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Aging-associated down-regulation of ClC-1 expression in skeletal muscle: phenotypic-independent relation to the decrease of chloride conductance

Sabata Pierno[a], Annamaria De Luca[a], Carol L. Beck[b], Alfred L. George Jr.[b], Diana Conte Camerino[a,*]

[a] *Unit of Pharmacology, Department of Pharmacobiology, Faculty of Pharmacy, University of Bari, Via Orabona 4, I-70125 Bari, Italy*
[b] *Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-2372, USA*

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Abstract In order to clarify the mechanism underlying the reduction of resting membrane chloride conductance (g_{Cl}) during aging, the levels of mRNA encoding the principal skeletal muscle chloride channel, ClC-1, were measured. Total RNA samples isolated from tibialis anterior muscles of aged (24–29 months old) and adult (3–4 months old) rats were examined for ClC-1 expression using Northern blot analysis, and macroscopic g_{Cl} was recorded from extensor digitorum longus muscle fibers from each adult and aged rat in vitro using a two intracellular microelectrode technique. Although interindividual variability was observed, aged rats exhibited a parallel reduction of both g_{Cl} and ClC-1 mRNA expression as compared to adult rats. A linear correlation exists between individual values of ClC-1 mRNA and g_{Cl}. These results provide evidence that ClC-1 is the main determinant of sarcolemmal g_{Cl} and demonstrate that the decrease of g_{Cl} observed during aging is associated with a down-regulation of ClC-1 expression in muscle.

Key words: Aging; Skeletal muscle; Electrophysiology; ClC-1 chloride channel; Northern blot analysis

1. Introduction

The aging process leads to profound impairment of skeletal muscle performance including decreases in muscle strength and speed of contraction. Factors underlying these impairments are complex and can be related to changes in the muscular apparatus [1] or in the nervous and endocrine systems [2,3]. The rate of synthesis of total skeletal muscle proteins is significantly slower in old subjects than in young adults [4] and studies in various animal tissues have suggested that this reduction may be due to age-related changes in both gene transcription and mRNA translation [5,6]. Additionally, alterations of muscle protein metabolism have been reported in the elderly, due to alterations of amino acid transport activity [7].

Alterations in membrane electrical properties occur in skeletal muscle of aged rats, including a specific reduction of resting chloride conductance (g_{Cl}) [8,9]. A large resting g_{Cl} is characteristic of adult fast-twitch muscle fibers and serves to control membrane excitability and muscle contractility [10]. In fact a dramatic decrease of g_{Cl} characteristic of some forms of hereditary myotonia is responsible for the abnormal generation of repetitive action potentials in this disease [10]. Sarcolemmal g_{Cl} is believed to be mediated predominantly by ClC-1, the major voltage-gated chloride channel expressed in skeletal muscle. This physiological role of ClC-1 is supported by the identification of mutations in the gene encoding this channel in humans affected by dominant and recessive myotonia congenita [11–14], as well as in goat and mouse forms of the disease [15–17]. However, the relationship between g_{Cl} and ClC-1 is still an open question. Studies performed using immunofluorescence microscopy have suggested that ClC-1 is confined to the surface membrane and it is not present on the transverse tubules of mouse muscle fibers [18]. On the basis of these results, the recent findings of Coonan and Lamb [19], showing a large 9-anthracene carboxylic acid (9-AC)-sensitive chloride conductance in the T-tubules of rat muscle fibers, have led to the hypothesis that a different chloride conducting channel, besides ClC-1, may be present in the T-system, and can contribute significantly to the total g_{Cl} [19].

The reason for reduced g_{Cl} in native muscle fibers of aged rats is unclear. Pharmacological studies led us to hypothesize that the decline of g_{Cl} may be due to concurrent events such as the impairment of intracellular signaling pathways involving calcium- and phospholipid-dependent protein kinase C, which are known to regulate muscle g_{Cl} [9,20]. However, a decrease in the number of conductive channels as a consequence of reduced protein synthesis could also explain this phenomenon [9]. It has been previously shown that during rodent postnatal development, a period in which the rate of protein synthesis is high, g_{Cl} increases rapidly. During the same period of life the increase of g_{Cl} is paralleled by an increase in the amount of mRNA encoding ClC-1 [21,22].

The aim of our study was to assess whether the reduction of g_{Cl} in skeletal muscle tissue of aged rats is due to a concomitant reduction in ClC-1 mRNA levels as well as to better clarify if resting g_{Cl} is sustained by ClC-1. Thus we determined in parallel both ClC-1 mRNA levels and g_{Cl} in representative fast-twitch muscles (tibialis anterior, TA and extensor digitorum longus, EDL) of aged and adult rats. We observed a strong linear correlation between the levels of these two parameters with advanced age, indicating that reduced g_{Cl} in aged animals is due to reduced ClC-1 expression. In parallel, in order to evaluate the presence of distinct phenotypes of aged rats, we measured another muscle functional parameter, i.e. the voltage threshold for contraction, and we searched for a possible correlation with either g_{Cl} or ClC-1 mRNA values.

*Corresponding author. Fax: (39) (80) 5442801.
E-mail: conte@farmbiol.uniba.it

Abbreviations: g_{Cl}, resting membrane chloride conductance; ClC-1, muscular chloride channel-1; 9-AC, 9-anthracene carboxylic acid; EDL, extensor digitorum longus; TA, tibialis anterior; UV, ultraviolet; G6PD, glucose 6-phosphate dehydrogenase; *R*m, membrane resistance; g_m, total membrane conductance; g_K, resting membrane potassium conductance; *R*, rheobase voltage

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2. Materials and methods

2.1. Animals and tissue preparations

Adult (3–4 months old) and aged (24–29 months old) Wistar rats (Charles River, Calco, Italy) were used for this study. Animals were in good health and exhibited normal locomotor activity [8]. Both EDL and TA muscles were dissected from rats under urethane anesthesia (1.2 g/kg i.p.) prior to their being killed. The EDL muscles were used for electrophysiological measurements of g_{Cl} while TA muscles and heart were immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation.

2.2. RNA and protein isolation

Total RNA was isolated from 10 adult and 12 aged rat TA muscles using a modified acid-phenol method [23]. Approximately 400 mg of each muscle was homogenized in 4 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and the homogenate was centrifuged at $12\,000\times g$ for 10 min. The aqueous supernatant containing the RNA was removed and the lower organic phase was saved for protein isolation. RNA was precipitated with 2-propanol, dissolved in diethylpyrocarbonate-treated water, and stored at −80°C until analysis. RNA isolated with this method was fully intact as determined by agarose gel electrophoresis. The amount of RNA recovered from the tissue was determined by UV spectrophotometry at 260 nm. Protein was isolated from the phenol phase according to the protocol supplied by the reagent supplier. Total protein concentration was determined by the Bradford assay using a commercial reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Northern blot analysis

Total RNA (10 mg) was size-fractionated on denaturing 1% agarose/6% (v/v) formaldehyde gels and transferred to a nylon membrane (Hybond-N, Amersham) as previously described [15]. Northern blots were sequentially hybridized with a rat ClC-1 cDNA probe (nt 298–638) and with either human glucose 6-phosphate dehydrogenase (hG6PD) cDNA or 18S ribosomal RNA radiolabeled probes as internal references in order to establish the relative amount of RNA in each sample. The ClC-1 and hG6PD probes were radiolabeled with [^{32}P]dCTP using the random priming method. Hybridizations were performed at 42°C for 16 h in 50% formamide/5×SSPE (1×SSPE is 0.18 M NaCl/10 mM Na_2HPO_4/1 mM EDTA)/1% SDS/0.05 M Tris-HCl, pH 7.5/5 mM EDTA/1% BSA and 1×10^{-6} cpm/ml ^{32}P-labeled cDNA probe. Blots were washed with a final stringency of 65°C in 0.1×SSC/0.1% SDS following hybridization with cDNA probes. A radiolabeled 18S RNA riboprobe was synthesized by in vitro transcription using an antisense control template (Ambion), T7 polymerase and [^{32}P]CTP. Hybridizations with 18S riboprobe were performed at 60°C with 0.5×10^{-6} cpm/ml ^{32}P-labeled probe in the same hybridization solution, followed by washes in 0.1×SSC/0.1%SDS at 68°C.

Hybridizing bands were visualized by autoradiography and quantified by phosphorimage analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. Electrophysiology

Using a computer-assisted two intracellular microelectrode technique in current clamp mode, the resting membrane chloride conductance (g_{Cl}) was measured in vitro from rat EDL muscle fibers. Soon after the removal from rat, the EDL muscle preparation, tied at the end of each tendon, was placed on a glass rod located in a muscle bath at 30°C and perfused with normal and chloride-free physiological solutions [9].

The normal (chloride-containing) physiological solution had the following composition (in mM): NaCl 148, KCl 4.5, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 12, NaH_2PO_4 0.44 and glucose 5.55. The chloride-free solution was made by equimolar substitution of methylsulfate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. All solutions were continuously gassed with 95% O_2 and 5% CO_2 (pH 7.2–7.3) [9]. The component resting membrane conductances to chloride and potassium ions were calculated from membrane resistance (Rm) values in normal and chloride-free solutions. Rm and the other cable parameters ($d$calc, calculated fiber diameter and Cm, total membrane capacitance) were obtained by injecting a hyperpolarizing constant square current pulse into the muscle fiber through one microelectrode and recording the resulting voltage deflection with a second microelectrode inserted at two different distances from the current electrode. The current pulse generation, acquisition of the voltage records and calculation of the fiber constants were done under computer control, as described in detail elsewhere [9]. The total membrane conductance g_m was $1/R$m in the normal physiological solution. The potassium conductance (g_K) was $1/R$m in the chloride-free solution. The mean chloride conductance, g_{Cl} was calculated as the mean g_m minus the mean g_K [9]. As already described [8,9] g_K values were higher in muscle fibers from aged rats as compared to the adults (data not shown). The data are expressed as mean ± S.E.M. and were calculated by standard methods from the variance of g_m and g_K, assuming no covariance [24]. Significance between mean values has been evaluated by an unpaired Student's t-test. Correlation coefficient has been calculated by standard linear regression analysis.

The mechanical threshold for contraction was determined using a two microelectrode point voltage clamp method, in the presence of 3 μM tetrodotoxin, as previously described [25,26]. The holding potential was set at −90 mV. Depolarizing current pulses of increasing durations (5–500 ms) were given repetitively at a rate of 0.3 Hz, while the impaled fibers were viewed continuously with a stereomicroscope. The command voltage was increased until contraction was just visible and the threshold membrane potential at this point was read from a digital sample-and-hold voltmeter. The threshold membrane potential V (mV) for each fiber was averaged at each pulse duration t (ms). A fit estimate of the rheobase voltage (R) and of the time constant to reach R was obtained by a non-linear least square algorithm using the following equation: $V=[H-R\exp(t/\tau)]/[1-\exp(t/\tau)]$ where H is the holding potential (mV), R is the rheobase (mV) and τ is the time constant [25,26]. The mechanical threshold values are expressed as the fitted rheobase (R) parameter ± S.E.M. which was determined from the variance-covariance matrix in the non-linear least square fitting algorithm.

3. Results

3.1. Total protein and total RNA content of aged rat skeletal muscle

We found a significant reduction in total protein content of aged rat TA muscle with respect to that found in adult rats (aged: 0.44 ± 0.08 mg/g muscle, $n=8$; vs adult: 0.82 ± 0.05 mg/g muscle, $n=8$; $P<0.001$). Similarly, the total RNA content of muscle was significantly decreased from 0.77 ± 0.03 mg/g muscle ($n=10$) in adult rats to 0.61 ± 0.05 mg/g muscle ($n=15$) in aged rats ($P<0.02$). These findings are consistent with the effects of aging determined in previous studies [4].

3.2. Membrane chloride conductance of skeletal muscle fibers from aged rats

In agreement with our previous findings [9] the chloride conductance (g_{Cl}) of EDL muscle fibers was significantly re-

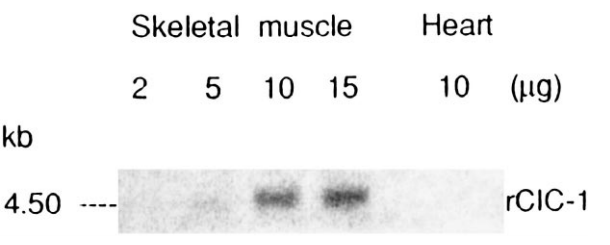

Fig. 1. Northern blot analysis of skeletal muscle RNA. The upper panel illustrates a representative autoradiograph showing hybridization of a rat ClC-1 probe (nt 298–638) to a 4.5 kb mRNA transcript in skeletal muscle, but not to RNA from heart. The quantity of total RNA loaded is given above each lane. Signal intensity was proportional to the amount of total RNA loaded. RNA size as determined by an mRNA molecular weight standard run on the same gel is given in kb to the left.

duced in the majority (8 of 12) of aged rats as compared with the adult animals. As previously reported [8], a high degree of interindividual variability was observed among the aged preparations, although the variability observed among the fibers of each aged animal was similar to that of the adult ones. Individual mean values of g_{Cl} in the eight muscle preparations exhibiting significant decreased g_{Cl} ranged from 653 ± 174 $\mu S/cm^2$ ($n = 13$ fibers) to 2341 ± 181 $\mu S/cm^2$ ($n = 14$ fibers), while four of 12 aged rats showed g_{Cl} values (mean: 2993 ± 158 $\mu S/cm^2$, $n = 39$ fibers) similar to those recorded in adults (2985 ± 130 $\mu S/cm^2$, $n = 60$ fibers). The mean value calculated from the eight animals with reduced g_{Cl} was 43% lower than the mean value of the 3–4 month old rats (aged: 1690 ± 136 $\mu S/cm^2$, $n = 127$; vs. adult: 2985 ± 130 $\mu S/cm^2$, $n = 60$; $P < 0.001$).

3.3. Expression of ClC-1 mRNA in aged and adult rats

Initially, to evaluate the specificity and sensitivity of our rat ClC-1 probe, Northern blot analyses of total RNA isolated from skeletal muscle (2, 5, 10, 15 μg per lane) and heart (10 μg) of an adult rat were performed. The blot was hybridized with a rat ClC-1 probe under high stringency conditions. The ClC-1 probe hybridized to an ~4.5 kb mRNA transcript in skeletal muscle with a little as 2 μg total RNA per lane, but did not hybridize to RNA from heart (Fig. 1). These observations demonstrate the specificity of our ClC-1 probe.

A comparison of ClC-1 mRNA content in skeletal muscle tissue of aged and adult rats was made using Northern blot analysis of total RNA (10 μg) extracted from TA muscles of 24–29 month old rats (12 samples) and from 3–4 month old rats (10 samples). The blot was sequentially hybridized with ClC-1 and 18S rRNA radiolabeled probes. The 18S probe was used as an internal reference rather than other 'housekeeping' genes, because reduced expression of G6PD was observed in the aged muscle preparations with respect to the adults (data not shown). In all TA muscle samples of both aged and adult rats, the 4.5 kb ClC-1 and 1.35 kb 18S rRNA bands were quantified by phosphorimage analysis (Fig. 2A). Expression of ClC-1 mRNA was significantly lower in the aged muscles as compared with adult muscle, while 18S rRNA was expressed equally in both groups of animals (Fig. 2A). The content of ClC-1 mRNA was normalized by dividing it by the 18S rRNA signal (Fig. 2B). In adult rats the normalized amount of ClC-1 mRNA exhibited a mean value of 30.2 ± 2.57 (arbitrary units $\times 10^4$), whereas higher variability was observed among aged rat preparations (mean of all 12 animals: 24.4 ± 3.11, range: 36.6–3.2, arbitrary units $\times 10^4$) (Fig. 3B). However, in aged rats with significantly reduced g_{Cl} ($n = 8$), the normalized value of ClC-1 mRNA was significantly lower than that of the adults. In these eight aged animals, the normalized mean value of ClC-1 mRNA was 19.4 ± 3.42 (arbitrary units $\times 10^4$; $P < 0.025$). Therefore,

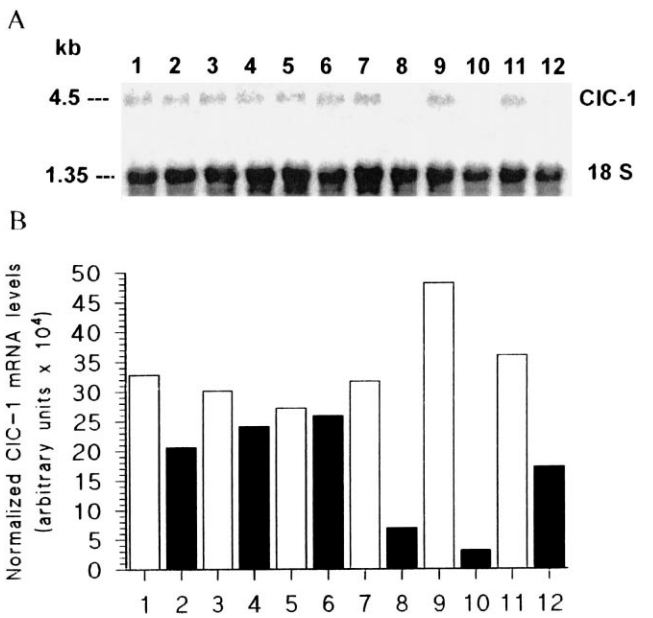

Fig. 2. Quantification of ClC-1 mRNA in skeletal muscle tissue of adult and aged rats. A: Representative Northern blot autoradiograph showing ClC-1 mRNA and 18S rRNA transcript levels in muscle of adult (3–4 months old) and aged (25–29 months old) rats. Each lane contains 10 μg total RNA from either adult (odd-numbered lanes) or aged (even-numbered lanes) rat TA muscles. Size standards are indicated to the left of the panel. B: The ClC-1 and 18S hybridization signals, shown in A, were quantified by Phosphorimage analysis. Vertical bars indicate the normalized level of ClC-1 mRNA (ClC-1 divided by 18S) in arbitrary units (adult, open bars; aged rats, filled bars).

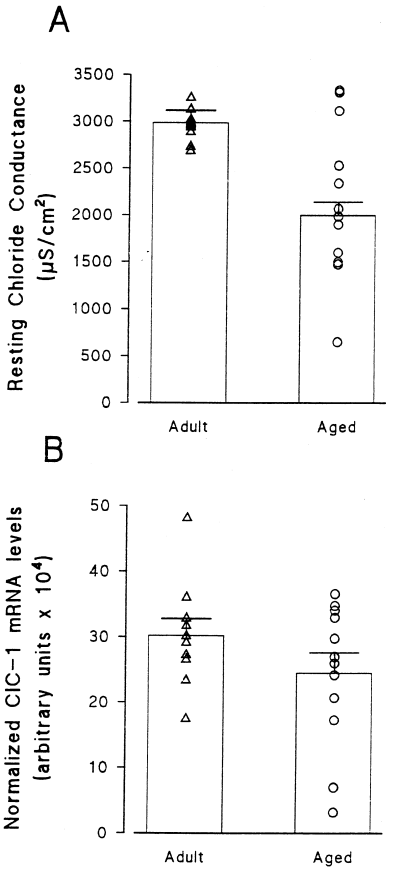

Fig. 3. Comparisons of resting g_{Cl} and normalized ClC-1 expression in adult and aged rats. A: Resting membrane chloride conductance (g_{Cl}) measured in the EDL muscle fibers of aged and adult rats. The height of the vertical bars represents the mean g_{Cl} values from 12 aged (166 fibers examined) and 10 adult (60 fibers examined) rats. Horizontal lines above each vertical bar indicate the S.E.M. B: Normalized ClC-1 mRNA levels measured in TA muscles of the same animals as illustrated in A. The height of the vertical bars represents the mean value of normalized ClC-1 mRNA level (arbitrary units). Horizontal lines above each vertical bar indicate the S.E.M.

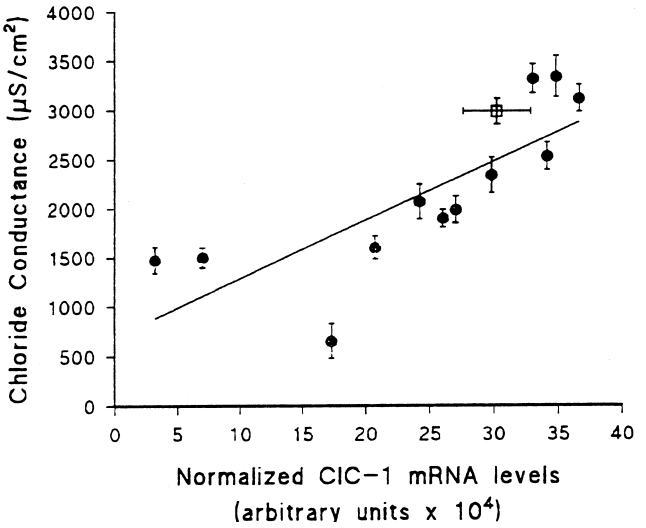

Fig. 4. Correlation between ClC-1 mRNA expression and resting g_{Cl} in skeletal muscle fibers of aged rats. Filled circles represent normalized ClC-1 mRNA levels in TA muscle of each aged rat plotted against the corresponding value for g_{Cl} measured in EDL muscle fibers of the same animal. The data are linearly related with a Pearson's correlation coefficient of 0.78 ($P < 0.01$). The single open square shows the mean ClC-1 mRNA level of 10 adult rats plotted against the mean g_{Cl} value from EDL muscles of the same 10 rats.

ClC-1 mRNA levels were 36% lower than in adults, parallel to the 43% reduction of g_{Cl} described above. The four aged rats exhibiting g_{Cl} values similar to those of the adults also had similar ClC-1 mRNA levels (mean: 34.6 ± 0.76, arbitrary units $\times 10^4$). The wide interindividual variability in g_{Cl} (Fig. 3A) and ClC-1 mRNA levels among the aged rats prompted us to examine whether these values were correlated. Fig. 4 illustrates the relationship between normalized ClC-1 mRNA levels measured in all 12 aged rats plotted against the g_{Cl} value measured in the EDL muscle from each corresponding animal. The two parameters exhibit a strong linear correlation ($r = 0.78$, $P < 0.01$, filled circles). The single filled square symbol in Fig. 4 represents the mean value of normalized ClC-1 mRNA and g_{Cl} measured in adult rats, and this point falls along the same linear relationship observed for the aged rats. These data suggest that g_{Cl} varies proportionally with expression of ClC-1.

3.4. Mechanical threshold of skeletal muscle fibers from aged rats

In line with previous results [25,26], muscle fibers of aged rats needed significantly less depolarization to contract with respect to that of adult, at each pulse duration. The rheobase voltage (R), estimated from the fit of the experimental points, was significantly different in adult and aged animals, being -58.6 ± 1.7 mV (85 fibers, 10 rats) and -69.2 ± 0.2 mV (97 fibers, 12 rats), respectively ($P < 0.001$). R was significantly more negative in each aged animal, in comparison to that of the adults, although also this parameter showed a certain interindividual variability. However, no linear correlation was found between R and the values of either g_{Cl} ($r = 0.15$, n.s.) or ClC-1 mRNA ($r = 0.03$, n.s.) measured in the same aged animals.

4. Discussion

Our study was planned to better understand the mechanisms underlying the reduction of skeletal muscle g_{Cl} during aging. The present study demonstrate that the primary mechanism for the reduction of g_{Cl} observed during aging in skeletal muscle is associated with a down-regulation of the expression of mRNA encoding the rat ClC-1 chloride channel. The basis for this reduced chloride channel expression could involve reduced transcription or alterations in mRNA processing or stability [27]. In this regard we also found a reduction of total RNA as well as of total protein content in skeletal muscle as already described by other authors [4]. In addition we observed a reduction of the amount of a constitutive enzyme, glucose 6-phosphate dehydrogenase, in skeletal muscle tissue of the aged vs. that of the adult rats, supporting the view that aging affects the expression of multiple genes.

Although the characteristic reduction of g_{Cl} has been observed in a large population of aged rats, a high degree of interindividual variability exists among aged muscle preparations with a small proportion of animals exhibiting values of g_{Cl} similar to that of the adults [8,9]. This variability may be related to either genetic [28] or metabolic factors [2]. It is generally recognized that the extent of age-related changes, such as the general loss of muscle mass or changes in muscle metabolism, varies from muscle to muscle. In addition the control by the motor neural system and the influence of external conditions such as exercise, immobility, nutrition and others may also contribute to the age-related decrease in muscle function [2]. Although the reduction of g_{Cl} is variable among aged animals, this reduction always occurs in parallel with a decrease of ClC-1 mRNA, in the same animal.

Sarcolemmal g_{Cl} plays an essential role in the control of normal excitability of mammalian muscle fibers and is generally thought to be mediated by the ClC-1 channel [22,29]. However, recent studies have hypothesized the presence of a chloride channel different from ClC-1, in the T-system, proposed as responsible for the substantial part of membrane g_{Cl} [19]. This hypothesis weakens the accepted theory that ClC-1 is the major chloride channel contributing for g_{Cl} of mammalian skeletal muscle fibers. Our study demonstrates a linear correlation between ClC-1 and g_{Cl} and strongly supports the hypothesis that ClC-1 is the main determinant of sarcolemmal g_{Cl}. Also we found that the reduction of muscle total protein content is more dramatic than that of the total mRNA. These data, together with the general reduction of protein synthesis, proposed by several authors [4,7], suggest that post-transcriptional events are more affected than transcription during aging, and are in favor of a consequent reduction of ClC-1 protein levels in parallel with its mRNA. The correlation between g_{Cl} and ClC-1 mRNA is consistent with studies of age related ClC-1 expression during early development. In rodent muscle, ClC-1 mRNA levels and g_{Cl} are steeply up-regulated during the first few weeks after birth [21,22,30] and both are reduced following denervation [31,16]. Moreover, lower g_{Cl} values have been observed in the slow-twitch soleus muscle than in the fast-twitch EDL [32] together with lower values of ClC-1 mRNA [16]. Both parameters are undetectable in adr myotonic mice, thus demonstrating the importance of ClC-1 in sustaining muscle g_{Cl} [17].

The genetically myotonic muscles, in which the decrease of g_{Cl} is the unique alteration, make it possible to appreciate the pivotal role of this parameter for excitability and contraction and more generally for muscle function. In fact the abnormally low g_{Cl} causes remarkable membrane hyperexcitability and prolonged contractions. In aged muscle the impact of the low g_{Cl} for muscle function is not as straightforward. In fact the excitation pattern is impaired [8,9] by concomitant changes in the function of other ion channels such as sodium [33] and potassium [34]. Furthermore, fiber contraction is affected by additional modifications in the intracellular structures devoted to calcium release and reuptake mechanisms [35,36]. This evidence implies that, although the degree of impairment of the muscle electrical and contractile characteristics can help to graduate the severity of aging process (i.e. the presence of more or less severe phenotypes), it is not possible to identify in aged muscle a functional parameter that is univocally impacted by g_{Cl}. The voltage threshold for contraction is a functional index that is always affected in aged rat muscle, being constantly shifted toward more negative potentials [25,26]. This change often occurs in parallel with the decrease in g_{Cl} [26]. However, in our experiments, we did not find a linear correlation between this functional index and either g_{Cl} or ClC-1 mRNA, suggesting that the change in ClC-1 expression is likely phenotypic-independent and only correlates with g_{Cl}.

It should be noted that although a linear correlation exists between ClC-1 mRNA and g_{Cl} for the entire group of animals, in a few aged rats the reduction of mRNA was not strictly proportional to the decrease of g_{Cl}. The basis for this disproportional relationship is not clear, but the concomitant occurrence of other factors, such as denervation [2] or alteration of the phosphorylation pathway controlling chloride channel [9], may differently affect g_{Cl} and ClC-1 expression in EDL and TA muscles in certain animals. Compensatory mechanisms acting on channel function may counteract the reduced expression of ClC-1 and adjust g_{Cl} towards the normal levels [17]. Although other channels of the ClC family are expressed, in lower amount in skeletal muscle (i.e. ClC-2) [22], and their contribution to g_{Cl} cannot be excluded, the general impairment of muscle protein synthesis occurring during aging and the strong correlation between ClC-1 expression and g_{Cl} makes it unlikely that different isoforms of chloride channel might contribute, in this situation, to the macroscopic g_{Cl}.

In summary, we have demonstrated that reduced expression of ClC-1 in skeletal muscle fibers of aged rats can account for the reduction in sarcolemmal g_{Cl} observed, which in turn may contribute to the alterations of excitability and contractility and partially justify the age-related decline of skeletal muscle performance. Drugs able to improve muscle protein synthesis may prove beneficial in this condition [9].

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