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The role of L- and T-type Ca^{2+} currents during the *in vitro* aging of murine myogenic (i28) cells in culture

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Abstract

The age-related decline in skeletal muscle strength could, in part, result from alterations in the mechanism of excitation–contraction coupling, responsible for muscle contraction. In the present work, we used the *in vitro* aging of murine myogenic (i28) cells as a model, to investigate whether the inefficiency of aged satellite cells to generate functional skeletal muscle fibres could be partly due to defective voltage-dependent Ca^{2+} currents. The whole-cell patch clamp technique was employed to measure L- and T-type Ca^{2+}currents in myotubes derived from the differentiation and fusion of these cells reaching replicative senescence. Our data showed that the expression and the amplitude of these currents decreased significantly during *in vitro* aging. Moreover, the analysis of the L-type current evoked in *young* and *old* cells by positive voltage steps, revealed no differences in the kinetics of activation, but significant alterations in the rate of inactivation. These effects of *in vitro* aging on voltage-dependent Ca^{2+} currents could also be related to their inability to fuse into myotubes. Taken together, our data support the hypothesis that age-related effects on voltage-dependent L- and T-type currents could be one of the causes of the failure of satellite cells to efficiently counteract the impairment in muscle force.

Keywords: L-type Ca^{2+} channels; T-type Ca^{2+} channels; *In vitro* aging; Skeletal muscle; Murine myogenic cells

1. Introduction

During the aging process, mammalian skeletal muscle undergoes significant changes that have been investigated by several groups in recent years (for review see [1–5]). Indeed, the mechanism underlying impaired motor performance in old age involves the central and peripheral nervous system and the muscle tissue itself. However, it is not still clear how nerve and muscles reciprocally influence gene expression, cellular mechanisms and age-related neurodegeneration [3]. In skeletal muscle, the age-related decline in force is a complex phenomenon linked to sarcopenia, an involuntary loss of muscle mass and strength [6]. One of the main causes of sarcopenia is the reduction of the pool of muscle satellite cells, the most specific myogenic cells available postnatally for skeletal muscle growth and repair (reviewed in [7–9]). Exhaustion of their proliferative ability in aged muscle, could thus be a consequence of the continuous demand on their activity for muscle tissue regeneration [10].

The deficit in specific contractile force (*i.e.* force normalised to muscle cross-sectional area) could not however, be explained by muscle atrophy [11]. Thus, other possible concomitant causes have been suggested, like changes of the sarcolemmal excitability [12,13], and alterations of the mechanisms controlling Ca^{2+} handling [14,15], also altered in the aging process of other tissues (see, for example [16]). In skeletal muscle, it is particularly important to consider alterations in the mechanism of excitation–contraction coupling (EC coupling), responsible for contraction [14,15]. The structural substratum for normal EC coupling is the mechanical interaction between the dihydropyridine-sensitive voltage-dependent L-type Ca^{2+} channels (dihydropyridine receptors: DHPRs), organized in tetrads at the sarcolemma T-tubules, and the ryanodine-sensitive Ca^{2+} channels (ryanodine recep-

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tors: RyR1s) located in the sarcoplasmic reticulum membranes ([17]; reviewed in [18]). Recently, it was shown that both mouse and human aged skeletal muscle fibres exhibit alterations in DHPR and RyR1 expression, which could lead to so-called EC uncoupling [14,15,19,20]. Moreover, in the elderly, muscle fibres derived from the proliferation and differentiation of *in vivo* aged satellite cells could also be characterized by EC uncoupling; thus, sarcopenia could be due, at least in part, to a reduced efficiency of aged satellite cells to generate functional skeletal muscle fibres [21].

The deficit in contractile force could also be a consequence of changes in sarcolemmal excitability. It has already been observed that a reduction in skeletal muscle Cl^- conductance occurs in aged rats [12], and a modification of K^+ channel activity is seen in old rats and humans [13,22,23]. Interestingly, an increase in Na^+ current density has been reported in old rats [24], but not in old mice [25]. Changes in muscle Na^+ channel expression or behaviour would be of great importance in the age-related impairment in muscle force, since voltage-gated Na^+ channels are responsible for the generation and propagation of the action potential in skeletal muscle fibres. The above alterations in sarcolemmal excitability could also involve changes in the properties of voltage-gated Ca^{2+} channels [26] that play an essential physiological role, not only in the function of normal adult skeletal muscle, but also in the process of myogenesis [27]. The DHPR, besides acting as a voltage-sensor to trigger the contractile machinery for EC coupling, produces a slow high voltage-activated (HVA) Ca^{2+} current identified as L-type (I_L). On the other hand, during myogenesis, also a low voltage-activated (LVA) Ca^{2+} current was identified as a transiently expressed T-type current (I_T), which most likely mediates the influx of Ca^{2+} strictly required for fusion between myoblasts or between myoblasts and myotubes [28,30]. Since it was shown [10] that senescent satellite cells fused more slowly and less efficiently, a possible role could also be suggested for a defective T-type channel.

A useful model of *in vivo* aging of satellite cells is the aging of the same cells *in vitro* under culture conditions [31]; this model would allow us to investigate if the replicative senescence affects their regenerating ability. The main goals of the present study were therefore (1) to reproduce the physiological muscle aging process that occurs *in vivo*, by maintaining mouse myogenic cells in culture until the stage of replicative senescence, determined by examining the effect of *in vitro* aging on their ability to differentiate and fuse into myotubes, and (2) to analyse the properties of L- and T-type Ca^{2+} currents in myogenic cells during *in vitro* aging, focusing in particular on the activation and inactivation kinetics of the L-type current, in order to study their possible involvement in age-associated alteration in Ca^{2+} homeostasis. To this end, the whole-cell patch clamp recording technique was employed to measure Ca^{2+} currents in myotubes derived from the differentiation and fusion of satellite cells reaching proliferative senescence.

2. Materials and methods

2.1. Cell culture

All the experiments were performed on myotubes derived from the *in vitro* differentiation and fusion of expanded primary mouse myoblasts (called i28), kindly supplied to us by Dr A. Wernig, Department of Physiology and Medical Policlinic, University of Bonn, Germany (see [32]). Myogenic cells could be maintained as exponentially growing myoblasts in the presence of HAM'S F-10 growth medium (GM) plus 20% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). The myoblasts were plated at a density of about 35,000 cells in 90 mm Petri dishes. To induce cell differentiation and fusion, mononucleated myoblasts were plated at a density of 70,000 cells in 35 mm matrigel-coated Petri dishes (matrigel kindly given to us by Drs A. Albini and D. Noonan, CBA, Genova) and 1 day after plating, the growth medium was replaced with a differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum and L-glutamine, penicillin and streptomycin as above. The differentiation medium was renewed every 3 days to avoid loss of nutrients and growth factors. Cultures were maintained at 37 °C in a humid air atmosphere containing 5% CO_2.

2.2. Cell senescence

The proliferative capacity of myoblasts was evaluated by counting the number of cells in culture at seeding and at harvesting. At each cell passage, the mean population doubling was calculated using the formula:

$$\mathrm{MPD} = \frac{\log(N_h/N_s)}{\log 2}$$

where N_h is the harvest cell number and N_s is the seeded cell number per Petri dish. The number of total divisions reached by the cell culture was derived from the sum of MPD calculated at each passage. The state of 'replicative senescence' (about 90 divisions) was determined by examining the effect of *in vitro* aging on the morphology of the plated myoblasts and the failure of such cells to differentiate, align and start to merge into multinucleated myotubes within 4 days in differentiation medium, renewed every 2 days of culture.

2.3. Differentiation index

At different population doublings, the efficiency of differentiation of i28 cultures was quantified by counting the percentage of myotubes and the mean number of nuclei per myotube. Nuclei were revealed under fluorescence microscopy by DAPI (4,6-diamino-2-phenylindole) staining; the cells plated on matrigel-coated coverslips were first fixed at room temperature in freshly prepared 3.7% paraformaldehyde in PBS for 15 min, permeabilized by a 5 min incubation in 100% methanol and then stained for 10 min with DAPI