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ATP Inhibition of CLC-1 Is Controlled by Oxidation and Reduction

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The effect of intracellular adenosine triphosphate (ATP) on the “common gating” of the CLC-1 chloride channel has been studied by several laboratories with controversial results. Our previous study on the channel expressed in Xenopus oocytes using excised inside-out patch-clamp methods showed a robust effect of ATP in shifting the open probability curve of the common gate toward more depolarizing voltages (Tseng, P.Y., B. Bennetts, and T.Y. Chen. 2007. J. Gen. Physiol. 130:217–221). The results were consistent with those from studying the channel expressed in mammalian cells using whole cell recording methods (Bennetts, B., M.W. Parker, and B.A. Cromer. 2007. J. Biol. Chem. 282:32780–32791). However, a recent study using excised-patch recording methods for channels expressed in Xenopus oocytes reported that ATP had no direct effect on CLC-1 (Zifarelli, G., and M. Pusch. 2008. J. Gen. Physiol. 131:109–116). Here, we report that oxidation of CLC-1 may be the culprit underlying the controversy. When patches were excised from mammalian cells, the sensitivity to ATP was lost quickly—within 2–5 min. This loss of ATP sensitivity could be prevented or reversed by reducing agents. On the other hand, CLC-1 expressed in Xenopus oocytes lost the ATP sensitivity when patches were treated with oxidizing reagents. These results suggest a novel view in muscle physiology that the mechanisms controlling muscle fatigability may include the oxidation of CLC-1.

INTRODUCTION

CLC-1, a chloride (Cl\(^-\)) channel contributing a dominating conductance to the skeletal muscle membrane, is important for the control of muscle excitability. We have recently demonstrated, using excised inside-out patch recording methods, that cytoplasmic ATP can reversibly inhibit the current of CLC-1 (Tseng et al., 2007). This ATP inhibition of CLC-1 is enhanced by acidic intracellular pH, and the effect appears to come from modulating the CLC-1 “common gating” that controls the opening of both protopenes of the channel. The inhibition is robust—ATP can shift the common gate opening curve (\(P_o\cdot V\) curve) toward more depolarized voltages by \(>100\) mV at a pH of 6.2. This mechanism was thought to play an important role in muscle fatigue; the inhibition of CLC-1 reduces the electrical shunting current on the surface membrane of skeletal muscles so that the partially inactivated Na\(^+\) channels are still able to initiate action potentials (Pedersen et al., 2004, 2005).

Although our results obtained from CLC-1 expressed in Xenopus oocytes were consistent with those obtained from the channel expressed in mammalian cells (Bennetts et al., 2005, 2007), a recent paper reported that intracellular ATP was unable to inhibit CLC-1 (Zifarelli and Pusch, 2008). The latter study, like ours, was performed on CLC-1 expressed in Xenopus oocytes using excised inside-out patch recording methods, so the discrepancy was surprising given that the same channel expression system and similar experimental techniques were used. To clarify the ATP inhibition mechanism, we have reexamined the ATP effect on CLC-1 in two channel expression systems. We found that the inhibition of CLC-1 common gating by the cytoplasmic ATP/H\(^+\) can be controlled by oxidation and reduction (redox). We suggest that the variation of the redox state of the channel may be the culprit underlying the discrepancy among the various studies. Oxidation has been known to affect the contraction of skeletal muscles (Lamb and Posterino, 2003; Smith and Reid, 2006; Ferreira and Reid, 2007; Peake et al., 2007). Oxidation of CLC-1 may thus be one of the mechanisms that control the muscle fatigability.

MATERIALS AND METHODS

Electrophysiological recordings were conducted using the Axopatch 200B amplifier and the Digidata 1320 A/D board controlled by pClamp8 software (MDS Analytical Technologies). Two channel expression systems were used, Xenopus oocytes and tsA201 cells. The human CLC-1 Cl\(^-\) channel inserted in the pTLN vector was provided by T.J. Jentsch (Leibniz-Institut für Molekulare Pharmakologie (FMP) and Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin, Germany) (Steinmeyer et al., 1991). The mRNA used to inject the oocytes was synthesized directly from this construct using SP6 mMessage mMachine kit (Ambion). The procedures for harvesting and injecting Xenopus oocytes were described previously (Chen, 1998; Li et al., 2005). Normally, the...
analyses and presentations were performed using the combination of Instruments) as described previously (Zhang et al., 2006). Data change was achieved by using the SF-77 solution exchanger (Warner et al., 2003). The experiments in tsA201 cells (see Figs. 3, 4, and 6 A), ATP was repeatedly applied for 10 s every min. For experiments on the common gate of CLC-1 (Bennetts et al., 2005). To compare our excised-patch experiments with those of whole cell recordings from mammalian cells, we tested the effects of various nucleotides/nucleosides in shifting the common gate $P_{o,c}$-V curve in acidic conditions. Fig. 1 A shows the common gate $P_{o,c}$-V curves of the wild-type CLC-1 in control (black symbols) and in the presence of various intracellular nucleotides/nucleosides.

The experiments were performed on the channels expressed in Xenopus oocytes using excised inside-out patch recording methods. It can be seen that ADP and AMP are at least as effective as ATP in shifting the $P_{o,c}$-V curve of CLC-1. On the other hand, cAMP (Fig. 1 A, left) and adenine (Fig. 1 A, right) have almost no effect, whereas adenosine and GTP are less effective than ATP. Two point mutations of CLC-1, H847A and L848A, have been reported to suppress the ATP inhibition of CLC-1 expressed in HEK293 cells. As shown in Fig. 1 (B and C), these two mutations indeed also reduced the extent of the shift of the $P_{o,c}$-V curve when the mutants were expressed in Xenopus oocytes, although the L848A mutation did not completely abolish the ATP effect as shown in Bennetts et al. (2005).

To further examine the ATP effect on the CLC-1 common gating, we examined channels in tsA201 cells using excised inside-out patch recording methods. To our surprise, 3 mM ATP had no effect on the steady-state current of CLC-1 at a pH of 6.8 (Fig. 2 A), as can also be seen from the nearly overlapped $P_{o,c}$-V curves obtained in the absence and presence of 3 mM ATP (Fig. 2 B). Because a robust ATP effect on CLC-1 was observed in whole cell recordings of mammalian cells (Bennetts et al., 2007), we hypothesized that the lack of the ATP effect in our excised-patch experiments might result from a loss of a cytosolic factor during the recording process. The experiment shown in Fig. 3 A, which was performed at a pH of 6.8, indeed supports this speculation; the ATP inhibition could be observed immediately after the patch excision, but the inhibition was reduced, or even disappeared, after several minutes. The time constant of the decay of the ATP effect was ∼2–3 min once the ATP sensitivity started decreasing ($n = 12$; Fig. 3 A, inset).
We suspected that the critical factor that maintained the ATP sensitivity of CLC-1 was the reducing power in cytoplasm, and the CLC-1 channel may have undergone oxidation upon the excision of the membrane patch into the regular bath solution. We thus excised the membrane patch into the same bath solution containing 100 μM β-ME. Fig. 3 B shows that such a maneuver can maintain the robust ATP sensitivity over the entire recording course of 20 min. Performing the experiment using 50 μM DTT also maintained the ATP sensitivity (not depicted). Thus, it appears that redox plays a significant role in controlling the ATP regulation of the CLC-1 common gating. We suspect that the target of the redox modulation is the free thiol group of cysteine because the ATP sensitivity of CLC-1 can also be maintained if the excised patch is exposed to methionine thiosulfonate (MTS) reagents immediately after the patch excision (Fig. 3 C). Likewise, studying the steady-state current in the presence of reducing reagents reveals that ATP indeed can shift the $P_{o,c}$-$V$ curve of the CLC-1 channel expressed in tsA201 cells (Fig. 3 D).

As the intracellular pH in skeletal muscles can vary significantly (Allen et al., 2008), we examined if the oxidation control of the ATP effect could occur at various pH$_i$’s. Fig. 4 shows similar experiments of monitoring the ATP sensitivity of CLC-1 at pH$_i$’s of 7.4 (Fig. 4 A) and 6.2 (Fig. 4 B). Under both pH conditions, if the patch was excised directly into the regular bath solution, the ATP sensitivity of CLC-1 was lost in several minutes. However, if the bath solution included small amounts of reducing reagents, the ATP sensitivity of CLC-1 was maintained for a long time.

Our excised patch experiments in CLC-1 channels expressed in mammalian cells thus reproduced the results obtained from whole cell recording experiments, when the channels were controlled in the reduced condition. Because we consistently observed a robust effect of ATP in our experiments performed in membrane patches excised from *Xenopus* oocytes (Fig. 5 A), the channels...
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before and after the CuPhe treatment indeed shows that the ATP-induced shift of the \( P_o \cdot V \) curve is greatly suppressed after the patch is treated with CuPhe (Fig. 5C). These results from the channels expressed in *Xenopus* oocytes again support the hypothesis that the ATP inhibition of the CLC-1 common gating is controlled by redox.

If oxidation of CLC-1 rendered the channel insensitive to ATP inhibition, and if the excised membrane from *Xenopus* oocytes were likely in the reduced condition. We therefore tested whether oxidizing reagents could suppress the ATP effect in channels expressed in *Xenopus* oocytes. The experiment in Fig. 5B shows that although ATP initially exerts a robust inhibition on the CLC-1 current, the inhibition is greatly reduced after treating the patch with 10 \( \mu M \) CuPhe for 2 min. Treating the patch with \( H_2O_2 \) can also reduce the ATP effect (not depicted). Comparing the \( P_o \cdot V \) curves of CLC-1 before and after the CuPhe treatment indeed shows that the ATP-induced shift of the \( P_o \cdot V \) curve is greatly suppressed after the patch is treated with CuPhe (Fig. 5C). These results from the channels expressed in *Xenopus* oocytes again support the hypothesis that the ATP inhibition of the CLC-1 common gating is controlled by redox.

If oxidation of CLC-1 rendered the channel insensitive to ATP inhibition, and if the excised membrane...
patch exposed the channels to oxidants in the recording solution, why did we consistently observe robust ATP inhibitions of CLC-1 in excised inside-out patches from *Xenopus* oocytes? We suspected that the patch obtained from *Xenopus* oocytes might have retained the reducing factor for quite a long time because the recording electrode had a large tip diameter (7–9 μm), which might have resulted in the inclusion of intracellular materials in the excised patch and prevented a quick washout of the reducing factor. We thus used the small-tip pipettes as those used in the experiments of tsA201 cells (tip diameter of ~1–3 μm), and, indeed, the loss of ATP sensitivity was observed (Fig. 5 D, top). However, if the small patch excised from *Xenopus* oocyte was immediately placed in the bath solution containing reducing reagents, the ATP sensitivity was maintained (Fig. 5 D, bottom). The loss of the ATP sensitivity as a function of time in small patches excised from *Xenopus* oocytes was independently observed in another laboratory (Dutzler, R., personal communication).

To further explore the redox control of the ATP sensitivity, we examined whether the loss of ATP sensitivity can be reversed by reducing reagents. Fig. 6 A shows an experiment in which the patch from tsA201 cells was excised into a solution without oxidation or reducing reagents. After the sensitivity to ATP inhibition was lost, the patch was treated with 10 mM DTT for 1 min. It can be seen that the channel regains ATP sensitivity after treating the channel with reducing reagents. In five experiments, the averaged ATP response before DTT treatment was ~20% of the first ATP response immediately after membrane excision. After a 1-min treatment of 10 mM DTT, the ATP response was recovered to ~80% of the initial ATP response (Fig. 6 A, inset). Similar experiments were performed on large membrane patches excised from *Xenopus* oocytes using small-tip electrodes. The voltages of the test pulse and the tail pulse were −40 and −100 mV, respectively. Notice that the spontaneous deterioration of the ATP sensitivity is much faster in the top panel (no reducing reagents in the bath solution) than that in the bottom panel (100 μM DTT in the bath solution).

**DISCUSSION**

Inhibition of the Cl− conductance on the surface membrane of skeletal muscles has recently been considered to be important in muscle fatigability (Pedersen et al., 2004, 2005). It has been proposed that the inhibition of CLC-1, the major Cl− conductance on the plasma membrane of skeletal muscles, reduces the membrane-shunting conductance, and, therefore, the firing of action potentials is still possible with the partially inactivated Na+ channels (Bennetts et al., 2007; Tseng et al., 2007). The inhibition was thought to result from a direct binding of ATP to CLC-1 because the C terminus of various CLC proteins contains potential ATP-binding sites. However, high-resolution structures of the C-terminal cytoplasmic domains of several vertebrate CLC proteins...
Bennetts et al. (2005) that the ATP modulation of CLC-1 common gating is not related to the energetic state of muscles. Nonetheless, the lack of ATP effect reported by Zifarelli and Pusch (2008) was alarming because their recording methods and channel expression system were similar to those used in our study. To address the discrepancy, we studied channels expressed in tsA201 cells as well as in *Xenopus* oocytes. We found that if the CLC-1 current was challenged with ATP immediately after the patch excision, the ATP effect could be observed in both systems. The ATP sensitivity, however, could reduce or even disappear within several minutes after the patch excision. This occurred in every excised patch from tsA201 cells (Fig. 3A). The ATP effect on the steady-state $P_{o c^{-}}$-$V$ curve was not observed in patches from tsA201 cells (Fig. 2) because we normally spent several minutes to complete one run of protocol A to construct the control $P_{o c^{-}}$-$V$ curve before the patch was tested with ATP. We suspected that the loss of the reducing power in the excised patch might be the underlying mechanism because the redox potential also controls the slow gating of a closely related channel, CLC-0 (Li et al., 2005). Indeed, we found that if the CLC-1 channel was exposed to a solution that contained small amounts of reducing agents (such as $\beta$-ME or DTT), the sensitivity to ATP was maintained (Fig. 3B). Most likely, this was due to the prevention of oxidation of cysteine residues because modifying CLC-1 with MTS could also maintain the ATP sensitivity (Fig. 3C). In contrast, the channel expressed in *Xenopus* oocytes, although sensitive to ATP in regular bath solutions, became less sensitive to ATP (Meyer and Dutzler, 2006; Markovic and Dutzler, 2007; Meyer et al., 2007) so far provide evidence of ATP binding only to the C-terminal domain of CLC-5. Therefore, the lack of an ATP effect on CLC-1 reported by Zifarelli and Pusch (2008) raised a doubt as to whether ATP directly binds to CLC-1. This consideration renders it imperative to clarify the ATP inhibition mechanism.

Although Zifarelli and Pusch (2008) suggested a possibility that the ATP effect on CLC-1 is indirect, there might be another explanation for their negative results. For example, CLC-1 might be subjected to an unidentified channel modulation mechanism varied among different studies. As mentioned above, our previous study (Tseng et al., 2007) and that of Bennetts et al. (2007) reached the same conclusion—that ATP can shift the $P_{o c^{-}}$-$V$ curve of CLC-1, and this effect is enhanced by acidic pH. In addition, we show that the effects of various nucleotides on the CLC-1 common gating and the ATP effect on the H847A and L848A mutants (Fig. 1) are largely consistent with those reported in the experiments using whole cell recordings in mammalian cells (Bennetts et al., 2005). These results make it less likely that a common artifact occurring in our study and in that of Bennetts et al. (2005) rendered CLC-1 sensitive to the ATP inhibition because the experiments were performed in different systems using different recording methods. Furthermore, our functional results are consistent with a previous biochemical study showing that ATP, ADP, and AMP bind to the ATP-binding site of CLC-5 with similar affinities (Meyer et al., 2007). Because ADP and AMP are as effective as ATP in shifting the $P_{o c^{-}}$-$V$ curve of CLC-1, we concur with the idea of Bennetts et al. (2005) that the ATP modulation of CLC-1 common gating is not related to the energetic state of muscles.

Nonetheless, the lack of ATP effect reported by Zifarelli and Pusch (2008) was alarming because their recording methods and channel expression system were similar to those used in our study. To address the discrepancy, we studied channels expressed in tsA201 cells as well as in *Xenopus* oocytes. We found that if the CLC-1 current was challenged with ATP immediately after the patch excision, the ATP effect could be observed in both systems. The ATP sensitivity, however, could reduce or even disappear within several minutes after the patch excision. This occurred in every excised patch from tsA201 cells (Fig. 3A). The ATP effect on the steady-state $P_{o c^{-}}$-$V$ curve was not observed in patches from tsA201 cells (Fig. 2) because we normally spent several minutes to complete one run of protocol A to construct the control $P_{o c^{-}}$-$V$ curve before the patch was tested with ATP. We suspected that the loss of the reducing power in the excised patch might be the underlying mechanism because the redox potential also controls the slow gating of a closely related channel, CLC-0 (Li et al., 2005). Indeed, we found that if the CLC-1 channel was exposed to a solution that contained small amounts of reducing agents (such as $\beta$-ME or DTT), the sensitivity to ATP was maintained (Fig. 3B). Most likely, this was due to the prevention of oxidation of cysteine residues because modifying CLC-1 with MTS could also maintain the ATP sensitivity (Fig. 3C). In contrast, the channel expressed in *Xenopus* oocytes, although sensitive to ATP in regular bath solutions, became less sensitive to ATP.
after the channel was treated with oxidizing reagents (compare Fig. 5 A with Fig. 5 B). It should be noticed that the oocyte membrane is known to have a large excess area from the membrane structures of wrinkles, folds, and ripples (Zhang and Hamill, 2000), and the existence of large unstirred layers on both sides of the membrane has been documented (Hill et al., 2005). Moreover, we have been using recording pipettes with a large tip size for the excised-patch experiments in *Xenopus* oocytes. These large excised patches were frequently found to be associated with intracellular materials, which likely contained reducing molecules. When a small-tip electrode was used, the loss of ATP sensitivity of the channels expressed in oocytes was indeed observed (Fig. 5 D), perhaps because a flatter membrane geometry of the small patch (Zhang and Hamill, 2000) rendered it easier for the cytoplasmic factor to be washed away. When the recording of the small patch of the oocyte membrane was made in the presence of reducing reagents, the ATP sensitivity was maintained for a long time (Fig. 5 D), just like the results from recording the excised patches from tsA201 cells.

The diffusion barrier near the membrane patch may create a larger problem for large molecules than for small hydrophilic molecules to leave or reach the surface of the membrane. In a recording on cyclic nucleotide-gated channels in excised olfactory cilia, for example, a cytosolic factor (later known to be calmodulin) controlling the channel’s affinity to cyclic nucleotides was stably maintained for tens of minutes even though small molecules such as cAMP or Ca\(^{2+}\) ions were repeatedly washed in and out of the cytoplasmic face of the channel (Kramer and Siegelbaum, 1992). In the giant patch experiments presented in this study, even the access of the cytoplasmic face of the channel to ATP is somewhat limited. Our solution exchanger can completely change the solution within several milliseconds (not depicted). It can be seen that the ATP inhibition and recovery rates shown in Fig. 3 (excised patches from tsA201 cells) and Fig. 5 (excised patches from *Xenopus* oocytes) are quite different—the former is much faster than the latter. The rates of recovering the ATP sensitivity by reducing reagents appear to be different between the patches from tsA201 cells and those from oocytes as well. These results indeed suggest that reaching the intracellular face of the channel may be more problematic in the membranes excised from oocytes than those excised from tsA201 cells. Conceivably, the applied oxidizing and reducing reagents, such as CuPhe and DTT, may not gain full access to the intracellular face of the channel. Thus, the remaining small ATP inhibition of CLC-1 after CuPhe treatment for two minutes (shown in Figs. 5 B and 6 B) could still come from channels located in regions that CuPhe does not reach.

Besides the dependence of the ATP effect of CLC-1 on the redox condition, we also notice that the properties of the common gating of CLC-1 in oxidized and reduced states appear to be different. Accardi (2008) pointed out that the steady-state \(P_o^c-V\) curve of the CLC-1 channels expressed in *Xenopus* oocytes from our previous study and from that of Zifarelli and Pusch (2008) appeared to be different in the half-activation voltage (\(V_{1/2}\)) and the minimal open probability. For example, the control \(P_o^c-V\) curve of CLC-1 in our study had a minimal \(P_o^c\) of \(\sim 0.4–0.5\) (Tseng et al., 2007), whereas that in the study of Zifarelli and Pusch (2008) had a minimal \(P_o^c\) of \(\sim 0.6–0.7\). It can be seen from Fig. 5 C (experiments in oocytes) that the control \(P_o^c-V\) curve of CLC-1 is shifted to the left and upper direction after the channel is treated with oxidizing reagents. Likewise, the \(P_o^c-V\) curves of the CLC-1 channel expressed in tsA201 cells show this consistent phenomenon—the channels that are insensitive to ATP (obtained in the absence of reducing agents; Fig. 2 B) have a \(P_o^c\) of \(\sim 0.7\) at \(-120\) mV, whereas those that are sensitive to ATP (obtained in the presence of \(\beta\)-ME; Fig. 3 D) have a \(P_o^c\) of \(\sim 0.5\) at the same voltage. These comparisons again suggest that oxidation of CLC-1 may indeed be the culprit underlying the discrepancy observed between our study and that of Zifarelli and Pusch (2008).

The observation that modifying CLC-1 with MTS reagents could prevent the loss of the ATP sensitivity suggests that cysteine residues on the channel protein may be involved in the ATP inhibition of the CLC-1 common gating. We have mutated the five cysteines located in the C-terminal cytoplasmic domain. However, these cysteine mutants still exhibited the time-dependent loss of ATP sensitivity in experiments where channels in small patches were examined in intracellular solutions without reducing reagents. We are currently mutating other cysteine residues in CIC-1, either singly or in combination, to identify the target of oxidation. So far, the experiments to identify the residue responsible for redox control of ATP inhibition discourage us from concluding at this stage that CLC-1 is the oxidation target. However, the results presented here support the idea that the ATP inhibition on the CLC-1 common gating likely results from a direct interaction between ATP and CLC-1 because two single-point mutations of CLC-1, H847A and L848A, can suppress the ATP effect.

The results shown here also suggest that redox may control muscle fatigue through regulating the ATP inhibition of CLC-1. Although skeletal muscle cells contain significant amounts of antioxidants such as glutathione and superoxide dismutase, the cells also generate lots of oxidizing anions, especially when a large quantity of oxygen is consumed during severe exercise (Allen et al., 2008). It was well documented that the cellular redox state controls the contractile force of the skeletal muscle (Lamb and Posterino, 2003; Smith and Reid, 2006; Ferreira and Reid, 2007; Peake et al., 2007). Redox of several proteins in skeletal muscles has been implicated...
in the redox control of muscle contraction (Thompson et al., 2006; Cooke, 2007; Phimister et al., 2007). The results shown here suggest that redox modulations of CLC-1 also play a role in muscle fatigue if the inhibition of CLC-1 common gating by ATP/H+ is critical to control muscle fatigability.

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