly modulated by adenosine ligands by showing that cytoplasmic ATP inhibits CLC-1 currents in HEK293 cells (Bennetts et al., 2005). This inhibition is the result of a dramatic right shift of the half-activation potential, $V_{1/2}$, of the common pore gate following ATP binding to the cytoplasmic domain of CLC-1. This study showed that ATP, ADP, AMP, and adenosine are all equally potent in shifting activation, and ATP hydrolysis is not required for inhibition since the nonhydrolyzable analogue ATP-γ-s is also equally effective. The binding site however is exquisitely sensitive to the molecular determinant of the headgroup: IMP is virtually ineffective. These results are in remarkable harmony and can be qualitatively explained in the context of the only known structure of a CLC cytoplasmic domain in complex with ATP, that of CLC-5 (Meyer et al., 2007). In this domain ATP binds to a cleft between the two CBS domains, with the adenine base and the ribose ring forming most of the stabilizing interactions with the protein; the phosphate groups are partly solvent exposed and weakly interact with the protein. Consistent with this organization ATP, ADP, and AMP all bind with roughly equal affinities. However, the cytoplasmic domains of two other CLCs, CLC-0 and

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Abbreviation used in this paper: CBS, cystathionine-β-synthase.

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CLC-Ka (Meyer and Dutzler, 2006; Markovic and Dutzler, 2007)—more homologous to CLC-1 than to CLC-5—do not bind ATP. Additionally, residues that had been implicated by functional and modeling work in ATP coordination in CLC-1 (Bennetts et al., 2005) appear to be on the opposite side of the ATP binding cleft of CLC-5, which in turn is poorly conserved in CLC-1 (Meyer et al., 2007). This could suggest that the CBS domains can accommodate ATP binding in multiple regions possibly by adopting different quaternary organizations. Despite these potential structural differences, adenosine ligands appear to bind to the CBS domains of the CLC proteins in a "head-on" configuration.

These nuggets of mechanistic and structural understanding of CLC-1 function and modulation by ATP, however, clash with the physiological behavior of the skeletal muscle chloride conductance, g_{Cl} (which is mediated by CLC-1), during prolonged muscular activity (Steinmeyer et al., 1991; Pedersen et al., 2004; 2005). Vigorous exercise of the muscle fibers leads to accumulation of extracellular K^+ and decreased excitability. This is partly counteracted by acidosis, which results in a reduction in g_{Cl} (Pedersen et al., 2004, 2005). This inhibition of g_{Cl} however was in direct contrast with the well-documented activation of heterologously expressed CLC-1 by acidic pH (Rychkov et al., 1996; Accardi and Pusch, 2000). Furthermore, even during prolonged stress, the total concentration of the adenosine ligands remains more or less constant, raising the question of the physiological role of the nonspecific inhibition of CLC-1 by ATP and its derivatives. Two recent papers have directly addressed this conundrum and unveiled an unexpected interplay between ATP and pH in regulating CLC-1 gating (Bennetts et al., 2007; Tseng et al., 2007). Both groups showed that the ATP-induced shift in the $V_{1/2}$ of CLC-1's common gate is greatly enhanced at acidic pHs. This result nicely ties together the above-described loose ends by offering us a simple and cohesive picture between the physiological effects on the skeletal muscle fibers and the biophysical properties of CLC-1.

In this issue of the *Journal of General Physiology* Zifarelli and Pusch (see p. 109) completely turn the table around by making a strong case that ATP does not modulate CLC-1 function, at least not directly! They overexpress CLC-1 in oocytes, excise inside-out patches, apply intracellular ATP, and see no inhibition, regardless of pH. They perform a thorough set of controls by changing the experimental solutions, the temperature at which the experiments are performed, and go as far as showing that their batch of ATP can stimulate CFTR. This constitutes an apparently insoluble conundrum: how can the same straightforward experiment (perfusing ATP on a patch excised from an oocyte) performed on the same channel (CLC-1) yield opposite and irreconcilable results? The only explanation is that, despite their intentions and extensive controls, the two groups did not actually perform the same experiment. Perhaps a hidden, uncontrolled, and so far silent modulator has been lurking in the dark all along during our investigations on CLC-1 and revealed itself only under these fortuitous circumstances. Where could this stowaway be hiding? At the moment we do not know, and more work is needed to identify this component and solve the riddle.

Other than the macroscopic difference in ATP modulation, are there other hints that something might be amiss? At a cursory inspection the currents shown in both papers Tseng et al. (2007) and Zifarelli and Pusch (2007) appear *kosher*: the pronounced instantaneous inward rectification, the crossover at negative voltages, and the deactivation kinetics are all hallmarks of well-behaved CLC-1 currents and in good qualitative agreement between the two studies. However, a closer look brings out some significant quantitative differences, which could bespeak of differences inherent in the two systems. The main difference lies in the half activation potential, $V_{1/2}$, measured in standard conditions (neutral pH and no ATP): Zifarelli and Pusch find a $V_{1/2}$ of ~ -90 mV while Tseng and colleagues report a $V_{1/2}$ of ~ -40 mV, a right shift of $\sim$50 mV. This difference is nontrivial and hard to interpret; looking back at the previously published literature there are several reports where the $V_{1/2}$ of CLC-1 varied between one extreme (Steinmeyer et al., 1991; Accardi and Pusch, 2000) and the other (Bennetts et al., 2001; Duffield et al., 2003). In these cases, however, the differences could be reconciled because the experimental conditions differed in the $[Cl^-]$ of the solutions (100 vs. 40 mM $[Cl^-]_{in}$), the expression system (oocytes vs. HEK293 cells), and technique (inside out patches vs. whole cell recordings). These justifications, however, fail to account for the difference seen here because the solutions, expression system, and technique are one and the same in all cases. The two papers disagree also on the minimal open probability reached by CLC-1 at negative voltages; the currents deactivate much more completely in the hands of Tseng et al. than in those of Zifarelli and Pusch, as evidenced by the more pronounced crossover (compare Fig. 1 B in Tseng et al. and Fig. 3 A in Zifarelli and Pusch, respectively). This difference appears to be mostly due to a change in the residual open probability of the common gate at very negative voltages, P_C^{min}, rather than an effect on the fast gate, P_C^{min}(Tseng) $\sim$0.3, whereas P_C^{min}(Zifarelli) $\sim$0.55. A third important difference between the two reports lies in the modulation of the common gate by pH. Although both groups show that CLC-1 is activated by acidic pH, Tseng et al. observe only a small effect in P_C^{min} (from $\sim$0.3 to $\sim$0.4), while Zifarelli and Pusch report a much more pronounced increase (from $\sim$0.55 to $\sim$0.8). Overall, these are not huge differences and under normal conditions would not trouble

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the sleep of the investigators. However, under the present circumstances they might be harbingers of an essential regulator of CLC-1 function that has so far escaped our detection and that is vital for the channel's sensitivity to ATP. These measurements were performed eschewing the complexities of a full cell in favor of the minimal system of an excised membrane patch so the possible culprits are limited: the solutions, the clones, the details of expression (oocyte incubation, growth solution, and skinning). The solutions are nominally identical (at least in some experiments) as are the clones (Zifarelli and Pusch go as far as fully sequencing their clone to rule out spurious mutations), and the oocyte expression protocols are standardized. Thus we are left with unlikely possibilities. For example, that trace amount of an unknown CLC-1 inhibitor might have contaminated some of the chemicals. The qualitative reproducibility of the inhibition in the hands of different groups (Bennetts et al., 2005, 2007; Tseng et al., 2007) and over the span of several years (2005–2007) would suggest otherwise. Another possibility is that small differences in the incubation and maintenance of the oocytes could directly (or indirectly) influence the channel's state through phosphorylation or ubiquitination, which in turn might influence its sensitivity to ATP.

Another hint that hidden actors might be playing a key role in this mystery comes from the comparison of the ATP dependence of CLC-1 in inside out patches from oocytes and whole cell recordings in HEK293 cells. Although the results from Bennetts et al. (2005, 2007) and Tseng et al. (2007) are in qualitative agreement, there are significant differences in the quantitative modulation of $V_{1/2}$ and P_C^{min}. At neutral pH in HEK293 cells, 3 mM ATP shifts $V_{1/2}$ by >40 mV and drastically reduces P_C^{min}, whereas in oocytes the shift is <10 mV and P_C^{min} seems to be virtually unaffected (at least at 1 mM ATP). Furthermore, by choosing the whole cell configuration Bennetts and colleagues (2005, 2007) cannot study the effect of a direct addition of ATP to the system but have to compare different cells, opening the door to the possibility that ATP might activate cellular pathways that in turn regulate CLC-1.

The resolution of this "perfect murder" will likely require a concerted effort from all the laboratories involved, possibly resulting in an exchange of clones, materials, and personnel among them. Ultimately, a brave investigator will have to address this question directly in the skeletal muscle fibers, where CLC-1 can be studied in its original physiological context and with its modulatory partners in place. Regardless of the final outcome on the specific question, does ATP directly modulate this channel or not, these studies hint at layers of yet undiscovered regulatory mechanisms of CLC-1, which will ultimately augment our understanding of the mechanism of function of this essential component of our muscular machinery and its involvement in contraction, exercise, and fatigue.

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