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Increased sarcolemma chloride conductance as one of the mechanisms of action of carbonic anhydrase inhibitors in muscle excitability disorders

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Bari, 2021/04/22

To Experimental Neurology
Editor

Dear Editor,

We are grateful to Reviewers for their positive comments. We are submitted the revised version of our manuscript with the corrections requested by reviewer #2.

We are very pleased that our study is of interest for Experimental Neurology.

Sincerely yours

On the behalf of the authors

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ORIGINAL ARTICLE

Increased sarcolemma chloride conductance as one of the mechanisms of action of carbonic anhydrase inhibitors in muscle excitability disorders

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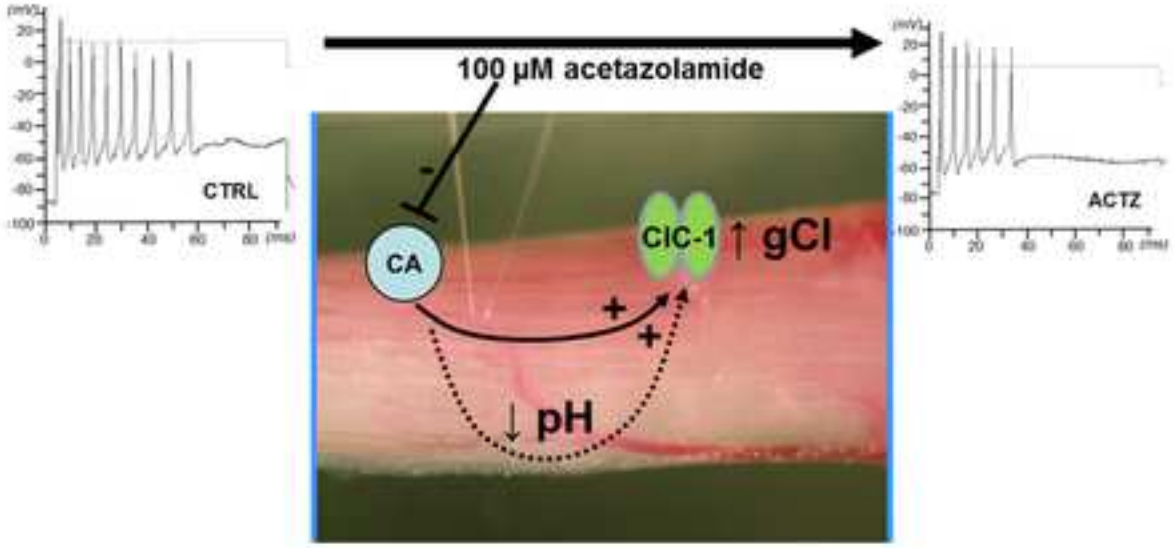
Declarations of conflict of interest

The authors declare no competing interests

Authors' reply to Reviewers' comments:

We are very grateful to both reviewers for their positive comments.
We made the corrections requested by reviewer #2 in the abstract and discussion section (page 14).

Graphical Abstract



Highlights

- Dichlorphenamide increases chloride currents in HEK cells expressing ClC-1 channel
- Dichlorphenamide increases sarcolemma chloride conductance in skeletal muscle fibers
- Acetazolamide reduces action potential firing in mouse skeletal muscle fibers
- Acetazolamide reduces intracellular pH in rat skeletal muscle fibers
- Carbonic anhydrase inhibitors may dampen muscle excitability though chloride channel activation

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Abbreviations: ACTZ: acetazolamide; BFT: bendroflumethiazide; CAI: carbonic anhydrase inhibitors; DCP: dichlorphenamide; EDL: *extensor digitorum longus*; gCl: chloride conductance; gK: potassium conductance

Abstract

To get insight into the mechanism of action of carbonic anhydrase inhibitors (CAI) in neuromuscular disorders, we investigated effects of dichlorphenamide (DCP) and acetazolamide (ACTZ) on ClC-1 chloride channels and skeletal muscle excitability. We performed patch-clamp experiments to test drugs on chloride currents in HEK293T cells transfected with hClC-1. Using the two-intracellular microelectrode technique in current-clamp mode, we measured the effects of drugs on the resting chloride conductance and action potential properties of sarcolemma in rat and mouse skeletal muscle fibers. Using BCECF dye fluorometry, we measured the effects of ACTZ on intracellular pH in single rat muscle fibers. Similarly to ACTZ, DCP (100 µM) increased hClC-1 chloride currents in HEK cells, because of the negative shift of the open probability voltage dependence and the slowing of deactivation kinetics. Bendroflumethiazide (BFT, 100 µM), structurally related to DCP but lacking activity on carbonic anhydrase, had little effects on chloride currents. In isolated rat muscle fibers, 50-100 µM of ACTZ or DCP, but not BFT, induced a ~20 % increase of the resting chloride conductance. ACTZ reduced action potential firing in mouse muscle fibers. ACTZ (100 µM) reduced intracellular pH to 6.8 in rat muscle fibers. These results suggest that carbonic anhydrase inhibitors can reduce muscle excitability by increasing ClC-1 channel activity, probably through intracellular acidification. Such a mechanism may contribute in part to the clinical effects of these drugs in myotonia and other muscle excitability disorders.

Key words: acetazolamide, dichlorphenamide, carbonic anhydrase inhibitors, ClC-1 chloride channel, myotonia, sarcolemma excitability; pH

1. Introduction

The carbonic anhydrase inhibitors (CAI), acetazolamide (ACTZ) and dichlorphenamide (DCP), have long been empirically used in skeletal muscle disorders. Off-label ACTZ was reported effective in hyperkalemic or hypokalemic periodic paralysis (Resnick et al., 1968; Griggs et al., 1970; Matthews et al., 2011; Dejthevaporn et al., 2013; Statland et al., 2018), as well as in nondystrophic myotonia due to sodium or chloride channel mutations (Griggs et al., 1978; Benstead et al., 1987; Trudell et al., 1987; Ferriby et al., 2006; Markhorst et al. 2014; Moreira et al., 2015; Avila-Smirnow et al., 2020). Effectiveness of DCP for prevention or blunting of weakness attacks in periodic paralyses was confirmed in randomized, placebo-controlled trials (Tawil et al., 2000; Sansone et al., 2016).

The mechanism of action of CAI in these neuromuscular disorders is not fully elucidated. It is commonly acknowledged that intracellular acidosis may mediate CAI effects through modulation of various metabolic processes (Vroom et al., 1975). The drugs also induce kaliuresis, which may help dampening skeletal muscle excitability. In addition, both ACTZ and DCP were shown to open directly skeletal muscle calcium-activated potassium channels, which may favor sarcolemma repolarization and contribute to their beneficial effects in periodic paralysis and possibly in myotonia (Tricarico et al., 2000; 2006). An effect of ACTZ was also found on recombinant human ClC-1 chloride channels (Eguchi et al., 2006). This channel is the main contributor to the large chloride conductance typical of resting sarcolemma, which stabilizes membrane excitability (Altamura et al., 2020). Loss-of-function mutations in the *CLCN1* gene encoding ClC-1 cause membrane overexcitability leading to clinical myotonia (Koch et al., 1992; George et al., 1993). Acetazolamide enhanced hClC-1 chloride currents in transfected HEK293T cells (Eguchi et al., 2006; Desaphy et al., 2013), an effect attenuated by

high intracellular concentration of pH buffer (HEPES), suggesting the contribution of intracellular acidification. Whether other CAI can exert a similar effect on hClC-1 channels and whether such effects actually occur in skeletal muscle fibers is unknown.

In the present study, we investigated the effects of CAI on heterologously expressed hClC-1 chloride channels, as well as on resting chloride conductance, sarcolemma excitability, and intracellular pH in skeletal muscle fibers of rat and mice.

2. Material and Methods

2.1 Chloride current measurements in HEK293T cells transfected with human ClC-1 chloride channels

The full-length cDNA encoding wild-type hClC-1 channels inserted into the pRC/CMV vector was a gift from prof. Alfred L. George (Northwestern University, Chicago, IL). The human embryonic kidney (HEK293T) cells were cultured according to supplier instructions (ECACC cell lines, Sigma-Aldrich). Cells (passages 4 to 10) were transiently transfected with a mixture of hClC-1 (0.5 µg/ml) and CD8 receptor (0.1 µg/ml) using the calcium-phosphate co-precipitation method, as previously described (Altamura et al., 2018a; 2018b). Cells decorated with CD8-antibody-coated microbeads (Dynal-Invitrogen, Milan, Italy) were used for patch-clamp experiments, 36-72 hours after transfection.

Whole-cell chloride currents were recorded at room temperature (20-22°C) using an axopatch 200B patch-clamp amplifier (Axon Instruments), as previously described (Altamura et al., 2018a; 2018b). With recording solutions, pipettes had resistance of 2-4 mOhm, and the equilibrium potential for chloride was -2.8 mV. Families of chloride currents were recorded using the protocol described in the Results section in control conditions and 2-4 minutes after application of the drug through a plastic capillary close to the cell.

2.2 Animal care

The experiments were performed in accordance with the Italian law for the protection of laboratory animals (D.Lgs 26/2014), which complies with EU Directive 2010/63/EU for animal experiments. The study received approval from the Ethical Committee (OPBA) of the University of Bari and the Directorate-General for Animal Health and Veterinary Medicinal Products of the Italian Ministry of Health (Authorization 647/2017-PR).

The male Wistar rats (3 months old) and male C57BL or FVB mice (2 months old) were purchased from Charles River Laboratories Italia (Calco, Italy). Animals were deeply anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). The *extensor digitorum longus* (EDL) muscles were surgically dissected tendon to tendon, and quickly used for electrophysiology or calcium fluorometry. Animals were killed immediately after surgery by anesthetic overdose.

2.3 Resting conductance and action potential measurements in rodent skeletal muscles

The rat or mouse EDL muscles were fixed by tendons in a recording chamber containing normal physiological (NP) solution at 30°C (*see* solution composition below). The two-microelectrode current-clamp method was used to measure sarcolemma electrical properties of single EDL fibers, as previously described (Camerino et al., 2016; Desaphy et al., 2020). Two electrodes were impaled within short distance in a single fiber on the surface of the muscle. Current was injected through the sharp microelectrode loaded with 3 M KCl and membrane potential was measured through the voltage-sensing microelectrode loaded with 3 M Kcitrate. To measure resting sarcolemma conductance, electrotonic potentials were elicited by square wave hyperpolarizing current pulse of 100-ms duration. The membrane resistance (Rm) was calculated in accordance with the infinite linear cable theory. The total resting conductance (gm=1/Rm) was

calculated in NP solution, while the component potassium conductance (gK) was calculated in chloride-free solution. The component chloride conductance (gCl) was then calculated as gm minus gK.

To measure action potentials, the membrane potential was clamped to -80 mV before to apply 100-ms depolarizing currents of increasing amplitude up to elicit a single action potential and then a train with the maximal number of action potentials (Desaphy et al., 2020). Off-line analysis of action potential recordings allowed the calculation of action potential amplitude (*AP*, in mV), the threshold current (I_{th}, in nA), the action potential latency (delay from the beginning of the current pulse to the onset of an action potential at threshold, *Lat*, in milliseconds), and the maximal number of elicitable action potentials (*N*-spikes).

Similar experiments were performed in presence of 100 µM 9-anthracene carboxylic acid (9-AC), an inhibitor of ClC-1 chloride channels, to mimic a myotonic condition (Desaphy et al., 2020; Dupont et al., 2020). The cross-bridge cycle blocker, N-benzyl-p-toluene sulfonamide (BTS, 50 µM), was preventatively added to NP solution to avoid muscle contraction (MacDonald et al., 2005). In these conditions, the resting potential (RP) was measured in control, in presence of 9-AC, and in presence of 9-AC + ACTZ (100 µM). Fibers with a RP less negative than -65 mV were discarded. Then, the membrane potential was clamped to -80 mV and a 200 ms-long depolarizing current was applied to elicit a train of action potentials. The stimulus was followed by a 1000-ms recording period to allow capturing action potential after-discharges (AfD), which are typical of the myotonic state. The parameters considered for analysis were the *N*-spikes, the current needed to elicit *N*-spikes (I_N, in nA), the percentage of fibers showing AfD, the number of spikes in the AfD, and the AfD duration (ms).

Data collection was performed in control conditions and after *at least* 20 min-incubation with drug. Data are given as mean ± S.E.M. from n fibers/N animals.

2.4 Dissection of native muscle fibers and measurement of intracellular pH

The rat EDL muscles were pinned in a dish containing NP solution. Small bundles of 10-15 fibers arranged in a single layer were dissected lengthwise (tendon-to-tendon) with the use of microscissors, as previously described (Fraysse et al., 2003). Experiments were performed at room temperature (20-22°C).

The intracellular pH (pHi) of muscle fibers was measured using the pH-sensitive dye, 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF). Bundles of muscle fibers were incubated for 1 hour in NP solution containing 10 µM BCECF-AM (BCECF-acetoxymethyl ester, Invitrogen^{TM}). After loading, muscle fibers were washed with bcecf-free NP solution and mounted in an experimental chamber (Warner Instrument Inc., Hamden, USA) on the stage of an inverted Eclipse TE300 microscope (Nikon, Japan) with a 40X Plan-Fluor objective (Nikon, Japan). Fluorescence measurements were made using a QuantiCell 900 integrated imaging system (Visitech International Ltd., Sunderland, UK), as previously described (Fraysse et al., 2003). The BCECF fluorescence was measured on background-subtracted images of muscle fiber portions at 510 nm wavelength after excitation at 490 and 440 nm (Graber et al., 1986). Ratiometric images (490/440 nm) were calculated for each muscle fiber of the preparation, before and during drug application using the QC2000 software. At the end of the experiments, the calibration of fluorescence signals with respect to pH was performed using the proton ionophore nigericin (Fuster et al., 2017). For this purpose, muscle fibers were exposed to solutions buffered at pH 6, 6.5, 7, 7.5, 8 and 8.5 in the presence of 10 µM nigericin (see "Solutions"). The calibration relationship between F490-to-F440 fluorescence ratio (R) and pH was fit to equation (1),

$$R = (R_{min} + R_{max} \bullet 10^{(pHi - pKa)}) / (1 + 10^{(pHi - pKa)}), \quad (1)$$

to determine the maximum and minimum ratio values, R_{max} and R_{min}, and the p*K*a value. The fit parameter values were then introduced in equation (2),

$$pH_i = pKa + \log[(R_{max} - R)/(R - R_{min})], \quad (2)$$

to convert the measured fluorescence ratio into pHi.

2.5 Experimental solutions and drugs

For patch-clamp experiments, the bath solution contained (in mM) 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES (pH 7.4 adjusted with NaOH). The pipette solution contained (in mM) 130 CsCl, 2 $MgCl_2$, 5 EGTA, and 1 HEPES (pH 7.4 adjusted with CsOH).

For recordings in muscle fibers, the normal physiological (NP) solution contained (in mM) 148 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 0.44 NaH_2PO_4, 12 $NaHCO_3$, and 5.5 sucrose. The chloride-free solution was made by equimolar substitution of methylsulphate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. The pH was adjusted to 7.3 by bubbling NP solution with 95% O_2 / 5% CO_2.

The pH calibration solutions contained 100 mM KCl, 1 mM $MgCl_2$, 0.01 mM nigericin, and 20 mM of the pH buffer. The various pH buffers were 2-(N-morpholino) ethanesulphonic acid (MES, pH 6-6.5), HEPES (pH 7.5 and 8), or 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIZMA, pH 8.5 and 9). The pH was adjusted with KOH.

Acetazolamide (ACTZ), dichlorphenamide (DCP), bendroflumethiazide (BFT), and 9-anthracene carboxylic acid (9-AC) were purchased from Sigma-Aldrich and dissolved in solutions at the desired concentration with a small quantity of dimethyl sulfoxide (not superior to 0.2% vol/vol). N-benzyl-p-toluene sulfonamide (BTS) was purchased from DBA Italia S.r.L., disosved in DMSO, and then in NP solution (final DMSO concentration was less than 0.5 % vol/vol).

3. Results

3.1 Effects of CAI on heterologously expressed hClC-1 channels

Whole-cell chloride currents were measured with patch-clamp technique in HEK293T cells expressing human ClC-1 chloride channels. The pH of the pipette solution was buffered at 7.2 with only 1 mM HEPES to allow intracellular pH variation in response to drugs. Families of chloride currents were recorded by applying 400 ms-long test pulses ranging from -200 to +120 mV, from the holding potential of 0 mV (Fig. 1A). At negative voltages, large instantaneous inward chloride currents deactivated to a steady state, while at voltages positive to -60 mV instantaneous currents remained merely stable for 400 ms. Inward rectification was observed at potentials greater than +50 mV due to saturation of chloride current amplitude. The average I-V relationships for instantaneous and steady-state currents are shown in Figure 1B-C. In addition, chloride currents measured at the beginning of the constant tail step at -105 mV were reported as a function of test pulse voltages (Fig. 1D) to allow calculation of the voltage dependence of channel activation (the apparent open probability, Po). The relationship was fit with equation (3),

$$Po(_V) = P_{min} + (P_{max}-P_{min}) / (1+\exp(V_{50}-V)/S)), \quad (3)$$

where P_{min} and P_{max} are the minimal and maximal open probabilities, V_{50} is the half-maximum activation potential, an S is the slope factor. Fit parameter values are reported in Table 1.

Application of 100 µM dichlorphenamide induced an increase of instantaneous and steady-state currents at negative voltages and slowed deactivation kinetics (Fig. 1A-C). At -80 mV, a voltage close to the resting membrane potential of skeletal muscle fibers, steady-state chloride currents were increased by 81 ± 30 % (n=8, P<0.006 with paired Student's t test) in presence of DCP. These effects developed gradually to reach a steady state in 2-4 minutes. Effects of DCP were less pronounced on outward currents. The voltage dependence of open probability was negatively shifted by -38.1 ± 8.9 mV (n=8, P<0.004 with paired Student's t test) (Fig. 1D). In

presence of 5 mM HEPES in the pipette solution, the shift induced by DCP was only -14.4 ± 2.5 mV (n=6, $P<0.003$ with paired Student's *t* test) (*not shown*). Effects of DCP were partially reversible upon DCP washout (*not shown*).

In similar conditions, application of 100 µM bendroflumethiazide (BFT), a bisulfonamide structurally-related to DCP but lacking activity on CA enzymes, had little effect on chloride currents (Fig. 1E-H). The voltage dependence of open probability was slightly but significantly shifted by -11.9 ± 2.4 mV (n=9, $P<0.002$ with paired Student's *t* test). At -80 mV, steady-state chloride currents were not modified in presence of BFT (+9.6 ± 5.3 %, n=9, $P=0.205$ with paired Student's *t* test).

Because DCP slowed down deactivation kinetics, we repeated the experiments using a test pulse duration of 800 ms (instead of 400 ms) to be sure that deactivation was complete before measuring open probability (Bennetts et al., 2007). In these conditions, 100 µM DCP still induced a significant negative shift of Po by 20.8 ± 3.9 mV (n=9, $P<0.001$ with paired Student's *t* test) (Table 1). Similarly, 100 µM ACTZ induced a significant negative shift of Po by 25.3 ± 3.6 mV (n=6, $P<0.001$ with paired Student's *t* test). Thus, the effects of DCP are reminiscent to those previously obtained with ACTZ (Eguchi et al., 2006; Desaphy et al., 2013), or intracellular acidification (Rychkov et al., 1996; Saviane et al., 1999; Accardi and Pusch, 2000). A limited but significant negative shift was obtained with 100 µM BFT (9.6 ± 3.7 mV, n=6, $P<0.05$ with paired Student's *t* test).

3.2 Effects of CAI on resting ion conductances in rat skeletal muscle fibers

The component resting ion conductances of sarcolemma were measured in rat EDL muscle fibers using the two-intracellular microelectrodes current-clamp method. In fast-twitch muscle fibers at rest, the chloride conductance (gCl) accounts for about 80 % of the total sarcolemma conductance (gm), while the remaining 20 % is due to potassium conductance (gK). The gCl is

mainly supported by ClC-1 channel activity (Steimeyer et al., 1991; Altamura et al., 2020). Incubation of muscles for 20 minutes with 50 or 100 µM of ACTZ, DCP, or BFT had no sign-ACificant effect on gK (Fig. 2). Conversely, ACTZ and DCP, but BFT, induced a significant increase of gCl by 12-20%. No significant difference was found between 50 and 100 µM of each drug, suggesting that maximal effect was reached with 50 µM. The effects of 50 µM DCP on gCl were reversible upon drug washout (*not shown*).

3.3 Effects of ACTZ on sarcolemma excitability in mouse skeletal muscle fibers

Similarly to rats, incubation of C57 mouse EDL muscle fibers with 100 µM ACTZ for 20 minutes had no effect on gK but significantly increased gCl by ~21 %, from 2053 ± 54 (n=15 fibers from N=5 rats) to 2482 ± 33 µS/cm^2 (n=17, N=5) (Fig. 3A). To measure excitability, the sarcolemma was clamped to -80 mV, and squared current waves of increasing amplitude were applied up to elicit a single action potential and then a train with the maximal number of action potentials (*N*-spikes) (Fig. 3B-C). In the presence of 100 µM ACTZ, the latency of the single action potential (*Lat*) was decreased from 8.5 ± 0.8 (n=15, N=5) to 6.2 ± 0.6 ms (n=21, N=5) (P<0.02 with unpaired Student's *t* test). The drug also decreased the *N*-spikes value from 10.9 ± 0.6 (n=15) to 7.5 ± 0.8 (n=21) (P<0.002 with unpaired Student's *t* test). No significant effect of ACTZ was found on *AP* (amplitude of single action potential) and I_{th} (threshold current to elicit a single action potential).

To mimic a myotonic condition, we incubated EDL muscles from FVB mice with 100 µM 9-AC, an inhibitor of ClC-1 chloride channels (Altamura et al., 2018b; Desaphy et al., 2020; Dupont et al., 2020). In presence of 9-AC, all the muscle fibers showed spontaneous electrical activity upon insertion of intracellular microelectrodes (not shown). Compared to control condition, the *N*-spikes was greatly increased and the current injected to elicit *N*-spikes, I_N, was greatly reduced (Fig. 4A-B; Table 2). In addition, while action potential firing stopped within the

end of the depolarizing stimulus in control conditions, all the fibers incubated with 9-AC continued to discharge action potentials for about 300 ms after the end of the depolarizing current, displaying the so-called myotonic after-discharges (AfD). Addition of 100 µM acetazolamide to 9-AC did not attenuate spontaneous electrical activity, did not affect I_N, but significantly reduced the *N*-spikes (Fig. 4C-D; Table 2). With ACTZ, AfD were observed only in about 70 % of fibers; the duration of these AfD was not significantly altered but the number of spikes was reduced. Neither 9-AC nor ACTZ had significant effect on the resting potential (Table 2).

3.4 Effects of ACTZ on intracellular pH in isolated rat skeletal muscle fibers

A previous study reported that ACTZ effects on recombinant hClC-1 chloride currents were attenuated by large intracellular concentration of proton buffer (30 mM HEPES), suggesting they are mediated by intracellular acidification (Eguchi et al., 2006). We thus measured pH variation in isolated rat skeletal muscle fibers in response to ACTZ or BFT application, using the fluorescent dye BCECF (Fig. 5). The calibration relationship fitted to equation (1) allowed the determination of p*K*a, R_{max} and R_{min} values needed for conversion of calculated fluorescence ratio to pH values using equation (2) (Fig. 5A). In control solution, the intracellular pH was 7.28 ± 0.07 (n=27). Application of 100 µM ACTZ to muscle fibers induced intracellular acidification, which was only partially reversed upon drug washout (Fig. 5B). Acetazolamide significantly decreased pHi from 7.28 ± 0.08 to 6.74 ± 0.08 (n=12, $P<0.0001$) (Fig. 5C). This effect was only partially reversible upon ACTZ washout (pH = 6.97 ± 0.08, n=12). In contrast, application of 100 µM BFT had no significant effect on pHi (n=11, P=0.58) (Fig. 5D).

4. Discusion

We report the functional effects of carbonic anhydrase inhibitors on heterologously-expressed voltage-gated ClC-1 chloride channels and skeletal muscle fiber resting chloride conductance and excitability. We first observed that dichlorphenamide exerts effects similar to acetazolamide on chloride currents recorded in HEK293T cells transfected with hClC-1 channels (Eguchi et al., 2006; Desaphy et al., 2013). These effects include the increase of chloride currents at negative voltages and the negative shift of the open probability voltage dependence, as well as the slowing of deactivation kinetics. The observation that bendroflumethiazide, which lacks carbonic anhydrase inhibiting activity, does not affect chloride currents, suggests that carbonic anhydrase inhibition may be involved in the effects of ACTZ and DCP on hClC-1 channels. The effects of ACTZ and DCP on ClC-1 channels in HEK293T cells are reminiscent to those of intracellular acidification recorded in *Xenopus* oocytes or Sf-9 insect cell expression systems (Rychkov et al., 1996; Saviane et al., 1999; Accardi and Pusch, 2000; Ryschkov et al., 2001). In HEK293 cells, the effects of ACTZ and DCP on chloride currents were attenuated in presence of an elevated intracellular concentration of pH buffer (Eguchi et al., 2006). Altogether, these results strongly support the hypothesis that carbonic anhydrase inhibitors increase ClC-1 chloride currents through intracellular acidification.

Effects of internal pH on ClC-1 channels have been challenged by Bennetts and collaborators, who reported that open probability voltage dependence was the most negative at pH 7.2, while both higher and lower intracellular pH resulted in a less negative voltage dependence (Bennetts et al., 2007). Yet, the effects of intracellular pH on ClC-1 channels are influenced by adenosine nucleotides and nicotinamide adenine dinucleotide (NAD), which bind to the intracellular CBS domains of the channel (Bennetts et al., 2005; 2007; 2012). The effects of intracellular pH, ATP, and NAD may depend on the redox state of ClC-1 channels channels (Zhang et al., 2008; Bennetts et al. 2012). In addition, extracellular protons and anions, including chloride itself, modulate ClC-1 gating (Rychkov et al., 1996; 1998; Chen and Chen, 2001). Thus,

apparent discrepancies between the various studies performed in heterologous expression systems may stem from subtle differences in experimental protocols.

Given the complexity of intracellular pH effects on ClC-1 channels and the different pattern of carbonic anhydrase isoenzymes expression in HEK cells and muscle fibers, it was important to verify the effects of acetazolamide on chloride channel function in skeletal muscle fibers. The chloride conductance (gCl) of sarcolemma is especially elevated, accounting for about 80 % of the total ion conductance at rest (Altamura et al., 2020). It is acknowledged that most of the gCl is supported by ClC-1 channels (Steinmeyer et al., 1991). Such a high gCl is crucial to maintain muscle excitability in a physiological range. According to what observed in HEK293T cells, the application of acetazolamide or dichlorphenamide, but not bendroflumethiazide, increased the gCl in single rat and mouse muscle fibers. The gCl increase was likely responsible for the shortening of single action potential latency and the reduced maximal number of elicitable spikes observed in mouse muscle. Such effect might be especially relevant for the beneficial effects of ACTZ in alleviating myotonia (Griggs et al., 1978; Benstead et al., 1987; Trudell et al., 1987; Ferriby et al., 2006; Markhorst et al. 2014; Moreira et al., 2015; Avila-Smirnow et al., 2020). In presence of 9-AC, a condition mimicking chloride channel myotonia, ACTZ was able to partially counteract hyperexcitability by reducing the number of fibers displaying after-discharges and by reducing the frequency of action potential firing. This is in accord with the antimyotonic effect of ACTZ reported by a few human carriers of loss-of-function ClC-1 mutations (Markhorst et al. 2014; Moreira et al., 2015). This also suggests that ACTZ may exert some antimyotonic effect through additional mechanisms not related to ClC-1 activation, which might include activation of potassium channels (Tricarico et al., 2000). Yet, the occurrence of spontaneous electrical activity, the current threshold, and the duration of AfD were not modified by ACTZ, suggesting a limited effect of ACTZ when ClC-1 channels are inhibited. Empirically, acetazolamide-responsive myotonia has been more often associated with sodium channel mutations. Although

ACTZ also induced intracellular acidification in muscle fibers, further studies are required to link definitively muscle intracellular acidification to gCl increase. Indeed, previous studies suggested that muscle acidification might reduce the gCl, thereby protecting muscle against fatigue during exercise (Pedersen et al., 2004; 2005; Bandschapp et al., 2012). Yet, these studies were performed in different experimental conditions, including elevated external K+ and intracellular acidification secondary to extracellular acidification. In a successive study, the same authors suggested that the reduction of gCl in working muscle might be due to a pH-independent, direct effect of lactic acid on the gCl (de Paoli et al., 2010). Conciliating this later observation and our results is the hypothesis that acetazolamide may worsen muscle fatigue in some conditions, an effect thus opposed to lactic acid (Garske et al., 2003; Gonzales and Scheuermann, 2013; Dominelli et al., 2018). It would be very interesting to verify whether such effect may involve ClC-1 chloride channels.

In conclusion, our study confirms that carbonic anhydrase inhibitors increase chloride currents carried by human ClC-1 channels in transfected HEK cells. Importantly, a similar effect occurs in skeletal muscle fibers resulting in increased gCl, which likely contribute to reduce excitability together with other mechanisms. Such effect may contribute to the clinical effects of acetazolamide and dichlorphenamide in humans affected by nondystrophic myotonia and other excitability disorders, although other additional mechanisms cannot be ruled out. In vivo studies in animal models are warranted to validate such hypothesis.

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Declarations of conflict of interest

The authors declare no competing interests

Author contributions

Conceptualization and Design of the study: S.P., J.-F.D.; Funding acquisition: J.-F.D.; Data acquisition: C.A., A.F., N.T., A.L., P.I.; Data analysis C.A., A.F., N.T., A.L., P.I., S.P., J.-F.D.; Writing original draft: C.A., J.-F.D.; Writing – review & editing: M.R.C., S.P.

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Table 1

Open probability parameter values of hClC-1 channels

drug	test duration (ms)	[drug] (μM)	V_{50} (mV)	*S* (mV)	Pmin
DCP (n=8)	400	0	-40.4 ± 6.8	36.6 ± 3.1	0.20 ± 0.03
		100	-78.4 ± 9.4*	48.2 ± 6.7*	0.26 ± 0.03*
BFT (n=9)	400	0	-51.3 ± 5.6	31.0 ± 2.9	0.14 ± 0.02
		100	-63.1 ± 4.6*	30.1 ± 3.8	0.13 ± 0.03
DCP (n=9)	800	0	-43.5 ± 7.7	31.1 ± 2.6	0.09 ± 0.03
		100	-64.3 ± 9.1*	31.6 ± 2.9	0.07 ± 0.02
ACTZ (n=6)	800	0	-55.1 ± 8.3	28.7 ± 0.8	0.07 ± 0.02
		100	-80.4 ± 11.2*	37.2 ± 4.6	0.04 ± 0.02
BFT (n=6)	800	0	-62.4 ± 3.1	24.0 ± 1.4	0.13 ± 0.03
		100	-72.0 ± 3.6*	22.8 ± 1.7	0.15 ± 0.02

DCP = dichlorphenamide; BFT = bendroflumethiazide; ACTZ = acetazolamide; V_{50} = half-maximum activation potential; *S* = slope factor; Pmin = minimal open probability. Values obtained as described in Fig. 1 are expressed as mean ± SEM from n cells. Statistical difference between drug and relative control was assessed using paired Student's *t* test: * at least $P<0.05$.

Table 2

Effects of acetazolamide (ACTZ) on resting potential and action potential firing in mouse skeletal muscle fibers in presence of 9-anthracene carboxylic acid (9-AC)

drug	CTRL	9-AC (100 µM)	9-AC + ACTZ (100 µM)
RP (mV)	-75.5 ± 2.5 (4)	-74.1 ± 1.5 (12)	-71.3 ± 1.1 (21)
N-spikes	4.8 ± 0.9 (4)	14.7 ± 0.9 (12)[b]	9.8 ± 0.8 (21)[a,d]
I_N (nA)	180 ± 14 (4)	80 ± 6 (12)[b]	83 ± 7 (21)[b]
AfD (% of fibers)	0 (4)	100 (12)	71.4 (21)
AfD (spike number)	0 (4)	13.8 ± 2.3 (12)[a]	4.2 ± 1.1 (15)[a,c]
AfD duration (ms)	0 (4)	317 ± 47 (12)[a]	290 ± 60 (15)[a]

RP = resting potential; *N*-spikes = maximal number of elicitable action potentials; I_N = injected current to elicit *N*-spikes; AfD = after-discharges. Values obtained as described in Fig. 4 are expressed as mean ± SEM from n muscle fibers (within brackets) from 4 FVB mice. Statistical difference between was assessed using unpaired Student's *t* test versus CTRL ([a]$P<0.03$; [b]$P<0.001$) or between 9AC+ACTZ and 9-AC ([c]$P<0.01$; [d]$P<0.001$).

FIGURE LEGENDS

Fig. 1 Effects of dichlorphenamide (DCP) and bendroflumethiazide (BFT) on chloride currents in HEK293T cells transfected with hClC-1 channels. (A) Representative chloride current families recorded in a HEK293T cells expressing hClC-1 chloride channels before (CTRL) and during application of 100 µM dichlorphenamide (DCP). Average instantaneous currents (B), steady-state currents (C), and open probability plotted against test pulse potentials were calculated along with S.E.M. from 8 cells in CTRL and during DCP application. The voltage dependence of channel activation (the apparent open probability, Po) was fit with equation (3) reported in the text. Fit parameter values are reported in Table 1. (E) Representative chloride current families recorded as in (A) before (CTRL) and during application of 100 µM bendroflumethiazide (BFT). Average instantaneous currents (F), steady-state currents (G), and open probability (H) were calculated along with S.E.M. from 4 cells in CTRL and during BFT application.

Fig. 2 Effects of carbonic anhydrase inhibitors on resting sarcolemma component conductances in rat skeletal muscle fibers. The cable properties of adult rat EDL muscle fibers were measured using two-intracellular microelectrode technique in current-clamp mode. The potassium and chloride conductance (gK and gCl) of sarcolemma at rest were calculated before (CTRL) and during application of acetazolamide (ACTZ), dichlorphenamide (DCP) or bendroflumethiazide (BFT). Each bar is the mean ± SEM from the number of muscle fibers indicated within brackets. Statistical analysis was performed using unpaired Student's t test vs relative CTRL (*P<0.01; **P<0.0001).

Fig. 3 Effects of acetazolamide on resting sarcolemma conductance and excitability in mouse skeletal muscle fibers. (A) The resting sarcolemma chloride conductance (gCl) was calculated from cable properties of mouse EDL muscle fibers measured using current-clamp intracellular microelectrode technique. Each bar is the mean ± SEM from the number of muscle fibers indicated within brackets. Statistical analysis between control (CTRL) and acetazolamide (ACTZ) was performed using unpaired Student's *t* test ($^{**}P<0.0001$). (B) Representative single action potentials elicited by threshold current, in absence (CTRL) and presence of 100 µM ACTZ. (C) Representative maximal action potential firing, in absence (CTRL) and presence of 100 µM ACTZ.

Fig. 4 Effects of acetazolamide (ACTZ) on excitability in overexcited mouse skeletal muscle fibers. Action potential firing was recorded in response to 200 ms-long depolarizing currents (black bar) in mouse EDL muscle fibers in control conditions (A, CTRL), in presence of 100 µM 9-anthracene carboxylic acid (B, 9-AC), and in presence of 9-AC + 100 µM acetazolamide (C and D, ACTZ). After-discharges (AfD) were observed in 100 % of the fibers in presence of 9-AC (B) and 70 % of fibers in presence of 9-AC + ACTZ (D).

Fig. 5 Effects of acetazolamide (ACTZ) on intracellular pH in rat skeletal muscle fibers. (A) Calibration curve of BCECF in isolated rat skeletal muscle fibers. Each data point is the mean fluorescence ratio ± S.E.M. recorded in nigericin-permeabilized muscle fibers perfused with calibration solutions (n=5 to 7 cells). The relationship was fit with equation (1) reported in the Methods section. The fit parameters were p*K*a = 6.54, R_{max} = 3.29, and R_{min} = 1.29. (B) Representative time course of intracellular pH variation recorded in a muscle fiber in response to 100-µM acetazolamide (ACTZ). (C, D). Box and whisker chart representation of the effects of 100 µM ACTZ (n=12 cells) or 100 µM bendroflumethiazide (BFT) (n=15 cells) and washout.

Median and 10th, 25th, 75th, and 90th percentiles are shown. Statistical analysis was performed using paired Student's *t* test (*at least $P<0.0005$ *vs* CTRL; $^{\#}P<0.005$ *vs* ACTZ).

Figure 1

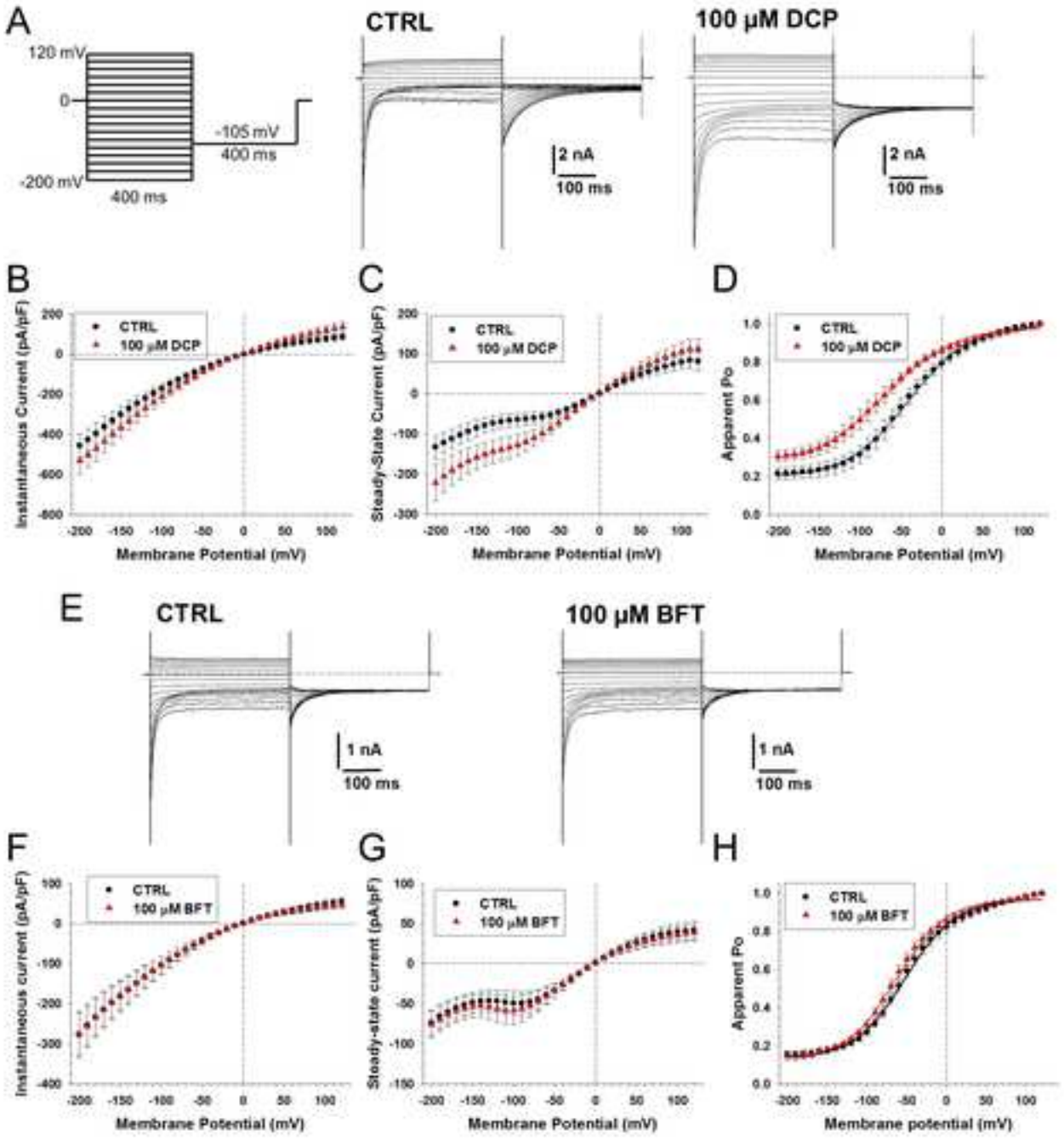

Figure 2

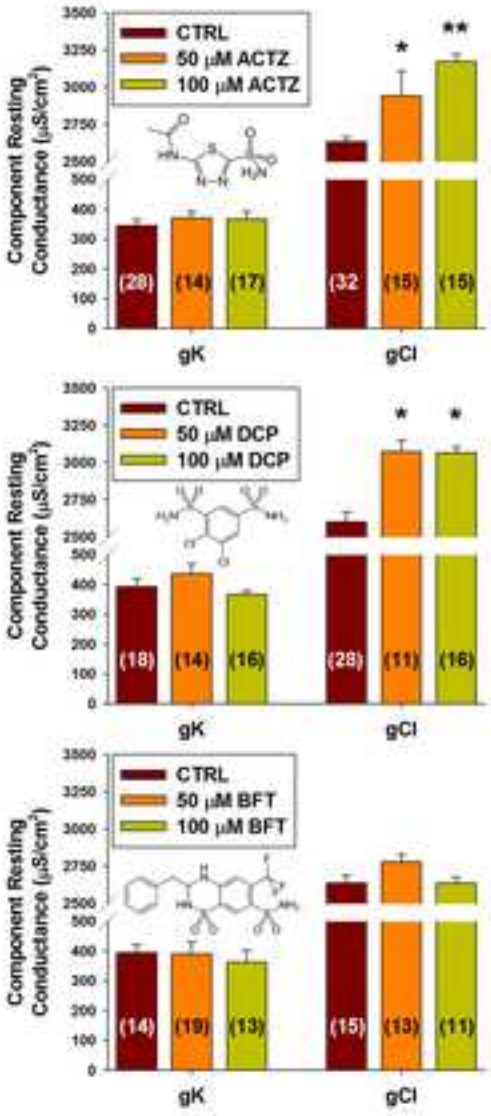

Figure 3

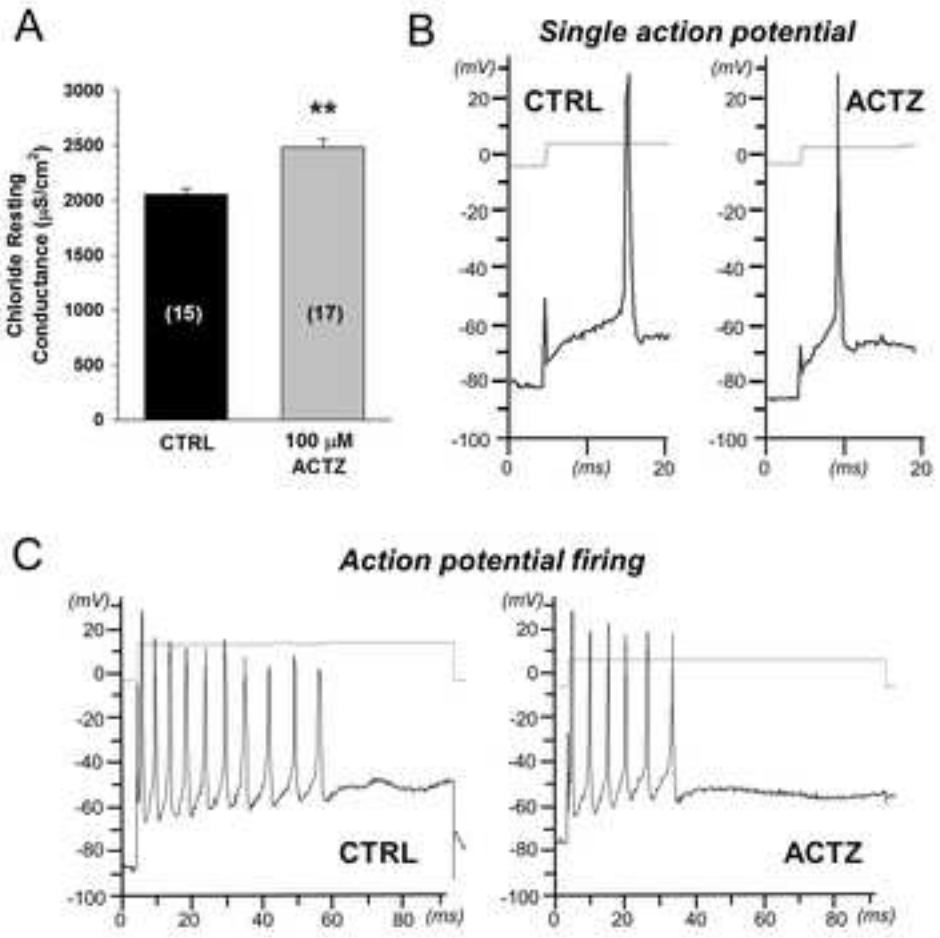

Figure 4

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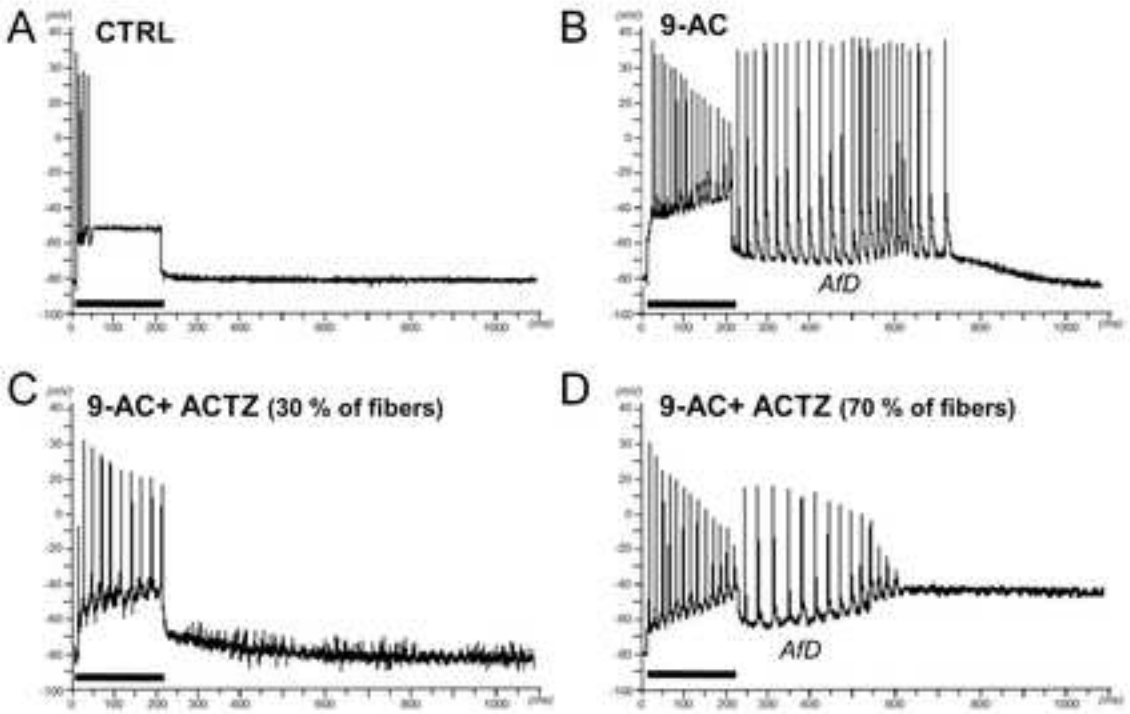

Figure 5