The Muscle Chloride Channel ClC-1 Is Not Directly Regulated by Intracellular ATP

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ClC-1 belongs to the gene family of CLC Cl⁻ channels and Cl⁻/H⁺ antiporters. It is the major skeletal muscle chloride channel and is mutated in dominant and recessive myotonia. In addition to the membrane-embedded part, all mammalian CLC proteins possess a large cytoplasmic C-terminal domain that bears two so-called CBS (from cystathionine-β-synthase) domains. Several studies indicate that these domains might be involved in nucleotide binding and regulation. In particular, Bennetts et al. (J. Biol. Chem. 2005. 280:32452–32458) reported that the voltage dependence of hClC-1 expressed in HEK cells is regulated by intracellular ATP and other nucleotides. Moreover, very recently, Bennetts et al. (J. Biol. Chem. 2007. 282:32780–32791) and Tseng et al. (J. Gen. Physiol. 2007. 130:217–221) reported that the ATP effect was enhanced by intracellular acidification. Here, we show that in striking contrast to these findings, human ClC-1, expressed in Xenopus oocytes and studied with the inside-out configuration of the patch-clamp technique, is completely insensitive to intracellular ATP at concentrations up to 10 mM, at neutral pH (pH 7.3) as well as at slightly acidic pH (pH 6.2). These results have implications for a general understanding of nucleotide regulation of CLC proteins and for the physiological role of ClC-1 in muscle excitation.

INTRODUCTION

ClC-1 is the major skeletal muscle chloride channel (Steinmeyer et al., 1991a,b). Its activity accounts for the large chloride conductance of the sarcolemma at rest that is necessary to stabilize the membrane potential. Mutations in the CLCN1 gene lead to dominant and recessive myotonia (Koch et al., 1992; George et al., 1993; Steinmeyer et al., 1994; Pusch, 2002). The channel is a member of the gene family of CLC Cl⁻ channels and Cl⁻/H⁺ antiporters (Zifarelli and Pusch, 2007), which are homodimeric proteins with independent ion permeation pathways in each subunit (Dutzler et al., 2002). In addition to the membrane-embedded part, all mammalian CLC proteins possess a large cytoplasmic C-terminal domain that bears two so-called CBS (from cystathionine-β-synthase) domains (Ponting, 1997). The precise role of the CBS domains is still unclear, but several recent reports indicate that CBS domains may be involved in the regulation of protein function by intracellular nucleotides like ATP, ADP, and AMP (Scott et al., 2004; Bennetts et al., 2005; Wellhauser et al., 2006; Meyer et al., 2007). Scott et al. (2004) showed that isolated CBS domains from CLC proteins are able to bind nucleotides. Similarly, Wellhauser et al. (2006) reported low affinity binding of ATP at the C terminus of ClC-5 that could be competed by 1 μM AMP. Moreover, nucleotides were found to be bound in the crystal structure of the C-terminal fragment of ClC-5 (Meyer et al., 2007). In agreement with these biochemical results, Bennetts et al. (2005) reported that the activity of ClC-1 is regulated by intracellular ATP and other nucleotides. They found that the presence of 5 mM ATP shifted the open probability (P_open) of the slow gate by ~50 mV toward positive potentials. Moreover, very recently Bennetts et al. (2007) and Tseng et al. (2007) reported that the effect of ATP was greatly enhanced by acidification of the intracellular solution.

The studies of Bennetts et al. (2005, 2007) were performed on human ClC-1 expressed in cultured cells and measured using the whole-cell configuration of the patch clamp technique. However, this method has several drawbacks. First, the effects of different intracellular solutions and their washout are not tested on the same cell. Rather, averages of different cells are compared. Second, indirect effects of ATP in the complex cellular environment cannot be excluded. Third, if large currents are measured, as is the case for ClC-1, the whole cell configuration allows only a relatively poor control of the ion concentrations close to the plasma membrane. For example, a reduction of the intracellular chloride concentration close to the plasma membrane may decrease inward currents after long hyperpolarizing pulses because of a reduced driving force for chloride. To find out if ATP has a direct effect on ClC-1 and if acidification may eventually modulate such an effect, we tested the nucleotide on inside-out patches. This method avoids the above mentioned problems. To our surprise, we could not detect any significant effect of ATP on ClC-1 activity even at acidic pH. During the elaboration of this manuscript, Tseng et al. (2007), in a...
study performed in inside-out oocytes patches, reported, in rough agreement with the findings of Bennetts et al. (2007), that intracellular ATP modulates CIC-1 activity and that intracellular acidification increases this effect. The origin of the discrepancy between the results reported by Tseng et al. (2007) and our results is unclear.

METHODS

WT hClC-1 (Koch et al., 1992) was expressed in Xenopus oocytes and currents were measured at 18°C (some control experiments were performed at 26°C to ensure that the temperature did not influence the results), 2–4 d after injection using the inside-out configuration of the patch clamp technique (Hamill et al., 1981) with an EPC-7 (List) amplifier and a custom acquisition program (GePulse, Visual C ++, Microsoft). Data analysis was performed using custom software (written in Visual C ++, Microsoft) and the Origin program (OriginLab Corporation).

The control internal solution contained (in mM) 100 NMDG-Cl, 2 MgCl₂, 10 HEPES, 2 EGTA, pH 7.3. The extracellular (pipette) solution contained (in mM) 100 NMDG-Cl, 5 MgCl₂, 10 HEPES, pH 7.3. Some control experiments were conducted with solutions identical to those used by Tseng et al. (2007) (120 mM NMDG-Cl, 1 MgCl₂, 10 HEPES, 1 EGTA, pH 7.4 or 6.2). In these experiments the pH 7.4 solution was also used as the extracellular (pipette) solution. Solutions containing ATP (adenosine 5' triphosphate bis(tris) salt dehydrate, Sigma-Aldrich) were prepared diluting the ATP solution and the efficiency of the perfusion system were evaluated on the ΔR-CFTR construct that was reconstituted from the injection of two separate RNAs encoding the N-terminal and C-terminal half of the protein, respectively. The gating of this channel does not require phosphorylation and depends only on ATP (Bompadre et al., 2005). The ΔR-CFTR was injected in the same batch of oocytes and measured before ClC-1.

The voltage protocol used to study CFTR-mediated currents consists of voltage steps ranging from −80 to 80 mV in 20-mV increments.

To study hClC-1-mediated currents we used a tail protocol consisting of a 100-ms test pulse to a variable potential ranging from 100 to −160 mV followed by a repolarization to −140 mV for 100 ms. To study in isolation slow gating transitions the test pulses were prolonged to 200 ms, and a 200-μs pulse to 160 mV, to fully activate the fast gating, was applied before the −140-mV repolarization. For measurements at pH 6.2 the length of the test pulse was prolonged to 300 ms as in Tseng et al. (2007). In some recordings, a 200-μs prepulse to 80 mV was applied before the test pulse (e.g., Fig. 2). The presence of this prepulse did not alter the gating parameters derived from the Boltzmann fits. The open probability was derived from tail currents fitting the latter with a monoexponential function and extrapolating the current values to the beginning of the tail pulse.

Open probabilities were derived from the tail currents by fitting a modified Boltzmann function (Pusch et al., 1995):

\[ I(V) = I_{\text{max}}(P_{\text{min}} + (1 - P_{\text{min}})/(1 + \exp((V_{1/2} - V)/k))) \]

where \( I_{\text{max}} \) is the (fitted) maximal current, \( P_{\text{min}} \) the residual open probability at negative voltages, \( V_{1/2} \) is the midpoint voltage of the open probability, and \( k \) the slope factor of the voltage dependence of the open probability.

To estimate the apparent \( K_d \) for ATP activation of the ΔR-CFTR channel, we perfused inside-out patches with different concentrations of ATP and measured the current at 80 mV. Current measured in the absence of ATP was subtracted. Since not all patches allowed the application of many concentrations and because most patches showed a slow rundown over time we bracketed all measurements by recordings with 1 mM ATP and normalized the response to the current measured with 1 mM ATP. Assuming that, for a single ideal patch without rundown, the ATP dependence can be described by

\[ I(c) = \frac{(c/K_d)^n}{1 + (c/K_d)^n} \]

where \( c \) is the concentration of ATP, \( K_d \) is the apparent dissociation constant, and \( n \) is the Hill coefficient, the normalized response, \( I_{\text{norm}} \), is given by

\[ I_{\text{norm}} = \frac{I(c)}{I(1 \text{ mM})} = \frac{(c/K_d)^n}{1 + (c/K_d)^n} \frac{1 + (1 \text{ mM}/K_d)^n}{(1 \text{ mM}/K_d)^n} \]

This equation was used to fit the data shown in Fig. 1. Data are presented as mean ± SEM except where noted.

Online Supplemental Material

The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200709899/DC1. We provide supplemental online information on the application of intracellular ATP on CIC-1 at 26°C (Figs. S1 and S2), demonstrating the lack of a significant effect also at this elevated temperature. Furthermore, we show the result of sequencing the hClC-1 insert in the expression vector with four primers (Fig. S3).

RESULTS

In this work we wanted to study the effect of intracellular ATP on hClC-1 expressed in Xenopus oocytes using inside-out patches. To test whether our perfusion system and the ATP containing solutions were appropriate for this task, we used as a positive control a CFTR construct lacking the regulatory domain (ΔR-CFTR) (Bompadre et al., 2005). In this construct, activation of the Cl⁻ conductance depends only on the presence of ATP on the cytoplasmic side and does not require activation by protein kinase. We expressed ΔR-CFTR in Xenopus oocytes and performed inside-out patch experiments, perfusing with control solution and with solutions containing 0.01 (\( n = 11 \)), 0.1 (\( n = 14 \)), 1 mM (\( n = 25 \)), and 5 mM ATP (\( n = 17 \)).

In the absence of ATP, we observed only very small background currents (Fig. 1 A, top left). However, perfusion with solutions containing increasing concentrations of ATP (Fig. 1 A) progressively activated larger Cl⁻ currents, with 1 mM being already a saturating concentration. Switching back to the control solution, devoid of ATP, currents returned to background levels (not depicted).

In particular, to make sure that the ATP was prepared and delivered at the appropriate concentrations, we performed a dose–response curve for ATP activation of
we adopted the voltage protocol devised by Accardi and Pusch (Accardi et al., 2001) (see Materials and methods). Also in this case, we did not observe a significant difference between currents recorded in the presence and in the absence of ATP (traces not shown). In fact, the voltage dependence of the open probability of the slow gate is virtually unaffected by the presence of 10 mM ATP as shown for a representative patch (Fig. 2C).

Fig. 3 displays the statistical evaluation of the parameters \( V_{1/2} \) and \( P_{\text{min}} \) derived as described for the representative measurements in Fig. 2. The mean values of \( V_{1/2} \) and \( P_{\text{min}} \) are not significantly changed by the presence of ATP when the overall gating of ClC-1 is considered (Fig. 3, A and B). The same conclusion holds when only the slow gating mechanism is taken into account (C and D).

Bennetts et al. (2007) and Tseng et al. (2007) recently suggested that the ATP effect was greatly enhanced by decreasing pH int. In particular at pH 6.2, ATP was reported to dramatically shift the open probability–voltage curve by >100 mV to more positive voltages, with 1 mM ATP being sufficient to reach a maximal effect. To test whether acidification of the intracellular solution could unmask an effect of ATP that was not evident at the neutral pH used in the previous experiments, we decreased the pH int from 7.3 to 6.2 and assessed the effect of 1 mM ATP. Fig. 4 shows representative current traces obtained at pH 7.3 (Fig. 4A), pH 6.2 (Fig. 4B), and pH 6.2 in the presence of 1 mM ATP (Fig. 4C). In agreement with previous
observations (Rychkov et al., 1996; Accardi and Pusch, 2000), switching from pH 7.3 to 6.2 slows down the deactivation kinetics and increases the steady-state value of the currents at negative potentials. Surprisingly, addition of 1 mM ATP had no detectable further effect on the kinetics and voltage dependence of the currents. The changes produced by acidification are quickly reversed upon wash with solution at pH 7.3 (Fig. 4 D). The voltage dependence of the $P_{\text{open}}$ derived for the representative patch shown in Fig. 4 (A–D) is practically unaffected by the presence of 1 mM ATP (Fig. 4 E). This is confirmed by the statistical evaluation of the $V_{1/2}$ and $P_{\text{min}}$ of the Boltzmann distribution describing the $P_{\text{open}}$ (Fig. 5, A and B), where average values of these parameters are shown.

The lack of any effect of ATP at acidic pH was confirmed by isolating the voltage dependence of the slow gate as already described for pH 7.3 (Fig. 4 F) and by the statistical evaluation of the $V_{1/2}$ and $P_{\text{min}}$ of the Boltzmann distribution (Fig. 5, C and D).

In the experiments described above we had used Tris-ATP and have added MgSO$_4$ at the same concentration, whereas Tseng et al. (2007) and Bennetts et al. (2007) had used MgATP. To test whether the discrepancies between our results and theirs could be due to this different source of ATP, we performed additional experiments with MgATP. Also with MgATP, $\Delta R$-CFTR could be reliably activated and no effect on CIC-1 currents could be detected (unpublished data), showing that the source of the nucleotide has no influence on our conclusions.

We also performed some experiments at 26°C to assess whether higher temperature could uncover an effect of ATP. In these experiments we employed recording solutions identical to those of Tseng et al. (see Materials and methods). At pH 7.4, the voltage dependence of opening is slightly shifted by $30 \text{ mV}$ to more positive voltages, compared with the recordings at 18 °C, pH 7.3 (see Figs. S1 and S2, available at http://www.jgp.org/cgi/content/full/jgp.200709899/DC1). This shift is probably caused by the combined effect of the higher temperature (Bennetts et al., 2001) and the slightly more basic pH. Interestingly, at pH 6.2 almost no effect of temperature on the gating parameters could be observed (Fig. S2). Most importantly, however, we could not observe any significant effect of 1 mM ATP on current magnitude or voltage dependence of gating at pH 7.4 or pH 6.2 (see Figs. S1 and S2).

**DISCUSSION**

In skeletal muscle, Cl$^{-}$ conductance ($g_{\text{Cl}}$) accounts for $\sim80\%$ of the overall membrane conductance at rest (Aromataris and Rychkov, 2006) and CIC-1 is the main determinant of this conductance (Lueck et al., 2007). The Cl$^{-}$ conductance is required to keep an appropriate resting potential and to repolarize the voltage following an action potential (Aromataris and Rychkov, 2006). This critical role is underscored by the fact that mutations in the gene coding for CIC-1 (Pusch, 2002) or aberrant splicing of CIC-1 (Charlet et al., 2002; Mankodi et al., 2002) produce muscle hyperexcitability. Several recent reports indicate that intracellular CBS domains may be involved in the regulation of CLC proteins by intracellular nucleotides like ATP, ADP, and AMP (Scott et al., 2004;
In stark contrast to these conclusions, we have demonstrated here that direct application of ATP to the intracellular side of excised inside-out patches does not exert a significant effect on the current amplitude or on the voltage dependence of the open probability of hClC-1 expressed in *Xenopus* oocytes at neither neutral nor slightly acidic pH (7.3 and 6.2). In particular, we were not able to detect any significant shift in the mid-point of the voltage dependence of the open probability, or on the residual open probability at negative voltages for both fast and slow gate. We observed practically no effect for concentrations of ATP up to 10 mM, even after perfusion for up to 5 min. Moreover, in contrast with Bennetts et al. (2007) and in agreement with previous studies (Rychkov et al., 1996; Accardi and Pusch, 2000), we find that acidic pH has at most a small effect on the $V_{1/2}$ of the $P_{open}$, and that inward currents are drastically increased at low pH.

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As already mentioned, Bennetts et al. (2007) reported a slight shift toward positive potentials of the P_{open} at acidic pH even in the absence of ATP. The estimated pK for the effect of protons was found to be compatible with the pK of histidine residues. On the basis of this observation and of a structural model, they suggested that a histidine residue (H847) is important to mediate the effect of pH-dependent ATP modulation. Residue H847 was suggested to interact electrostatically with the phosphate of ATP, however the mutational analysis of Bennetts et al. failed to support this hypothesis as changing this residue to either A or R similarly affected ATP dependence. Finally, the mutant L848A that was reported in the first work of Bennetts et al. (2005) to have the largest impact on ATP effect was not investigated in their latest work, nor in the work of Tseng et al. (2007).

The striking contrast with the results of Bennetts et al. (2007) and Tseng et al. (2007) prompted us to thoroughly control the experimental condition used in our study. We proved that we efficiently apply active ATP because we could robustly and reversibly activate currents mediated by ΔR-CFTR, a channel that is gated by intracellular ATP (Bompadre et al., 2005). Moreover, we quantitatively controlled the concentration of ATP delivered to the patches by performing a dose–response curve of the ATP activation of ΔR-CFTR, obtaining a K_{d} in agreement with published results (Bompadre et al., 2005).

To exclude possible sequence variations of the cDNA construct we fully sequenced the open reading frame of our hClC-1 clone and found it to be in complete agreement with the published hClC-1 cDNA (see Fig. S3, available at http://www.jgp.org/cgi/content/full/jgp.200709899/DC1).

To exclude any influence of the type of ATP salt used, we tested both Tris salt and Mg^{2+} salt without observing any difference. Moreover, we used pulse protocols of variable length (see Materials and methods) to ensure that the current reached a steady state during our experiments.

Finally, we proved that temperature in the range between 18 and 26°C did not influence the lack of effect of ATP on ClC-1–induced currents. Hence, we conclude that intracellular ATP does not directly affect the function of ClC-1 at both neutral and acidic pH.

The conflicting conclusions of Bennetts et al. (2005, 2007) might result from the different electrophysiological methods used and/or the different expression system adopted. In this respect, it is important to note that the whole-cell configuration used by Bennetts et al. presents some problematic aspects for the type of study that we are considering. Most importantly, in the whole-cell configuration it is normally not possible to change the internal solution, implying that the comparison between different ATP concentrations cannot be performed directly on the same cell. Furthermore, the interior of the cell, even after internal access has been established by the patch pipette, remains probably a complex and biochemically active milieu in which ATP can potentially activate signaling cascades that may indirectly modulate channel function. For example, ClC-1 activity could be regulated by protein kinases (Chen and Jockusch, 1999; Rosenbohm et al., 1999).

To assess whether the difference in the apparent ATP sensitivity of ClC-1, is caused by the different expression systems, we attempted to measure ClC-1 currents from inside-out patches of transfected HEK cells. Unfortunately, as also reported by Bennetts et al. (2007), we were not able to obtain a sufficient expression level to allow reliable measurements.

It is more difficult to explain the difference with the study of Tseng et al. (2007) in which the same expression system (Xenopus oocytes) and excised inside-out patch clamp recordings were used. We are not able to provide any reasonable explanation for the discrepancy between our study and the one of Tseng et al. Independent studies from other research groups should help to clarify this important issue.

Our results have implications for the interpretation of recent results from x-ray crystallography of CLC proteins and for the physiological role of ClC-1. In accordance with the lack of functional effects on ClC-1, Meyer and Dutzler (2006) could not detect ATP binding to the cytoplasmic domain of the highly homologous ClC-0 channel, and no nucleotides could be detected in the crystal structure of ClC-0.
the C terminus of the ClC-Ka channel (Markovic and Dutzler, 2007). Interestingly however, the same group found that nucleotides bind with 100 μM affinity to the CBS domains of C terminus of the Cl / H + antiporter ClC-5 (Meyer et al., 2007), in agreement with biochemical data on isolated C-terminal protein fragments (Scott et al., 2004; Wellhauser et al., 2006). Of course our results do not exclude that nucleotides can bind to the C terminus of ClC-1. However, the lack of direct functional alteration of the channel by ATP shows that if nucleotides have any effect in the physiological regulation of the skeletal muscle chloride conductance, it is of indirect nature.

Regarding the physiological role of ClC-1, an important finding is that macroscopic $g_{Cl}$ is reduced by intracellular acidification (Pedersen et al., 2004, 2005), an effect that, in principle, could be mediated by a direct pH dependence of ClC-1. However, previous reports (Rychkov et al., 1996; Accardi and Pusch, 2000) have shown that low pH activates rather than inhibits ClC-1, a finding that we plainly reproduce (see current traces in Fig. 4), suggesting an indirect mechanism of acidosis-induced reduction of $g_{Cl}$. In contrast to these findings, from apparent shifts of activation curves, Bennetts et al. (2007) deduced that ClC-1 currents are inhibited by low pH, and speculated that acidosis directly inhibits ClC-1 and leads to hyperexcitability. Moreover, they speculated that their finding of increased effect of ATP on ClC-1 upon intracellular acidification (Bennetts et al., 2007) would explain the so far elusive link between acidification, decrease in $g_{Cl}$, and recovery of muscle excitability (Nielsen et al., 2001; Pedersen et al., 2004; Pedersen et al., 2005) because acidification, potentiating the effect of ATP, which is to shift the $P_{open}$ toward positive potentials, would greatly decrease $g_{Cl}$ facilitating muscle excitation (Bennetts et al., 2007). Our measurements do not support any of these speculations and point to an indirect role of ClC-1 and a more complicated mechanism of regulation of $g_{Cl}$ that can probably be thoroughly understood only in the context of active skeletal muscle tissues.

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