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# Partial Recovery of Skeletal Muscle Sodium Channel Properties in Aged Rats Chronically Treated with Growth Hormone or the GH-Secretagogue Hexarelin[1]

JEAN-FRANÇOIS DESAPHY, ANNAMARIA DE LUCA, SABATA PIERNO, PAOLA IMBRICI and DIANA CONTE CAMERINO
*Unit of Pharmacology, Department of Pharmacobiology, Faculty of Pharmacy, University of Bari, Bari, Italy*
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**ABSTRACT**

This study was aimed at investigating the effects of chronic treatment of aged rats with growth hormone (GH, 8 weeks) or the GH-secretagogue hexarelin (4 weeks) on the biophysical modifications that voltage-gated sodium channels of skeletal muscle undergo during aging, by means of the patch-clamp technique applied to fast-twitch muscle fibers. Two phenotypes of aged-rat fibers could be discriminated on the basis of channel conductance. In the young phenotype, sodium channels present a conductance of 18 pS as in young-adult rats. In the aged phenotype, channels present a conductance of 9 pS while ensemble average currents activate and inactivate more slowly. Nevertheless, in all situations, sodium channels shared a number of biophysical properties, such as open probability, mean open time, steady-state inactivation and use-dependent inhibition. Furthermore, channel density on extrajunctional sarcolemma was higher in aged rats, a result independent of the phenotype. Chronic treatment of aged rats with either GH or hexarelin restored current kinetics but not channel conductance and density. These results confirm the specific age-related changes in sodium channel behavior and show that treatment with either GH or hexarelin has partial restorative effects. Moreover, hexarelin restored the firing capacity of fast-twitch muscle fibers, as did GH in previous studies. These findings support the possible therapeutic value of the synthetic peptide in cases of GH deficiency, as in the elderly.



Voltage-gated sodium channels are responsible for the initial rise and the subsequent conduction of action potential in excitable tissues as skeletal muscle (Hille, 1984). These channels are closed at the resting potential, open in response to membrane depolarization and then close rapidly in less than 1 ms, entering a fast-inactivated state. Moreover, prolonged depolarization pushes sodium channels to a slow-inactivated state. Fast and slow inactivation are relieved by membrane hyperpolarization. Modification of sodium channel gating can modify sarcolemma excitability and, as a consequence, can alter contractile properties of skeletal muscle. For example, most of the mutations in the gene encoding the skeletal muscle sodium channel $\alpha$-subunit in a set of inherited neuromuscular disorders (hyperkalemic periodic paralysis, paramyotonia congenita and the potassium aggravated myotonias) impair the inactivation process of the channel and lead to sarcolemma hyperexcitability (for review, see Cannon, 1997).

An impairment of muscle performance, including decrease in muscle strength and speed of contraction, is also observed during aging (Gutmann *et al.*, 1971; Larsson *et al.*, 1979; Caccia *et al.*, 1979). Before dramatic macroscopic alterations of muscle structure such as loss of muscle mass (Ermini, 1976; Carlsen and Walsh, 1987) and denervation (Gutmann and Hanzlikova, 1975; Pettigrew and Gardiner, 1987), early events have been shown to include modification of the calcium-sequestering activity of sarcoplasmic reticulum (De Coster *et al.*, 1981; Carlsen and Walsh, 1987; Larsson and Salviati, 1989), impairment of excitation-contraction coupling (De Luca and Conte Camerino, 1992; Delbono *et al.*, 1995), and alteration of sarcolemma excitability (De Luca *et al.*, 1990). The latter can be related to the complex age-related changes in ion channels present in the plasma membrane. In rat fast-twitch muscle fibers, the enhanced activation of protein kinase C that occurs during aging leads to reduction of the macroscopic chloride conductance (De Luca *et al.*, 1992; 1994a). Biophysical and pharmacological properties of ATP-sensitive potassium channels are modified by

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**ABBREVIATIONS:** GH, growth hormone; IGF-1, insulin-like growth factor 1; Hex, hexarelin; TTX, tetrodotoxin; *N*/*n*, number of rats/number of fibers; FDB, flexor digitorum brevis; EDL, extensor digitorum longus; RP, resting potential; $I_{th}$, injected-current threshold to elicit an action potential; AP, amplitude of the action potential; *n* spikes, maximum number of action potentials elicited by an injected current of high intensity.

age-related redox potential change (Tricarico and Conte Camerino, 1994), whereas the activity of calcium-activated potassium channels increases with advancing age (Tricarico *et al.*, 1997), resulting in an increase of the macroscopic potassium conductance (De Luca *et al.*, 1994b; Tricarico *et al.*, 1997). In addition, dihydropyridine-sensitive calcium currents are reduced in skeletal muscle fibers of aged humans and mice (Delbono *et al.*, 1995; Messi *et al.*, 1997). We also found, in a preliminary study, that single sodium channel conductance is reduced in some fibers of the fast-twitch FDB muscle of aged rats but that the number of available sodium channels in extrajunctional sarcolemma is generally greater, which results in enhanced sodium currents (Desaphy *et al.*, 1997).

The physiopathological process of aging is complex and results from multiple factors. GH may play an important role among them, because its secretion is markedly reduced in the elderly (Cocchi, 1992; Ho and Hoffman, 1993). Thus GH replacement therapy was proposed for aged persons and was shown to result in beneficial effects (Rudman *et al.*, 1990). Accordingly, chronic treatment of aged rats with GH improves the macroscopic chloride conductance and sarcolemma excitability of skeletal muscle (De Luca *et al.*, 1994b). This effect is mimicked *in vitro* by IGF-1, which suggests that this peptide is the mediator of GH at the muscular level for its effect on chloride channels (De Luca *et al.*, 1997). In the short term, the peptide may stimulate a serine-threonine phosphatase that is able to counteract the enhanced age-related activation of protein kinase C and then to increase the chloride conductance (De Luca *et al.*, 1997). Furthermore, IGF-1 may induce the neosynthesis of chloride channels in the long term (De Luca *et al.*, 1997). However, administration of GH requires injection, which results in nonphysiological prolonged elevation of hormone serum levels and can induce undesirable side effects (Papadakis *et al.*, 1996). Recent studies have stimulated increasing interest in synthetic GH secretagogues that show oral biodisponibility and act at the level of the hypothalamus-pituitary gland axis, releasing GH in a physiological pulsatile manner (Argente *et al.*, 1996). Because their effects seem little influenced by aging, these compounds are of potential therapeutic interest in treatment of the elderly (Argente *et al.*, 1996). One of these compounds is the hexapeptide hexarelin (His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lys-$NH_2$) which is currently the object of clinical studies (Ghigo *et al.*, 1996).

The present study was aimed at investigating in more detail the biophysical modifications that muscle voltage-gated sodium channels undergo during aging by means of the patch-clamp technique applied to skeletal muscle fibers freshly dissociated from the FDB muscle of young-adult and aged rats. Experiments were performed after chronic treatment of aged rats with growth hormone (8 weeks) or hexarelin (4 weeks). The results confirm that specific age-related changes occur on sodium channels and show that the pharmacological treatment has partial restorative effects. We also measured the macroscopic excitability parameters of the EDL muscle fibers of hexarelin-treated aged rats by means of the standard two-intracellular-microelectrode technique. As expected from its effect on sodium channels, hexarelin treatment also had beneficial results, as did GH in previous studies (De Luca *et al.*, 1994b; De Luca *et al.*, 1997).

## Materials and Methods

**Animal care and treatment procedure.** We used 18 control young-adult (4–6 months) and 30 aged (21–30 months) male Wistar rats for all the experiments. Randomly chosen among the aged group, four rats received 150 $\mu$g/kg rat growth hormone s.c. (provided by the National Hormone and Pituitary Program, NIDDK, N.I.H., Bethesda, MD) 6 days a week for 8 weeks, and eight other rats received 80 $\mu$g/kg hexarelin s.c. (kindly provided by Pr. D. Cocchi, Institute of Pharmacology, University of Brescia, Brescia, Italy) 6 days a week for 4 weeks. Most of the 18 control aged rats received an equivalent volume of saline s.c. as placebo. All animals survived the treatment. We could not find any correlation between evolution in body weight and treatment. Muscle functional activity was controlled before animal sacrifice; one control aged rat showed paralysis of hind limbs and was eliminated from the study.

**Measure of excitability parameters.** Some excitability parameters of the skeletal muscle fibers were measured in vitro in seven hexarelin-treated aged rats and in seven aged and eight young-adult rats randomly taken in the control groups. The effect of the treatment with GH has been investigated in other studies and the results already published (De Luca *et al.*, 1994b, 1997). Briefly, the EDL muscles were dissected from animals under urethane anesthesia (1.2 g/kg i.p.) and placed in a 25-ml muscle bath maintained at 30°C and continuously perfused with a 95/5-$O_2$/$CO_2$ gassed physiological solution (148 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.44 $NaH_2PO_4$, 5.5 mM glucose, pH 7.3). The RP was measured with a single standard microelectrode. Excitability parameters related largely to the sodium permeability were measured in current clamp mode by means of the standard two-intracellular-microelectrode technique. A steady holding current was first injected into the fiber to clamp the membrane potential at −80 mV before application of depolarizing current pulses 100 ms in duration (De Luca *et al.*, 1994b). The following excitability parameters were then measured: $I_{th}$, AP and $n$ spikes.

**Recording of sodium currents.** Animals were killed either by decapitation or by an overdose of urethane (i.p. injection). The FDB muscles of the hind feet were promptly removed and placed in Ringer's solution (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM MOPS, 5 mM glucose, pH 7.3) supplemented with 2.5 to 3 mg/ml collagenase (3.3 I.U./ml, type XI-S, Sigma, St Louis, MO). They were shaken at 70 $min^{-1}$ for 1 to 3 hours at 32°C under a 95% $O_2$/5% $CO_2$ atmosphere. All along the incubation, dissociated cells were sampled and rinsed several times with bath recording solution (145 mM CsCl, 5 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.3) before being transferred into the RC-11 recording chamber (Warner Instrument, Hamden, CT). Most of the fibers appear intact with visible sarcomere striation under an 400×-inverted microscope (Axiovert 100, Zeiss, Germany). However, some fibers isolated from aged-rat muscles appeared clearly atrophied and were discarded.

Single-channel currents were recorded at room temperature (21 ± 2°C) in the cell-attached and the inside-out configurations of the patch-clamp method (Hamill *et al.*, 1981) with the AxoPatch 1D amplifier and the CV-4-0.1/100U headstage (Axon Instruments, Foster City, CA). Pipettes were formed from Corning 7052 glass (Garner glass, Claremont, CA) with an automatic puller (Zeitz Instruments, Augsburg, Germany). They were coated with sylgard 184 (Dow Corning, Belgium) and heat-polished on a microforge (MF-83, Narishighe, Tokyo, Japan). Pipettes had resistance ranging from 2.0 to 3.3 MΩ when filled with recording pipette solution (150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.3).

Voltage-clamp protocols and data acquisition were performed on a PC-compatible computer with Pclamp 6.0 software (Axon Instruments, Foster City, CA) through a 12-bit AD/DA interface (digidata 1200, Axon Instruments, Foster City, CA). Currents elicited by pulses at a frequency of 2 Hz were low-pass-filtered at 2 kHz (−3 dB) by the amplifier four-pole Bessel filter and digitized at 40 kHz. In the

cell-attached mode, patches were stimulated by a depolarizing test pulse applied from −100 to −20 mV at a frequency of 2 Hz during at last 5 min before initiating recordings in order to allow fiber membrane potential to stabilize. In the CsCl-rich bath solution, membrane potential was depolarized to −7.6 ± 1.0 mV as measured on 45 FDB muscle fibers of young-adult rats by means of a single intracellular microelectrode. Thus we used the same voltage-clamp protocols in both cell-attached and inside-out modes to measure single-channel conductance. All other parameters were measured only in the inside-out configuration.

Data analysis was performed with Clampfit and Fetchan programs (Pclamp 6.0 software package). Subtracting the average of blank sweeps from the records eliminated capacity transients and leak. For a given depolarization, sodium current depends on the product of the single-channel conductance, the number of channels ready to open $N$ and the open probability $P_o$. The single-channel conductance is voltage-independent and was determined as the slope of the single-channel current-voltage relationship. Single-channel current amplitudes $i$ were evaluated either by eye or by all-points amplitude histogram when satisfactory resolution was reached. $N$ was estimated at −100 mV by measuring the maximum peak current amplitude elicited by depolarizing the membrane from −100 to −20 mV (Kimitsuki *et al.*, 1990). For comparison between patches, $N$ was normalized by the square of pipette conductance, which is assumed to be linearly correlated to the patch membrane area (Sakmann and Neher, 1983). We performed patches on extrajunctional membrane far from the fiber endplate to avoid heterogeneous distribution of sodium channels along the sarcolemma (Ruff and Whittlesey, 1993). Availability of sodium channels is voltage-dependent and this voltage dependence can be described by the steady-state inactivation curve as follows: Macroscopic-like ensemble average currents were constructed from at least 100 test pulses applied every 0.5 s to −20 mV, varying the holding potential from −120 to −70 mV. The ensemble average peak currents were normalized with respect to the maximum peak current, $I_{max}$, obtained at −120 or −110 mV and were reported as a function of the holding potential. Experimental points were fitted with the Boltzmann equation

$$I/I_{max} = 1/\{1 + \exp[(V - V_{h_{1/2}})/K]\}$$

where $V_{h_{1/2}}$ is the potential to have half of the channels inactivated and $K$ is the slope factor. The open probability of sodium channels is voltage-dependent. We measured $P_o$ at −20 mV, the potential at which it may have reached its maximum (Kimitsuki *et al.*, 1990), by dividing the time integral of the ensemble average current by $iN$. We also studied the time dependence of sodium currents by measuring the fast-kinetics parameters of the ensemble average current—*i.e.*, the time necessary to reach the peak current (time to peak) and the time constant of the current decay ($\tau$h). When patches contained fewer than six channels, analysis of channel open time was performed. Open-time histograms were constructed using the half-amplitude threshold criterion (Colquhoun and Sigworth, 1983). Overlapping events were discarded and also closed times less than 0.4 ms, which were considered as burst activity. Histograms were well fitted with a mono-exponential by the simplex Maximum Likelihood fitting routine of the Pstat program (Pclamp 6.0 software package). Because patches always contained more than one channel, closed-time analysis was not performed. Finally, we studied the use-dependent inhibition that we observed when the membrane patch was repetitively depolarized from −100 to 0 mV, which is due to cumulative slow inactivation of sodium channels (Wang and Wang, 1997).

**Statistical analysis.** Average results are given as mean ± S.E.M. ($N$, number of rats/$n$, number of fibers). Statistics were performed by $\chi^2$ test or Student's $t$ test for unpaired data with $P < .05$ taken to indicate the minimum significant difference.

## Results

Membrane depolarization elicited sodium channel openings in more than 90% of the patches in both young-adult and aged rats. Both single openings and overlapping events occurred generally at the beginning of the test pulse. Long openings, late openings and reopenings were also observed along the 50-ms pulse. Interpulse duration of 0.45 s (stimulation frequency of 2 Hz) at a holding potential of −100 mV allowed complete recovery of the sodium channels inactivated during the test pulse of 50 ms when the inside-out patch potential was kept negative. Thus stable recordings were obtained when the test pulse was −20 mV, as illustrated in figure 1. By contrast, when the patch membrane was repetitively depolarized to 0 mV, sodium currents exhibited a use-dependent inhibition with reduction of the number of channel openings (fig. 1). Such a use-dependent inhibition was shown to be due to cumulative slow inactivation of sodium channels at depolarized potentials, which cannot recover during the hyperpolarized interpulse (Wang and Wang, 1997). Use-dependent inhibition was observed at 0 mV in young-adult and aged-rat fibers (fig. 1) and developed at a similar rate in all fiber types (table 1). As a consequence, sodium currents elicited at 0 mV from the holding potential of −100 mV were totally inhibited in about 500 pulses. Hyperpolarization a few minutes long at −100 mV was required for the initial current amplitude to be recovered (not shown). All these behaviors were constantly observed in inside-out patches of aged rats, regardless of the pharmacological treatment (table 1) and of the fiber phenotype (see below).

**Effect of GH and hexarelin on conductance, availability and open probability of sodium channels.** We observed that skeletal muscle fibers of aged rats could be classified in two groups on the basis of the single sodium channel conductance measured in the inside-out patch-clamp mode (Desaphy *et al.*, 1997). We defined 1) a young phenotype composed of the aged-rat fibers showing a sodium channel conductance close to 18 pS and overlapping that observed in young-adult rat fibers and 2) an aged phenotype composed of the aged-rat fibers presenting a lower sodium channel conductance of 9 pS. The same feature was presently found in the cell-attached mode (fig. 2). Combined results obtained in cell-attached and inside-out modes give a single-channel conductance of 18.0 ± 0.5 pS (14 rats/19 fibers) in young-rat fibers. In 15 control aged rats, conductance was distributed among two mean values, 18.7 ± 0.5 pS (15/20) for the young phenotype and 9.7 ± 0.4 pS (15/15) for the aged phenotype. The conductance level of aged-rat fibers belonging to the aged phenotype is significantly smaller than the conductance levels of young-adult rat fibers and aged-rat fibers belonging to the young phenotype ($P < .001$). Treated aged rats showed a similar binomial distribution of fibers, with conductance mean values of 19.5 ± 0.7 pS (4/7) and 8.6 ± 0.8 pS (4/6) for GH and 17.9 ± 0.9 pS (8/13) and 9.4 ± 0.7 pS (8/6) for hexarelin. The incidence of aged-phenotype fibers was similar in the control aged rats and the GH-treated aged rats (fig. 2D). In hexarelin-treated animals, the incidence of the aged phenotype appeared slightly lower compared with control aged animals, but the difference was not significant (fig. 2D). Both phenotypes were observed in 7 out of 10 aged rats where more than two fibers were investigated for single-channel conductance.

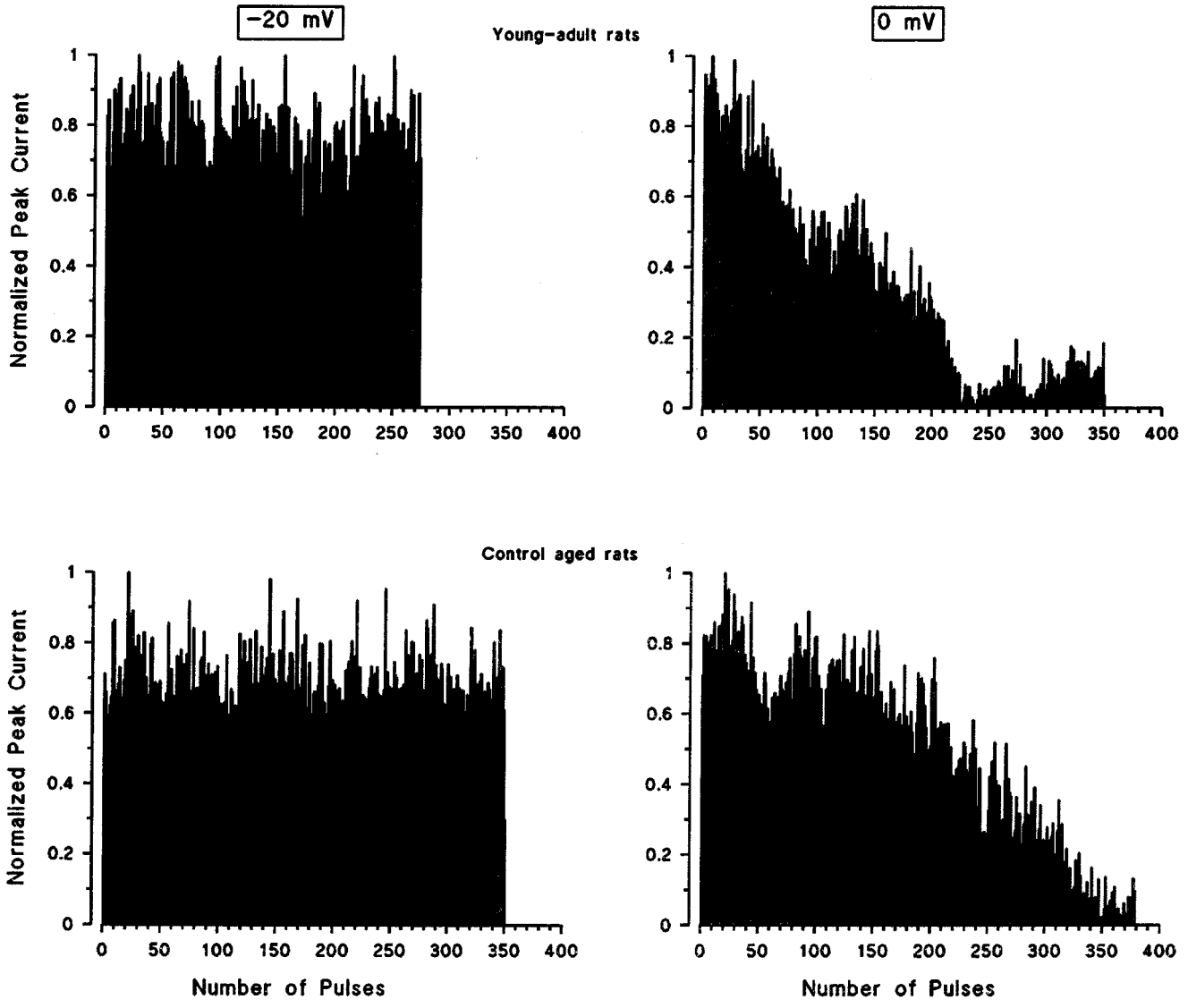


**Fig. 1.** Use-dependent inhibition of sodium currents in inside-out patches from rat skeletal muscle fibers. Peak sodium current was measured for each 50-ms depolarizing pulse applied from −100 to −20 mV (left column) or 0 mV (right column) at the frequency of 2 Hz, normalized by the maximum peak current and reported as a function of the number of pulses. Linear regression of the graph gives a measure of the rate of use-dependent inhibition. Representative patches for young-adult rat fibers presented a use-dependent inhibition rate of −0.29 and −2.40 per $10^3$ pulses at −20 and 0 mV, respectively. Representative patches for control aged rat fibers presented a use-dependent inhibition rate of −0.09 and −2.10 per $10^3$ pulses at −20 and 0 mV, respectively. Such behavior was observed independently of the conductance-based phenotype and of the pharmacological treatment.

TABLE 1
Use-dependent inhibition rate of sodium current at −20 and 0 mV

| Group of rats | *N*/*n* | Rate at −20 mV | *N*/*n* | Rate at 0 mV |
|---|---|---|---|---|
| Young-adult | 4/5 | −0.03 ± 0.16 | 6/7 | −1.89 ± 0.55* |
| Control aged | 6/7 | −0.02 ± 0.06 | 7/8 | −1.88 ± 0.24** |
| GH-treated aged | 3/3 | −0.01 ± 0.09 | 2/3 | −2.04 ± 0.33*** |
| Hex-treated aged | 5/7 | +0.08 ± 0.24 | 2/3 | −1.65 ± 0.29*** |

The use-dependent inhibition rate of sodium currents recorded in inside-out patches was calculated by linear regression of the normalized peak current on the number of pulse as illustrated in figure 1 and is given as mean ± S.E.M. per 1000 pulses. *N*/*n*: number of rats/number of fibers. For a given potential, no significant difference was found among fiber types, but the rate observed at 0 mV is significantly higher than the rate observed at −20 mV (*P < .05; **P < .01; ***P < .005).

Aged-rat fibers showed a significantly (P < .05 and less) greater number of available channels per membrane area at −100 mV than young-rat fibers, a result independent of the pharmacological treatment (table 2). This difference remained when, on the basis of single-channel conductance measurement, we discriminated aged-rat fibers between young and aged phenotypes. One exception was for young-phenotype fibers of control aged rats that showed no significant difference from young-adult fibers (.05 < P < .10), a result that may be due to the limited number of fibers investigated. Neither drug treatment showed any sign of inducing recovery of the available channel number, which was unchanged, or in one case was significantly greater in fibers of treated aged rats than in control aged-rat fibers (table 2). Moreover, the voltage dependence of sodium channel availability (steady-state inactivation) was not modified by aging either in fibers belonging to the young phenotype or in fibers belonging to the aged phenotype (fig. 3).

The open probability of sodium channels measured at −20 mV was not significantly altered by aging (table 3), nor did chronic treatment with GH or hexarelin induce any change (table 3).

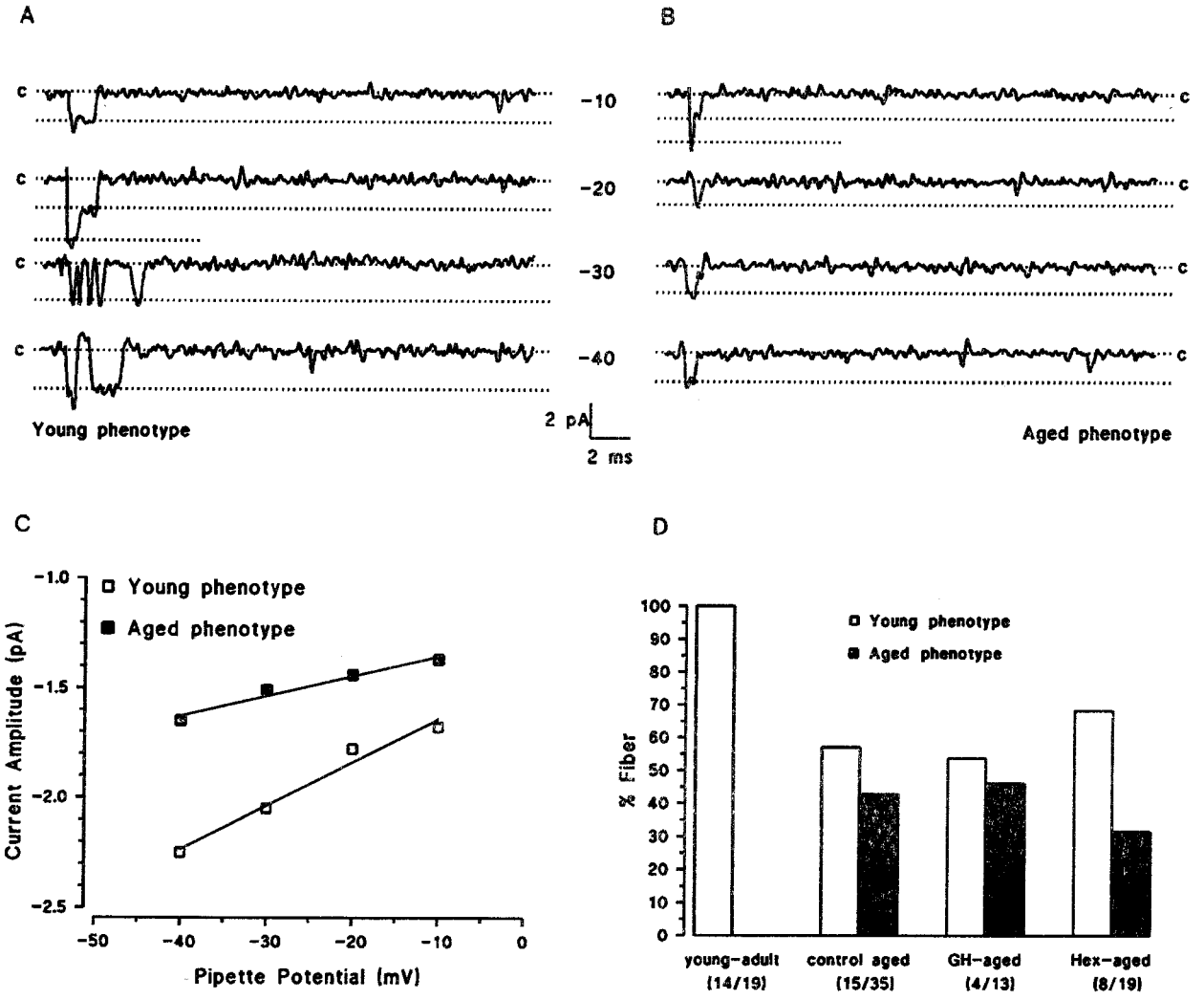


**Fig. 2.** Sodium channel conductance in skeletal muscle fibers of aged rats. Openings of sodium channels were elicited in the cell-attached mode by depolarizing the patch membrane to the indicated potentials at the frequency of 2 Hz. The holding potential here was −70 mV in order to reduce the number of overlapping events. Representative patches for young (panel A) and aged (panel B) phenotypes, observed in the same aged rat, contained at least three and seven channels, respectively. C) The slopes of linear regressions of current-voltage relationships give conductances of 19.8 pS and 9.1 pS for the patches shown in panels A and B, respectively. D) No significant difference in the incidence of either phenotype of conductance was found between control aged rats and growth hormone-treated aged (GH-aged) rats ($\chi^2 = 0.425$, $P > .50$), between control aged rats and hexarelin-treated aged (Hex-aged) rats ($\chi^2 = 0.670$, $P > .25$) or between GH-aged rats and Hex-aged rats ($\chi^2 = 1.552$, $P > .10$). Numbers in parentheses indicate *n* fibers investigated in *N* rats of each group as (*N*/*n*).

**Effect of GH and hexarelin on time dependence of sodium currents.** Aging modifies the fast kinetics of ensemble average sodium currents recorded in the inside-out configuration. Sodium currents of aged-phenotype fibers of control aged rats activated and inactivated more slowly than those of young-adult rat fibers and those of young-phenotype fibers of control aged rats (fig. 4A, and B). The time to peak and the decay time constant of aged-phenotype fibers of control aged rat were significantly higher than those of young-phenotype fibers of the same animals at −50, −40 and −30 mV (fig. 4C, and D). At −20 mV the results tended to be similar, although they were not significant. At 0 mV we were unable to find any modification. This suggests a voltage dependence of the aging-induced modification of fast kinetics, although it may be argued that at the depolarized potentials of −20 and 0 mV, the kinetics of sodium currents are so fast that it becomes difficult to demonstrate any significant change. Interestingly, both drug treatments shortened the current kinetics at −50, −40, −30 and −20 mV toward values similar to those observed in the young-phenotype fibers of the same animals (fig. 4C, and D).

The ensemble average sodium current is a combination of the all latency distribution (latencies for a channel first to open and then to reopen) and the mean open time (Aldrich *et al.*, 1983; see also Wang *et al.*, 1996). No difference in the mean open time of sodium channels was found between young and aged phenotypes of control aged rats (table 4). Latencies for first opening and reopenings were not measured because of the constant presence of more than one channel in the patch and because of the limited number of collected events. Modification of gating mode may also affect the fast kinetics of ensemble average and macroscopic currents (Zhou *et al.*, 1991; Chang *et al.*, 1996). In fact, sodium channels show a complex gating behavior that can be simplified in two main gating modes (Zhou *et al.*, 1991). The fast-gating mode that occurs the most often is characterized by

TABLE 2
Number of available sodium channels per membrane area at −100 mV

| Group of rats | Phenotype | *N*/*n* | *N*/membrane area |
|---|---|---|---|
| Young-adult | Total | 17/32 | 30.6 ± 7.2 |
| Control aged | Total | 14/33 | 62.0 ± 9.1** |
| | Young | 9/8 | 52.4 ± 9.8 |
| | Aged | 9/12 | 79.1 ± 12.2*** |
| GH-treated aged | Total | 4/18 | 68.7 ± 16.5* |
| | Young | 2/3 | 140.9 ± 52.8*** |
| | Aged | 2/3 | 112.5 ± 7.2*** |
| Hex-treated aged | Total | 8/28 | 56.9 ± 10.7* |
| | Young | 7/10 | 62.8 ± 15.4* |
| | Aged | 7/6 | 96.6 ± 28.6*** |

The number of available sodium channels (*N*) per membrane area was calculated from inside-out patches as described under "Materials and Methods" and is expressed as mean ± S.E.M. for the total number of fibers (Total) of young-adult rats, control aged rats, GH-treated aged rats and hexarelin-treated aged rats. In control and drug-treated aged rats, the mean ± S.E.M. was also calculated for fibers demonstrated to belong to the young phenotype or the aged phenotype (discrimination based on the measure of single-channel conductance). *N*/*n*: number of rats/number of fibers. Statistics were done with Student's *t* test for unpaired data between each group of aged-rat fibers (total, young phenotype, and aged phenotype) and the fibers of young-adult rats (*P < .05; **P < .01; ***P < .005). No significant difference was found between corresponding phenotypes of each group of aged rats, except between young-phenotype fibers of control and GH-treated aged rats (P < .05).

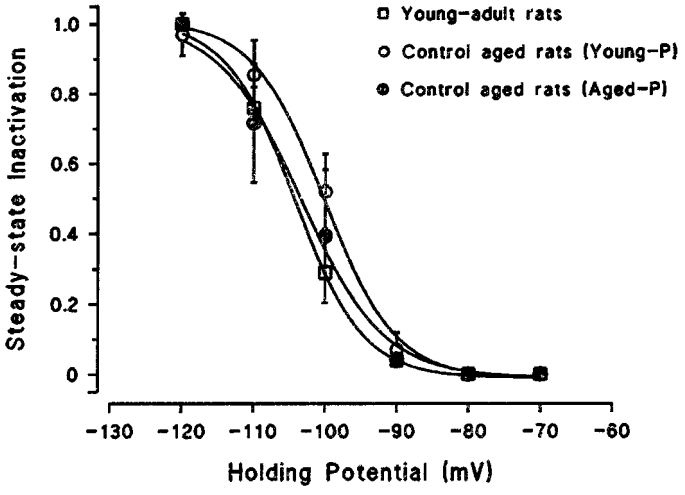


**Fig. 3.** Steady-state inactivation of sodium currents in inside-out patches from skeletal muscle fibers of young-adult and control aged rats. Ensemble sodium currents are averaged from at least 200 test pulses applied at −20 mV every 0.5 s. Peak currents are normalized and reported as a function of the holding potential ranging from −120 to −70 mV. Symbols represent the mean ± S.E.M. for each fiber group. The curves fit the experimental points to the Boltzmann equation as described under "Materials and Methods." The mean potential for having half of the channels inactivated $V_{h_{1/2}}$ is −105.0 ± 1.5, −100.2 ± 0.51 and −103.4 ± 0.78 mV, and the mean slope factor *K* is 4.0 ± 0.6, 4.1 ± 0.5 and 5.0 ± 0.0 mV for young-adult rat fibers (number of rats/number of fibers: 4/4), young-phenotype (Young-P) aged-rat fibers (5/4) and aged-phenotype (Aged-P) aged-rat fibers (5/3), respectively. No significant difference in the voltage-dependence of steady-state inactivation was found.

rapid opening upon depolarization followed by rapid inactivation and very few reopenings. The slow-gating mode, which occurs relatively rarely, is characterized by bursting activity of long duration with numerous reopenings. Channels switch normally between the two gating modes, and the balance between modes is influenced by modulating factors. Accordingly, we observed both gating modes in our experiments, but we did not see any apparent difference in their relative frequency of occurrence between young-adult and control aged rats or between young and aged phenotypes of control aged rats (not shown). Also, we saw no evidence of any change in gating mode in fibers of drug-treated aged rats. Thus such a mechanism was not responsible for the alteration of fast kinetics that we observed in this study. We discarded bursting gating-mode events from open-time histograms so the mean open times presented in table 4 correspond exclusively to the fast gating mode.

TABLE 3
Open probability of sodium channels at −20 mV

| Group of rats | Phenotype | *N*/*n* | $P_o$ |
|---|---|---|---|
| Young-adult | | 6/7 | 0.22 ± 0.04 |
| Control aged | Young | 8/7 | 0.27 ± 0.02 |
| | Aged | 8/7 | 0.32 ± 0.06 |
| GH-treated aged | Young | 2/6 | 0.26 ± 0.07 |
| | Aged | 2/3 | 0.19 ± 0.09 |
| Hex-treated aged | Young | 3/3 | 0.24 ± 0.11 |
| | Aged | 3/3 | 0.17 ± 0.04 |

The open probability ($P_o$) of sodium channels recorded in inside-out patches was calculated by dividing the time integral of ensemble average currents by the single-channel current *i* and the number of available channels *N*. Ensemble average currents were constructed from at least 200 depolarizing pulses from −100 to −20 mV applied every 0.5 s. *N*/*n*: number of rats/number of fibers. Average results are given as mean ± S.E.M. No significant difference was found between young-phenotype or aged-phenotype fibers of each group of aged rats and the young-adult rat group, between the two phenotypes in the same aged-rat group or between corresponding phenotypes of each group of aged rats (Student's *t* test for unpaired data).

**Effect of hexarelin on the macroscopic excitability parameters of EDL muscle fibers.** EDL muscle fibers of eight young-adult and seven aged rats randomly taken in the control groups and seven hexarelin-treated aged rats were investigated for the excitability parameters related to the sodium current (table 5). These parameters are the injected-current threshold needed to elicit an action potential ($I_{th}$), which depends on sodium and chloride conductances; the amplitude of AP, which depends on sodium and potassium conductances; and *n* spikes, which depends on sodium, potassium and chloride conductances. The control aged rats showed the classical aging-related modifications of excitability. The $I_{th}$ and the *n* spikes were lower than those of young-adult rats (P < .01). The membrane RP was also significantly reduced (P < .005). On the other hand, the AP was slightly increased in these aged rats. Treatment of aged rats for 4 weeks with hexarelin demonstrated some beneficial effects on excitability parameters (table 5). The *n* spikes value was restored to that observed in young-adult rats (P = .138 *vs.* young-adult rat fibers and P < .005 *vs.* control aged-rat fibers), and the $I_{th}$ showed a tendency to increase (P = .060 *vs.* young-adult rat fibers), although it was still not significantly different from that observed in control aged-rat fibers (P = .073). The RP was also partly restored in hexarelin-treated rat fibers (P = .068 *vs.* young-adult rat fibers and P = .073 *vs.* control aged-rat fibers). In addition, the AP became significantly higher than that of young-adult rat fibers (P < .01). The sensitivity of control aged rat EDL fibers to TTX was also evaluated by measuring the fibers' ability to generate action potential in response to current stimulation. In the presence of 100 nM TTX in the bath solution, the muscle excitability of control aged rats was drastically inhibited.

## Discussion

**Modification of skeletal muscle sodium channels by aging.** We observed that skeletal muscle fibers of the fast-twitch FDB muscle of aged rats can be discriminated in two phenotypes on the basis of single sodium channel conductance measured in the inside-out configuration of the patch-

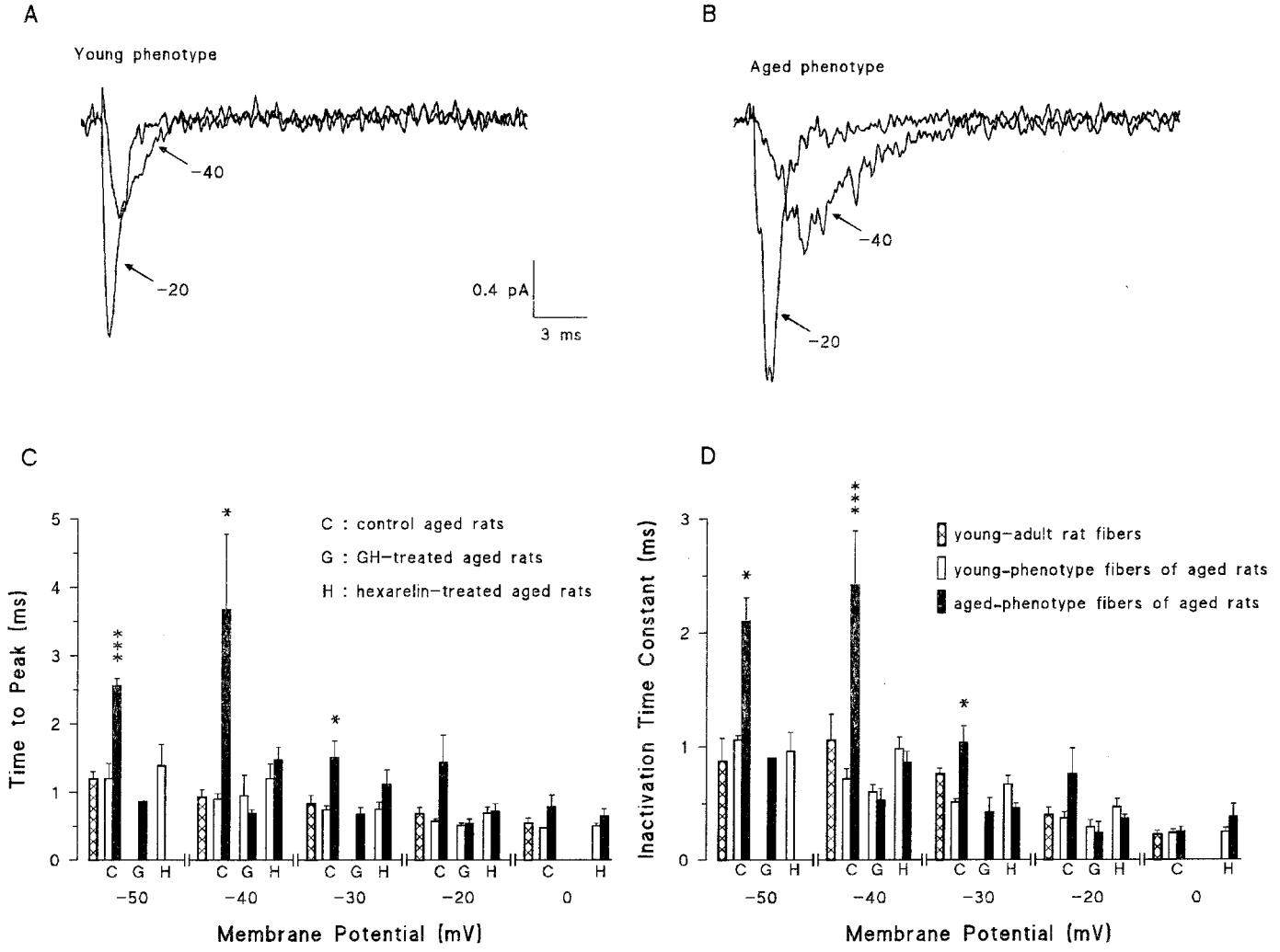


**Fig. 4.** Slower activation and inactivation of sodium currents in control aged-rat skeletal muscle fibers. Ensemble average sodium currents were constructed from at least 100 depolarizing pulses applied from a holding potential of −100 mV to potentials of −50 to 0 mV every 0.5 s. Representative currents at −40 and −20 mV for young (A) and aged (B) phenotypes were obtained in inside-out patches of control aged-rat fibers containing at least seven and nine channels with a single conductance of 17.1 pS and 7.4 pS, respectively. Time to peak (panel C) and decay time constant (panel D) are reported as the mean ± S.E.M. of 1 to 10 patches from young-adult rat fibers (cross-filled bars) and young phenotype fibers (open bars) and aged phenotype fibers (solid bars) of control aged rats (C), GH-treated aged rats (G) and hexarelin-treated aged rats (H). In control aged rats, aged phenotype fibers showed significantly longer time to peak and decay time constant than young phenotype fibers, at −50, −40, and −30 mV (*P < .05; ***P < .005) but not at −20 and 0 mV. Chronic treatment of aged rats with GH or hexarelin enabled the sodium-current fast kinetics of aged-phenotype fibers at all tested potentials to recover toward those of young-phenotype fibers of the same animals.

TABLE 4
Mean open time of sodium channels at −40 and −20 mV

| Group of rats | Phenotype | *N*/*n* | Mean Open Time at −40 mV (ms) | *N*/*n* | Mean Open Time at −20 mV (ms) |
|---|---|---|---|---|---|
| Young-adult | | 6/9 | 0.321 ± 0.109 | 8/11 | 0.453 ± 0.078 |
| Control aged | Young | 7/4 | 0.385 ± 0.053 | 7/5 | 0.399 ± 0.086 |
| | Aged | 7/5 | 0.356 ± 0.092 | 7/4 | 0.423 ± 0.102 |

The mean open time of sodium channels recorded in inside-out patches was calculated as the exponential time constant of the open-time histograms as described under "Materials and Methods" and is expressed as mean ± S.E.M. *N*/*n*: number of rats/number of fibers. No significant difference was found among fiber types.

clamp method (Desaphy *et al.*, 1997). We defined a young phenotype characterized by a conductance of 18 pS, which overlaps that found in young-adult rat fibers and closely corresponds to that of the adult form of the mammalian skeletal muscle sodium channel SkM1 (Trimmer *et al.*, 1989) measured in similar conditions (Franke and Hatt, 1990; Ruff, 1996). In addition, about 50% of the aged-rat fibers showed a conductance of approximately 9 pS; we defined this as the aged phenotype. In the present study, we confirm this result by measuring single-channel conductance in both inside-out and cell-attached modes. Thus the "half-conductance" was not due to the potential stress that the patch membrane could suffer during patch excision. During development *in vitro* and *in vivo* (Lombet *et al.*, 1983; Weiss and Horn, 1986) and during denervation (Pappone, 1980), skeletal muscle fibers express a juvenile form of sodium channel, namely SkM2 (Kallen *et al.*, 1990), which has a low conductance and a low sensitivity to TTX. We sought to exclude denervation from our experiments by controlling the functional muscle activity of aged rats before each sacrifice and by patching only the fibers that did not show any sign of denervation under the 400× magnification of our inverted microscope, such as atrophy or abnormal fiber diameter or length. Moreover, in the presence of 100 nM TTX, excitability of control aged-rat fibers was drastically inhibited, which suggests that

TABLE 5
Effects of hexarelin on macroscopic excitability parameters

| Group of rats | *N*/*n* | $I_{th}$ (nA) | AP (mV) | *N*/*n* | *n* Spikes | *N*/*n* | RP (mV) |
|---|---|---|---|---|---|---|---|
| Young-adult | 8/33 | 113.0 ± 4.1 | 96.8 ± 1.7 | 8/30 | 4.2 ± 0.3 | 2/9 | 69.7 ± 2.4 |
| Control aged | 7/30 | 84.4 ± 5.8** | 101.9 ± 5.0 | 7/27 | 2.7 ± 0.5** | 7/30 | 60.3 ± 1.2*** |
| Hex-treated aged | 7/40 | 99.3 ± 5.8 | 108.1 ± 3.7** | 7/29 | 5.5 ± 0.8† | 7/33 | 64.1 ± 1.6 |

The excitability parameters related largely to the sodium current were measured by means of the standard two-intracellular-microelectrode technique performed on EDL muscles. These parameters are the injected-current threshold needed to elicit an action potential ($I_{th}$), the amplitude of the action potential (AP), and the maximum number of action potentials elicited by raising the injected-current intensity (*n* spikes). Moreover, the resting membrane potential (RP) was measured by means of a single intracellular microelectrode. *N*/*n*: number of rats/number of fibers. Results are expressed as mean ± S.E.M. and compared with unpaired Student's *t* test. **P < .01; ***P < .005 for comparison between control aged rats or hexarelin-treated aged rats and young-adult rats. †P < .005 for comparison between hexarelin-treated aged rats and control aged rats.

aged-rat fibers did not express TTX-resistant sodium channels. In addition, young and aged phenotypes shared a number of voltage- and time-dependent biophysical properties, including steady-state inactivation, open probability, mean open time and use-dependent inhibition at 0 mV. All of this suggests that the same channel, modulated during aging, accounts for both phenotypes.

Young-phenotype and aged-phenotype fibers of control aged rats, differing in single-channel conductance, differed also in the fast kinetics of macroscopic-like currents. Indeed, fibers showing the low single-channel conductance showed slower activation and inactivation of ensemble average sodium currents. This effect was significant only at the weaker depolarizations, which suggests a possible dependence on voltage. Slower current activation suggests that the first latency for the channel to open is longer. Slower inactivation can come from a longer channel mean open time and/or from a greater number of reopenings. However, we show that mean open time was not significantly altered in fibers belonging to the aged phenotype, and we did not see any apparent modification of the equilibrium among the fast-gating and slow-gating modes that sodium channels can enter. Therefore, it seems that the slowing of ensemble average sodium current inactivation in control aged-rat fibers of the aged phenotype resulted from the slowing of activation overlapping the current decaying phase rather than from a true alteration of the sodium channel fast-inactivation process. We do not yet know what is responsible for these modifications, but sodium channel gating is for example sensitive to phosphorylation, lipid environment, channel protein glycosylation and interaction with the associated $\beta_1$-subunit, which might be altered in aged-rat muscle.

Independently of the conductance-based phenotype, the number of available channels for a given membrane area measured at −100 mV was higher in aged-rat fibers than in young-adult rat fibers. Because the voltage dependence of channel availability was not modified, this indicates a greater number of physical channels in the extrajunctional sarcolemma of aged-rat fibers. This may be due to the age-related alteration of protein synthesis (Ermini, 1976; Carlsen and Walsh, 1987), leading to an increased sodium channel expression, and/or to the reorganization of sarcolemma observed during aging (Gutmann *et al.*, 1971), leading to the redistribution of sodium channels along the membrane.

**Pathophysiological relevance of age-related modifications of sodium channel properties.** Age-related reduced performance and weakness of skeletal muscle was shown to be primarily due to alterations in the motor unit, including modification of sarcolemma excitability. In mammalian fast-twitch skeletal muscle fibers, a large macroscopic chloride conductance stabilizes the resting membrane potential and thus controls membrane excitability (Bretag, 1987). In some muscle disorders, reduction of the chloride conductance results in exacerbated firing of action potentials, inducing sustained contraction referred to as myotonia (Lehmann-Horn and Rüdel, 1996). In aged rats, the chloride conductance is markedly reduced and the membrane becomes more excitable so that the electrical threshold necessary to initiate a first action potential is lowered but firing capacity is reduced (De Luca *et al.*, 1990; De Luca *et al.*, 1994b). In addition, the potassium conductance is higher in aged-rat muscle compared to muscle in young-adult rats (Tricarico *et al.*, 1997). The control aged rats investigated in the present study showed modifications of the muscle excitability parameters similar to those reported in previous studies. Because sodium channel properties also appeared to be modified in these aged rats, it is likely that sodium channels may also participate in the aging-induced modification of muscle excitability. The higher number of available sodium channels on the sarcolemma of aged-rat fibers, together with the reduced chloride conductance, would contribute to increased membrane excitability, reducing the stimulation needed to initiate an action potential. The higher sodium current may also account for the larger AP observed in the aged rats of the present study. However, increased entry of sodium ions in the fiber, together with the age-related increase in potassium conductance, would accelerate and promote accumulation of potassium ions in the transverse tubular system, quickly leading to the muscle flaccid paralysis and weakness observed in the elderly. The reduced rate of sodium current activation and the higher potassium conductance may both contribute to the reduction of firing activity in aged-rat skeletal muscle fibers. However, the effects of the slowing of sodium current kinetics, which occurred in only half of the control aged-rat fibers, may have been underestimated because excitability parameters were averaged from the total population of aged-rat fibers. Also, it is important to note that comparison between macroscopic excitability parameters and microscopic sodium current properties must be limited to qualitative consideration, because the former were measured in intact fibers and the latter in collagenase-isolated fibers. In conclusion, although the relative participation of the different ion permeabilities is difficult to establish, combined modifications of chloride, potassium and sodium conductances may explain the age-related modification of muscle excitability, which may contribute, together with the reported aging-induced alteration of excitation-contraction coupling (Delbono *et al.*, 1995; Messi *et al.*, 1997), to the perturbation of contraction that occurs in the skeletal muscle of aged subjects.

**Effects of chronic treatment of aged rats with GH or hexarelin on age-related modifications of sodium channel properties.** GH is one of the hormonal factors the reduced secretion of which has been associated with aging process (Cocchi, 1992; Ho and Hoffman, 1993). Chronic treatment of aged rats with GH has been shown partially to restore the macroscopic chloride conductance of skeletal muscle sarcolemma as well as the macroscopic potassium conductance (De Luca *et al.*, 1994b; De Luca *et al.*, 1997). The longer the treatment, the better the recovery, but significant effect was already obtained with a treatment performed for 6 to 8 weeks. In the present study, we show that an 8-week treatment did not allow recovery of the sodium channel conductance, the aged phenotype occurring with a similar incidence in all aged rats. Availability of sodium channels was still higher in skeletal muscle fibers of GH-treated aged rats. By contrast, GH allowed the recovery of fast-kinetics parameters toward adult values in aged-phenotype fibers. This is of particular interest because 1) it supports the hypothesis that young-phenotype and aged-phenotype conductances are carried by the same channel isoform, and 2) it indicates that conductance and fast-kinetics parameters are modified by aging through two distinct pathways or by the same pathway but with distinct sensitivities. Maybe a longer treatment is required to reverse completely the modifications that sodium channels undergo during aging. The mechanism by which GH may influence sodium channel kinetics is difficult to establish. Our previous studies suggest that the effect of GH on chloride conductance is likely to be mediated, at least in part, by IGF-1 through an okadaic acid-sensitive phosphatase (De Luca *et al.*, 1997). However, GH exerts a wide action on protein synthesis, which may result in a variety of effects through a variety of pathways. As a result, the combined recovery of sodium channel fast kinetics and chloride and potassium conductances induced by GH treatment allows the recovery of firing capacity of skeletal muscle fibers of aged rats. The treatment of aged rats during 4 weeks with the GH secretagogue hexarelin mimicked the treatment with GH at the sodium channel level. The peptide also increased $I_{th}$ and AP and allowed the total recovery of the firing capacity of the fast-twitch muscle fibers, as GH did in previous studies (De Luca *et al.*, 1994b; De Luca *et al.*, 1997). This supports the hypothesis that modification of sodium current kinetics may contribute to the impairment of the firing capacity observed in aged rats. By contrast, the reduction of the number of extrajunctionnal sodium channels did not parallel the recovery of $I_{th}$, which suggests that another GH target, perhaps the chloride conductance, is the limiting factor for the age-related reduction of $I_{th}$. In addition, hexarelin partially restored the resting membrane potential of aged-rat muscle fibers toward that of the young-adult muscle fibers. All these results provide another argument for the therapeutic use of hexarelin in treatment of the elderly.

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**Send reprint requests to:** Prof. Diana Conte Camerino, Unità di Farmacologia, Dipartimento Farmaco-Biologico, Facoltà di Farmacia, Università degli Studi di Bari, via Orabona 4, campus, I-70125 Bari, Italia.