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## Effects of carnosine on contractile apparatus $\text{Ca}^{2+}$ sensitivity and sarcoplasmic reticulum $\text{Ca}^{2+}$ release in human skeletal muscle fibers

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**Dutka TL, Lamboley CR, McKenna MJ, Murphy RM, Lamb GD.** Effects of carnosine on contractile apparatus  $\text{Ca}^{2+}$  sensitivity and sarcoplasmic reticulum  $\text{Ca}^{2+}$  release in human skeletal muscle fibers. *J Appl Physiol* 112: 728–736, 2012. First published December 15, 2011; doi:10.1152/jappphysiol.01331.2011.—There is considerable interest in potential ergogenic and therapeutic effects of increasing skeletal muscle carnosine content, although its effects on excitation-contraction (EC) coupling in human muscle have not been defined. Consequently, we sought to characterize what effects carnosine, at levels attained by supplementation, has on human muscle fiber function, using a preparation with all key EC coupling proteins in their in situ positions. Fiber segments, obtained from vastus lateralis muscle of human subjects by needle biopsy, were mechanically skinned, and their  $\text{Ca}^{2+}$  release and contractile apparatus properties were characterized.  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus was significantly increased by 8 and 16 mM carnosine (increase in  $\text{pCa}_{50}$  of  $0.073 \pm 0.007$  and  $0.116 \pm 0.006$  pCa units, respectively, in six type I fibers, and  $0.063 \pm 0.018$  and  $0.103 \pm 0.013$  pCa units, respectively, in five type II fibers). Caffeine-induced force responses were potentiated by 8 mM carnosine in both type I and II fibers, with the potentiation in type II fibers being entirely explicable by the increase in  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus caused by carnosine. However, the potentiation of caffeine-induced responses caused by carnosine in type I fibers was beyond that expected from the associated increase in  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus and suggestive of increased  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. Thus increasing muscle carnosine content likely confers benefits to muscle performance in both fiber types by increasing the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus and possibly also by aiding  $\text{Ca}^{2+}$  release in type I fibers, helping to lessen or slow the decline in muscle performance during fatiguing stimulation.

contractile apparatus; ryanodine receptors; fiber types;  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release

CARNOSINE, AN ENDOGENOUS CYTOPLASMIC dipeptide ( $\beta$ -alanyl-L-histidine), has been shown to be important in a myriad of myocellular processes such as pH buffering, membrane stabilization, and acting as an osmotic shock protector, anti-oxidant, and anti-aging agent (1, 2, 4, 8, 20, 36, 42). We have previously shown that carnosine (4–16 mM) increases the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in rat extensor digitorum longus (EDL) fibers in a concentration-dependent manner (14). Carnosine has also been shown to increase the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in chemically skinned fibers from a variety of nonmammalian species (29). However, the effects of carnosine on contractile properties in human muscle have not been examined, including the question of whether the effects of carnosine differ between different fiber types. It has

also been suggested that carnosine directly stimulates  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR), increasing the  $\text{Ca}^{2+}$  sensitivity of ryanodine receptors (RyRs) and potentiating  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR). These suggestions were based on findings of studies with SR vesicles from rabbit hindlimb muscle (7) and isolated porcine RyRs and chemically skinned fibers of human vastus lateralis muscle (48). However, in all these cases, the  $\text{Ca}^{2+}$  release and RyR activation was elicited in the presence of abnormally low cytoplasmic free  $[\text{Mg}^{2+}]$ , and hence the channels were not subject to the normal strong resting inhibition exerted by cytoplasmic  $\text{Mg}^{2+}$  (26, 30, 33). Furthermore, the RyRs were not coupled as normal to the voltage-sensor proteins [dihydropyridine receptors (DHPRs)] in the adjacent transverse tubular (t-system), coupling that likely also exerts a strong resting inhibitory effect on the channels (49). In a previous study, our laboratory could find no evidence that carnosine heightens  $\text{Ca}^{2+}$  release in near physiological conditions in rat EDL (type II) fibers (14), but its actions on  $\text{Ca}^{2+}$  release in human muscle fibers remain to be examined. Since the aforementioned effects of carnosine on muscle processes and EC coupling could potentially be important in helping to slow the development of muscle fatigue in humans (2, 17, 39), these remain important issues for understanding the potential ergogenic and therapeutic actions of carnosine.

Carnosine levels in muscle can be increased either directly by L-carnosine supplementation or indirectly by  $\beta$ -alanine supplementation, whereby carnosine synthase rapidly converts  $\beta$ -alanine to carnosine inside muscle fibers (Refs. 3, 4, 6, 13, 46; see reviews in Refs. 12, 40). Carnosine content in human vastus lateralis muscle seemingly remains unchanged with 4–16 wk of various resistance or endurance training regimes (see review in Refs. 5, 12), whereas numerous studies have shown that carnosine content in various human leg muscles (including vastus lateralis) can be markedly increased by supplementation (12, 40).

It is also known that the carnosine content in human muscle is fiber-type dependent, with fast-twitch (type II) fibers containing approximately twice as much carnosine as slow-twitch (type I) fibers. The normal physiological concentration of carnosine in human vastus lateralis muscle ranges from  $\sim 10.5$  to  $17.8$  mmol/kg dry muscle mass (dm) in type I fibers and  $\sim 23.2$  to  $32.6$  mmol/kg dm in type II fibers (18, 19, 44). L-carnosine and  $\beta$ -alanine supplementation can raise the vastus lateralis carnosine content  $\sim 1.5$ - to  $2$ -fold, from  $\sim 17.8$  to  $34.3$  mmol/kg dm in type I fibers and from  $\sim 29.6$  to  $46.6$  mmol/kg dm in type II fibers (19). In another study that measured carnosine content in human vastus lateralis muscle (not fiber typed), 4 wk of L-carnosine supplementation increased muscle carnosine content from  $23.2$  to  $39.5$  mmol/kg dm, and similarly

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4 wk of  $\beta$ -alanine supplementation increased muscle carnosine content from 24.2 to 35.3 mmol/kg dm (17). Carnosine is a cytoplasmic dipeptide, and when the aforementioned carnosine contents in human vastus lateralis muscle are expressed relative to fiber cytoplasmic volume, the ranges equates to  $\sim 4$ – $7$  mM in type I fibers and  $\sim 9$ – $13$  mM in type II fibers, with supplementation increasing both by  $\sim 7$ – $8$  mM to  $\sim 14$  mM and  $\sim 18$  mM in type I and II fibers, respectively.

Using mechanically skinned muscle fibers from human vastus lateralis biopsies, it is possible to determine the direct effects of carnosine on the properties of the contractile apparatus and on SR  $\text{Ca}^{2+}$  release with the key EC coupling proteins in their in situ position and under broadly physiological conditions. Importantly, in this preparation, carnosine can be precisely and rapidly added or removed from the fiber, making it possible to examine the effects of both normal physiological carnosine levels and levels attained via supplementation on the individual EC coupling steps, with each fiber acting as its own control. We hypothesized that the presence of carnosine at physiological concentrations would cause an increase in the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in a concentration-dependent manner in both type I and type II fibers from human vastus lateralis muscle. Additionally, we also sought to determine whether the presence of carnosine sensitizes either fiber type to CICR when the RyRs remain coupled to the DHPRs and with the cytoplasmic free  $[\text{Mg}^{2+}]$  maintained at its normal physiological level ( $\sim 1$  mM).

## MATERIALS AND METHODS

**Muscle biopsy, subject details.** Five healthy subjects, 4 men and 1 woman (age  $28 \pm 3$  yr; height,  $173 \pm 6$  cm; body mass,  $70 \pm 9$  kg) gave written, informed consent and participated in this study. These subjects were all recreationally active, i.e., performed regular physical activities such as jogging and cycling (30–60 min, 2–3 times/wk) but were not specifically trained in any sport. All protocols and procedures performed were approved by the Human Research Ethics Committees at Victoria University and La Trobe University. After a local anaesthetic was injected into the skin and fascia [1% lidocaine (Xylocaine)], a small incision was made in the middle third of the vastus lateralis muscle of each subject, and a muscle sample was taken using a Bergstrom biopsy needle (32). An experienced medical practitioner took all biopsies at approximately constant depth. The excised muscle sample was rapidly blotted on filter paper to remove excess blood and placed in paraffin oil (Ajax Chemicals, Sydney, Australia) at  $10^\circ\text{C}$  for 45 min before individual muscle fibers were dissected.

**Fiber mounting.** The muscle biopsy was pinned at resting length in a petri dish lined with Sylgard 184 (Dow Corning, Midland, MI) and immersed in paraffin oil and kept cool ( $\sim 10^\circ\text{C}$ ) on an icepack. Using jeweler's forceps, segments of individual fibers were mechanically skinned (as described in Ref. 26) and mounted at 120% of resting length on a force transducer (AME801, Horten) with a resonance frequency of  $>2$  kHz. The fiber was then transferred to a 2-ml Perspex bath containing standard  $\text{K}^+$ -based solution that broadly mimics the intracellular milieu (see below).

**Solutions.** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. The standard K-HDTA solution used for examining SR  $\text{Ca}^{2+}$  release experiments contained (in mM) 50 hexa-methylene-diamine-tetraacetate ( $\text{HDTA}^{2-}$ ; Fluka, Buchs, Switzerland), 8 total ATP, 37  $\text{Na}^+$ , 126  $\text{K}^+$ , 8.5 total  $\text{Mg}^{2+}$  (giving 1 mM free  $[\text{Mg}^{2+}]$ ), 10 phosphocreatine (CP), 0.05 total EGTA, 90 HEPES, pH 7.1, and pCa ( $-\log_{10}[\text{Ca}^{2+}]$ ) 7.0, except where stated. The large concentration of HEPES in all solutions

clamps the pH at 7.1. Where required, the SR of the skinned fiber was totally depleted of releasable  $\text{Ca}^{2+}$  by using a K-HDTA solution with 30 mM caffeine, 0.05 mM free  $\text{Mg}^{2+}$  (total  $\text{Mg}^{2+}$  adjusted to 2.15 mM), and 0.5 mM EGTA (pCa 8.0) to chelate released  $\text{Ca}^{2+}$  (16, 22). When the properties of the contractile apparatus were examined, it was necessary to use heavily  $\text{Ca}^{2+}$ -buffered solutions in which all HDTA was replaced with EGTA (i.e., relaxing solution) or CaEGTA (i.e., maximum  $\text{Ca}^{2+}$ -activating solution), as described previously (24). The pCa of solutions (for pCa of  $<7.2$ ) was measured with a  $\text{Ca}^{2+}$ -sensitive electrode (Orion Research, Boston, MS). In addition, a strontium-based solution (at pSr 5.3) was made by mixing relaxing solution (50 mM EGTA) with Sr-EGTA solution similar to the maximum  $\text{Ca}^{2+}$ -activating solution; this solution was used to functionally determine the likely fiber type (pSr 5.3 force level of  $<5\%$  for fast-twitch and  $>60\%$  for slow-twitch fibers; see Refs 9, 31, 45). The osmolality was  $295 \pm 5$  mosmol/kg $\text{H}_2\text{O}$  for all solutions except for the carnosine stock solutions.

An 80 mM  $\text{K}^+$ -carnosine stock solution was made similarly to the K-HDTA solution by adding 80 mM carnosine and reducing the [HEPES] from 90 to 81 mM, with the  $[\text{HDTA}^{2-}]$ ,  $[\text{Mg}^{2+}]$ , [ATP], [CP], etc. all kept the same as in the standard K-HDTA solution. At pH 7.1, carnosine is  $\sim 33\%$  positively charged, and total charge compensation meant that the  $[\text{K}^+]$  in the stock solution was reduced (by  $\sim 27$  mM). The osmolality of this carnosine stock solution was increased from the standard level of  $\sim 295$  to  $\sim 346$  mosmol/kg $\text{H}_2\text{O}$ . The changes to the osmolality were quite small in the final solutions when using either 8 or 16 mM carnosine (i.e., the differences in osmolality in the final solutions were  $\sim 5$  and 10 mosmol/kg $\text{H}_2\text{O}$  for 8 and 16 mM carnosine, respectively). The relative changes to ionic strength were even less (i.e., 0.6 and 1.1 mM for final concentrations of carnosine of 8 and 16 mM, respectively). The effects of these small changes to ionic strength and osmolality would not have appreciably altered the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus (27). Also, carnosine negligibly binds to  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  at pH 7.1. The effect of carnosine on the properties of the contractile apparatus was also examined in four fibers from one of the subjects after chemically skinning the fibers with the detergent Triton X100 in relaxing solution (1% vol:vol, 10-min treatment followed by two separate 1-min wash periods) to remove all membranous structures such as the SR.

**$\text{Ca}^{2+}$ -release experiments.** After mounting the skinned fiber segment on the force transducer and equilibrating it briefly (i.e., 2 min) in K-HDTA intracellular solution, the SR of the fiber was depleted of all of its releasable  $\text{Ca}^{2+}$  by exposing the fiber to the "full release" solution (containing 30 mM caffeine, 0.05 mM free  $[\text{Mg}^{2+}]$ , 0.5 mM EGTA, pCa 8), eliciting a force response that was used as an estimate of the endogenous amount of  $\text{Ca}^{2+}$  (22). The fiber was then washed in the standard K-HDTA solution to remove the 30 mM caffeine and to raise the free  $[\text{Mg}^{2+}]$  back up to 1 mM. The SR of the fiber was then reloaded with  $\text{Ca}^{2+}$  to various levels by bathing the fiber in a "load" solution (pCa 6.7, 1 mM total CaEGTA-EGTA) for different times (5–60 s). The SR was then again depleted with the full release solution, and the fiber was washed again. This load-release-wash cycle was repeated several times to determine a load period that produced a similar size force response to that found initially with the endogenous level of SR  $\text{Ca}^{2+}$  (see Fig. 3) and then to load the SR to that level, or above or below, as desired. When the amount of  $\text{Ca}^{2+}$  taken up into the SR after a given load time (as in Table 2) was estimated, it was assumed to increase approximately in proportion with the load time, provided that the SR was loaded well to its maximum capacity (see Ref. 45).

The  $\text{Ca}^{2+}$  sensitivity of the RyRs was tested using a submaximal caffeine solution made by adding 8 mM caffeine to the standard K-HDTA intracellular solution; note that this solution had free  $[\text{Mg}^{2+}]$  maintained at 1 mM, not lowered to 0.05 mM level as in the full release solution, and also it had only weak  $\text{Ca}^{2+}$  buffering by EGTA (50  $\mu\text{M}$  total) to allow possible self-reinforcement of any  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR). By using such a submaximal



stimulus, it was possible to readily determine whether carnosine modified the responsiveness of the RyRs to CICR. After each release of  $\text{Ca}^{2+}$  in the 8 mM caffeine solution (15-s exposure), the SR was totally depleted of any remaining releasable  $\text{Ca}^{2+}$  by exposing the fiber to the full release solution. The cycle was then repeated with, and then again without, 8 mM carnosine present in both the wash (i.e., preequilibration, 30 s) and the 8 mM caffeine solutions.

**Contractile apparatus experiments.** After the  $\text{Ca}^{2+}$ -handling properties of the fiber had been examined, the effects of 8 and 16 mM carnosine on the contractile apparatus were examined by directly activating the contractile apparatus with a series of heavily  $\text{Ca}^{2+}$ -buffered solutions of different known pCa with and without carnosine present. The range of progressively higher free  $[\text{Ca}^{2+}]$  in solutions (0.1–20  $\mu\text{M}$ ) was produced by mixing appropriate mixtures of the 50 mM EGTA and the 50 mM CaEGTA solutions. This sequence of solutions elicited force responses resembling a staircase (see Fig. 2). When a given carnosine concentration was added to a sequence of test solutions, an equivalent volume of the standard K-HDTA solution was added to the matching control solutions (i.e., no carnosine). Thus, for the cases with 8 mM carnosine, all solutions (both control and carnosine) contained 45 mM EGTA-CaEGTA and 5 mM HDTA. Fibers were preequilibrated in carnosine for 2 min in relaxing solution before examining its effects on  $\text{Ca}^{2+}$  activation.

For each given condition, the isometric force responses elicited at each pCa were expressed as a percentage of the corresponding maximum  $\text{Ca}^{2+}$ -activated force (max) plotted against the pCa value and a Hill curve fitted using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

**Western analysis.** After the physiological study, each fiber examined was placed in an Eppendorf tube containing 10  $\mu\text{l}$  of relaxing solution (50 mM EGTA pCa of >9 described above) with 2:1 vol/vol of 3 $\times$  solubilizing buffer, which contained 0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8. Fibers were stored at  $-80^\circ\text{C}$  until analyzed. Western blots were performed to determine the myosin heavy chain (MHC) and troponin C (TnC) isoforms present, as described previously (35). Both fast and slow TnC isoforms are detected with the same antibody, and they are distinguished by their differing mobilities on SDS-PAGE gels, with the fast TnC isoform travelling more slowly (i.e., at a larger molecular weight) than the slow TnC isoform (see Fig. 1). Total denatured protein from individual fiber segments was separated on 4–15% Criterion TGX Stain Free gels

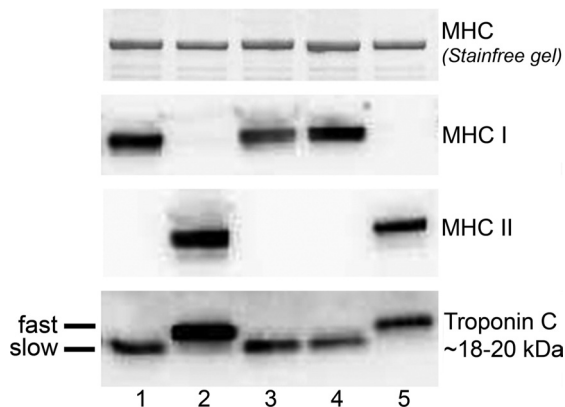


Fig. 1. Fiber-typing of vastus lateralis muscle fibers by Western blotting. Western blots for myosin heavy chain isoforms (MHC I and MHC II) and for troponin C (TnC) in a single segment of each of five vastus lateralis fibers (lanes 1–5) following completion of the physiological measurements on each segment. Top: MHC band imaged on stain-free gel before transfer of proteins to the membrane (band density indicative of amount of fiber loaded in each lane); membrane probed first for troponin C and then reprobed for MHC I and then MHC II.

(Bio-Rad, Hercules, CA; and see Ref. 35) and then transferred to nitrocellulose. Membranes were exposed to mouse primary antibodies diluted in 1% BSA in PBST [anti-MHC I: 0.19  $\mu\text{g}/\text{ml}$ , mouse monoclonal IgM, clone A4.840; anti-MHC II: 0.15  $\mu\text{g}/\text{ml}$ , mouse monoclonal IgG, clone A4.74; both purchased from Developmental Studies Hybridoma Bank (DSHB), University of Iowa; and anti-TnC: 0.2  $\mu\text{g}/\text{ml}$ , rabbit polyclonal, sc-20642, Santa Cruz, CA], following which the appropriate secondary antibody (i.e., either goat anti-mouse or goat anti-rabbit) conjugated with horseradish peroxidase was added to the membranes. Bands were visualized using West Femto chemiluminescent substrate (ThermoScientific), and images were captured using Quantity One software (Bio-Rad).

**Statistics.** All values are presented as means  $\pm$  SE, with  $n$  denoting the number of individual fibers examined. Statistical significance ( $P < 0.05$ ) was determined using paired Student's one or two-tailed  $t$ -tests where appropriate.

## RESULTS

In this study, the following experiments were carried out in the indicated order on each individual skinned muscle fiber: 1) the endogenous SR  $\text{Ca}^{2+}$  content was estimated in relative terms by emptying the SR of all its releasable  $\text{Ca}^{2+}$  and then reloading to that level, or above or below, as desired; 2) the effects of carnosine on RyR sensitivity to a submaximal caffeine stimulus was tested at various levels of SR  $\text{Ca}^{2+}$  content; 3) the effects of carnosine on the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus was examined using heavily  $\text{Ca}^{2+}$ -buffered solutions, and also the  $\text{Sr}^{2+}$  sensitivity was examined as an indicator of fiber type; and 4) the MHC and TnC isoforms present in the given fiber segment were determined by Western blotting.

The full set of the above characteristics were examined in each of 11 vastus lateralis muscle fibers from four human subjects. Figure 1 shows the MHC and TnC isoforms detected in five of the fibers examined (from two different subjects). Of the total fiber set examined, six fibers contained only the MHC I isoform, and the other five contained only the MHC II isoform, and accordingly they were classified as type I and type II fibers, respectively. In every case, the TnC isoforms present were in accord with the MHC isoform, being almost exclusively the slow isoform of TnC in the type I fibers and the fast isoform in the type II fibers (see Fig. 1, bottom). Both type I and type II fibers were obtained from all three of the male subjects, but type II fibers only were obtained from the female subject. The two type II fibers obtained from the female subject were not noticeably different from the type II fibers obtained from the three male subjects in regard to the effects of carnosine on both the response to caffeine and the properties of the contractile apparatus. All of the type I fibers examined here had broadly similar SR loading and  $\text{Ca}^{2+}$  release properties, which differed appreciably from those of the type II fibers examined (see later for details); there was no apparent difference in these measures between the type I fibers obtained from different subjects, nor between the type II fibers obtained from different subjects.

**Effect of carnosine on contractile apparatus properties in human vastus lateralis muscle fibers.** The properties of the contractile apparatus in each fiber were determined by directly activating the contractile apparatus in the skinned segment with heavily-buffered high  $[\text{Ca}^{2+}]$  solutions (see MATERIALS AND METHODS). As shown in Fig. 2A, each fiber was exposed to the pCa 4.7 solution (i.e., 20  $\mu\text{M}$  free  $\text{Ca}^{2+}$ ) to ascertain maximum  $\text{Ca}^{2+}$ -activated force (max.) and then exposed to a  $\text{Sr}^{2+}$ -

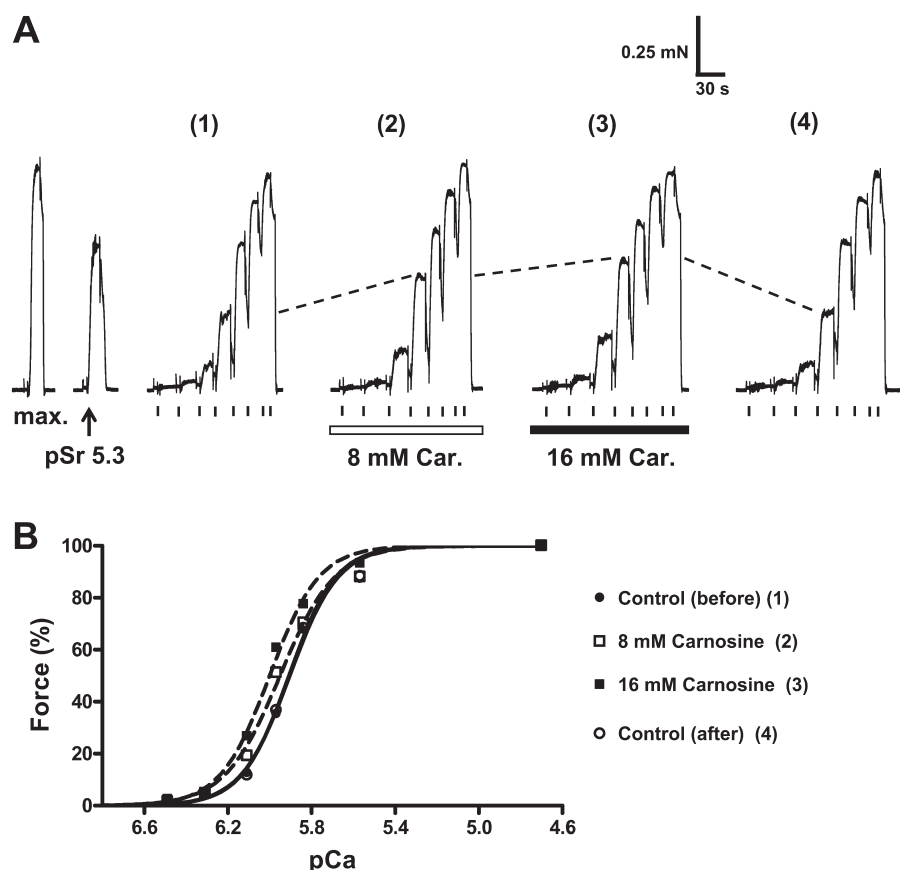


Fig. 2. Carnosine increases  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in human vastus lateralis fibers. *A*: force responses elicited in a human type I fiber when directly activating the contractile apparatus with heavily  $\text{Ca}^{2+}$ -buffered solutions with progressively higher free  $[\text{Ca}^{2+}]$  (pCa of successive solutions: >9.00, 6.49, 6.31, 6.11, 5.97, 5.84, 5.57, 4.7, then back to >9.00, marked by ticks under each force trace) and responsiveness to solution at pSr 5.3. Force-pCa staircases elicited before carnosine addition (control) in the presence of 8 and 16 mM carnosine (Car.) and again after washout of carnosine. Broken line joins force levels produced at pCa 5.97 in absence and presence of carnosine. *B*: Hill fits to force-pCa staircases from *A*. Values before (●) and after addition of carnosine (○) are joined by overlapping black lines, and values for 8 mM (□) and 16 mM (■) carnosine are joined by broken lines. The  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus was increased in carnosine (i.e., force-pCa curve shifted to left).

containing solution at pSr 5.3 to determine whether the fiber predominantly contained the fast or the slow isoform of TnC (9, 31, 45). In each of the 11 human vastus lateralis fibers examined here, the response to  $\text{Sr}^{2+}$  was totally consistent with the TnC isoform expression subsequently identified by Western blotting (e.g., Fig. 1).

The  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in each fiber was then examined, in both the absence and the presence of 8 and 16 mM carnosine (Fig. 2). In every type I and type II fiber examined, the presence of 8 mM carnosine caused an increase in the force level produced at submaximal  $[\text{Ca}^{2+}]$ , as seen by comparing the force levels in force-pCa staircases for runs 1 and 2 for the type I fiber shown in Fig. 2A, where the broken line indicates the force reached at pCa 5.97 in each condition. Submaximal force increased even further in the presence of 16 mM carnosine (e.g., staircase 3 in Fig. 2) and

dropped back to the original control level upon washout of the carnosine (staircase 4). Maximum  $\text{Ca}^{2+}$ -activated force on average was unchanged or only marginally increased in the presence of 8 or 16 mM carnosine (Table 1). Fitting Hill curves to the force-pCa data in each fiber (e.g., Fig. 2B) highlighted how carnosine increases the  $\text{Ca}^{2+}$ -sensitivity of the contractile apparatus by increasing the  $\text{pCa}_{50}$  (the pCa at half-maximal force) without significantly affecting the Hill coefficient (the steepness of the force-pCa relationship). On average, the presence of 8 and 16 mM carnosine significantly increased the  $\text{pCa}_{50}$  by approximately +0.07 and +0.12 pCa units, respectively, in type I fibers and by approximately +0.06 and +0.10 pCa units, respectively, in type II fibers ( $P < 0.05$ ; see Table 1). There was no significant difference between the fiber types in the effects of carnosine, and in both fiber types the increase in  $\text{pCa}_{50}$  was significantly greater with 16 mM carnosine than

Table 1. Carnosine increases the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in human vastus lateralis fibers

Treatment	Max, % Control	$\Delta\text{pCa}_{50}$	$\Delta h$	<i>n</i>
Type II				
8 mM carnosine	102 ± 3	+0.063 ± 0.018*	+0.3 ± 0.3	5
16 mM carnosine	101 ± 3	+0.103 ± 0.013*,†	+1.3 ± 0.7	5
Type I				
8 mM carnosine	103 ± 1*	+0.073 ± 0.007*	+0.1 ± 0.2	6
16 mM carnosine	101 ± 1	+0.116 ± 0.006*,†	+0.4 ± 0.2	6

Values are means ± SE for maximum  $\text{Ca}^{2+}$ -activated force (Max) and change ( $\Delta$ ) in  $\text{pCa}_{50}$  and Hill coefficient (*h*) compared with bracketing control levels. *n*, The number of fibers examined. \*Response in the presence of carnosine is significantly different from control level (i.e., no carnosine) ( $P < 0.05$ ). †Values for 8 mM carnosine are significantly different from those with 16 mM carnosine (Student's paired two-tailed *t*-tests;  $P < 0.05$ ). Mean control  $\text{pCa}_{50}$  and *h*: 5.802 ± 0.059 and 3.3 ± 0.2, respectively, for type II fibers (*n* = 5) and 5.841 ± 0.027 and 3.3 ± 0.2, respectively, for type I fibers (*n* = 6).

with 8 mM carnosine (Table 1). Although when given on the pCa scale these  $\text{Ca}^{2+}$ -sensitivity increases may seem small, they actually represent very considerable functional changes, with the increased sensitivity meaning that a fiber producing ~40% of maximal force at a particular intracellular  $[\text{Ca}^{2+}]$  in the absence of carnosine would produce >60% of maximum force at that same  $\text{Ca}^{2+}$  in the presence of 16 mM carnosine (e.g., see Fig. 2B).

In an additional experiment using the same heavily  $\text{Ca}^{2+}$ -buffered solutions as above, the effect of carnosine on the properties of the contractile apparatus was examined in fibers chemically skinned with Triton-X100 (see MATERIALS AND METHODS). In the four fibers examined this way, 16 mM carnosine caused a mean increase in the pCa<sub>50</sub> by  $0.111 \pm 0.019$  pCa units with no change in either maximum  $\text{Ca}^{2+}$ -activated force (mean max =  $102 \pm 2\%$  of control level) or the Hill coefficient (mean  $\Delta h = 0.2 \pm 0.2$ ). These four fibers consisted of two type I and two type II fibers, with the changes in pCa<sub>50</sub> being +0.063 and +0.113 pCa units for the two type II fibers and +0.109 and +0.158 pCa units for the two type I fibers. Thus the effect of carnosine on the properties of the contractile apparatus in chemically skinned fibers lacking a functional SR was not detectably different from that in mechanically skinned fibers.

**Endogenous SR  $\text{Ca}^{2+}$  content in type I and II human vastus lateralis muscle fibers.** The relative endogenous SR  $\text{Ca}^{2+}$  content of each fiber was estimated by releasing the  $\text{Ca}^{2+}$  initially present in the SR by exposing the fiber to full release solution and then performing load-release cycles with a range of set  $\text{Ca}^{2+}$  loading times until a similar size force response was elicited (22, 45) (as in Fig. 3, A and B, for type II and I fibers, respectively). Using this procedure, it was found that the  $\text{Ca}^{2+}$  loading period required to reload the SR to approximately its initial endogenous level was quite similar for the human type I and II vastus lateralis fibers [ $\sim 22 \pm 3$  s and  $\sim 28 \pm 4$  s for type I ( $n = 6$ ) and type II fibers ( $n = 5$ ), respectively].

**Effect of carnosine on caffeine-induced force responses in human vastus lateralis fibers.** We then sought to determine whether carnosine either directly elicits  $\text{Ca}^{2+}$  release or increases the caffeine sensitivity of the RyRs when the RyRs are in their normal in situ positions and with the normal level of  $\text{Mg}^{2+}$  present (i.e.,  $\sim 1$  mM; see Refs. 22, 26 and DISCUSSION). The sensitivity of the RyRs to submaximal caffeine concentrations is closely related to their sensitivity to  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) and is highly dependent on the amount of  $\text{Ca}^{2+}$  present in the SR (15, 22). The latter could be varied as desired in this experimental preparation (Fig. 3).

The effect of 8 mM carnosine caffeine-induced force responses was examined for a range of different SR load levels, as seen in Fig. 4. The examples shown are those of the same two fibers for which the SR loading characteristics are presented in Fig. 3. A first important observation was that application of 8 mM carnosine did not elicit a measurable force response in the absence of caffeine in any fiber with any of the SR load levels examined in the present study. In the type II fiber in Fig. 4A, when the SR was loaded with  $\text{Ca}^{2+}$  back to a level somewhat above its original endogenous level (see 30-s loading in Fig. 3A), the submaximal (8 mM) caffeine stimulus failed to elicit any force response in either the control conditions or in the presence of 8 mM carnosine (see Fig. 4A and Table 2 for mean data). In all of the type II fibers examined, the

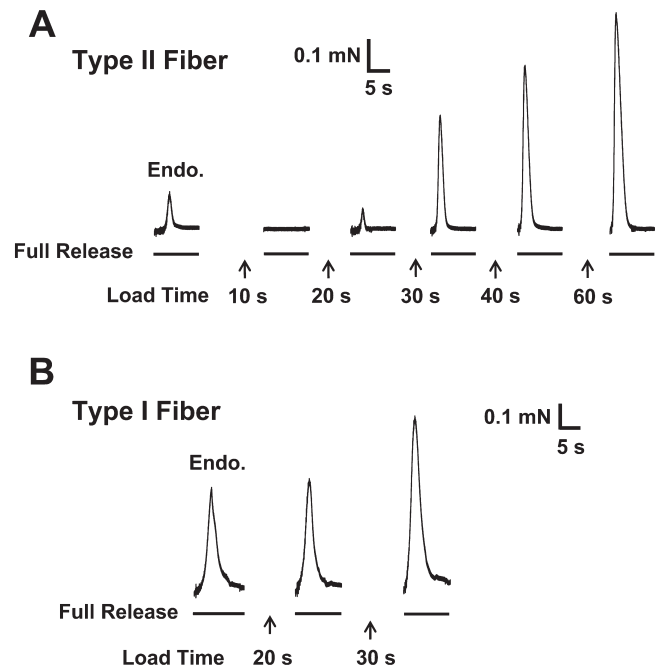


Fig. 3. Emptying the sarcoplasmic reticulum (SR) of all its releasable  $\text{Ca}^{2+}$  using full release solution. A: force responses in a type II fiber elicited when emptying the SR of all its releasable  $\text{Ca}^{2+}$  in the full release solution (in mM: 30 caffeine, 0.05 free  $\text{Mg}^{2+}$ , and 0.5 EGTA); the first such response (Endo) is indicative of the endogenous level of SR  $\text{Ca}^{2+}$  present at the start of the experiment. The fiber was then placed in the load solution for a set loading time (10–60 s) to reload the SR to the desired level, and then all the SR  $\text{Ca}^{2+}$  were again released with the full release solution. After each full release, the fiber was washed for 30 s with standard K-HDTA solution (1 mM total EGTA) to remove caffeine and raise  $[\text{Mg}^{2+}]$  back to 1 mM before reloading (see MATERIALS AND METHODS). B: responses in a type I fiber using similar protocol as in A, showing just two loading times. Time required to reload SR to endogenous level was between 20 and 30 s in fiber in A and  $\sim 20$  s in fiber in B.

SR had to be loaded substantially above the endogenous level in order for the 8 mM caffeine stimulus to elicit a self-reinforcing force response in these conditions (where the free  $[\text{Mg}^{2+}]$  was 1 mM and the free  $[\text{Ca}^{2+}]$  was weakly buffered at  $\sim 100$  nM). When the type II fiber shown in Fig. 4A was loaded for 60 s, the caffeine stimulus elicited a transient force response peaking at  $\sim 70\%$  of maximum  $\text{Ca}^{2+}$ -activated force, and in the presence of carnosine this response was potentiated by  $\sim 10\%$  (mean data in Table 2). Importantly, this potentiating effect of 8 mM carnosine on caffeine-induced force responses in the type II fiber shown was entirely explained by its effect on increasing the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus [compare the peaks of the caffeine-induced force responses with (white arrow) and without (black arrow) 8 mM carnosine to the force-pCa measurements made afterward in the same fiber (shown at right)]. The potentiation of caffeine-induced force responses seen with carnosine in the type II fibers examined (mean potentiation with 8 mM carnosine equated to the peak force of the caffeine response increasing by  $\sim 6 \pm 3\%$  of maximum  $\text{Ca}^{2+}$ -activated force; Table 2) was entirely explained by the increase in  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in every case and did not indicate any additional potentiating effect of carnosine on CICR.

In contrast to the type II fibers, four of the six type I fibers examined produced a force response to the 8 mM caffeine

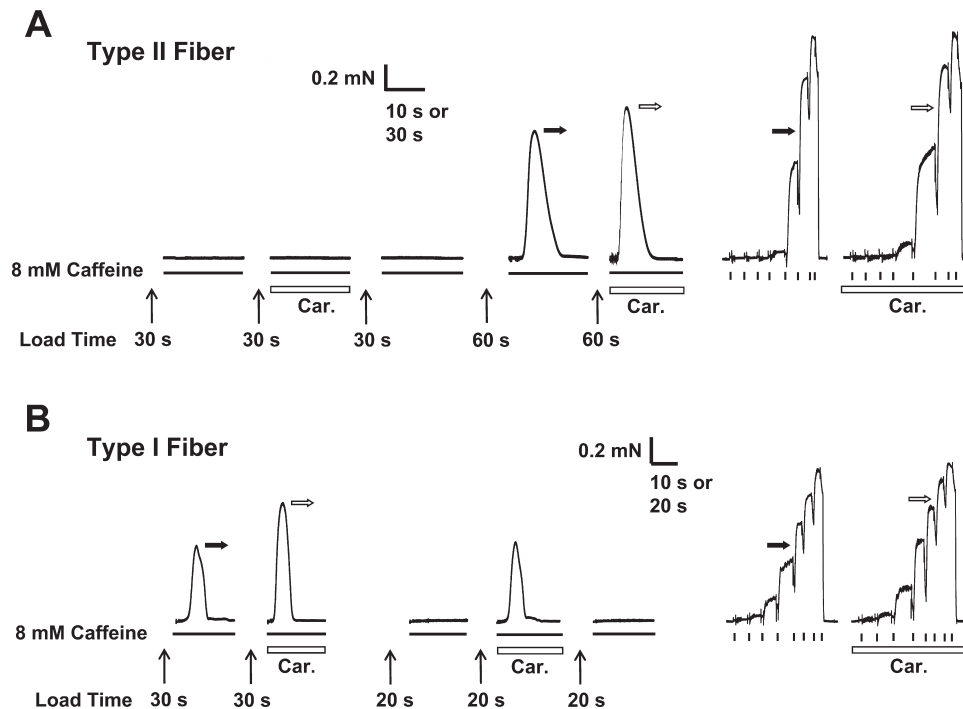


Fig. 4. Effect of carnosine on caffeine-induced responses in human type I and II fibers. *A*: SR of skinned type II fiber (same fiber in Fig. 3A) loaded with Ca<sup>2+</sup> for 30 s and then exposed to 8 mM caffeine (with 1 mM free Mg<sup>2+</sup>, 50  $\mu$ M EGTA). After each 15-s exposure to 8 mM caffeine solution, the SR was depleted of any remaining releasable Ca<sup>2+</sup> with the full release solution, as in Fig. 3 (force responses not shown, but summarized in Table 2). With 30-s load, the 8 mM caffeine solution failed to elicit any force response in either the presence or absence of 8 mM carnosine (Car.). However, after 60-s loading, the peak of the response elicited by 8 mM caffeine was moderately potentiated in the presence of 8 mM carnosine. The potentiation was explicable by the increase in Ca<sup>2+</sup> sensitivity of the contractile apparatus, as seen from the force responses elicited when subsequently directly activating the contractile apparatus with heavily Ca<sup>2+</sup>-buffered solutions (*right*; performed as in Fig. 2). Horizontal arrows indicate peak force to 8 mM caffeine in absence (solid arrows) and in presence of 8 mM carnosine (open arrows). *B*: similar procedure in a type I fiber (same fiber in Fig. 3B). Caffeine-induced force responses could be elicited with less SR loading in type I fibers (see Table 2). The potentiation of force responses to 8 mM caffeine were not explicable simply by the increased Ca<sup>2+</sup> sensitivity of the contractile apparatus. Timescale: 10 s for caffeine-induced force responses in *A* and *B*, and 30 s or 20 s for force-pCa staircases in *A* and *B*.

stimulus even when the SR was loaded with considerably less Ca<sup>2+</sup> than present endogenously (e.g., with *load level 2* in Table 2), indicating that the sensitivity of the RyRs to caffeine-induced and Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release was substantially

higher in the type I fibers than in the type II fibers. Furthermore, the presence of carnosine substantially potentiated the responses to 8 mM caffeine in the type I fibers, which was particularly noticeable when the SR was loaded at or below its

Table 2. Effect of carnosine on caffeine-induced force responses

Treatment	Initial Relative SR Ca <sup>2+</sup> Content (% Endo.)	Peak Force to 8 mM Caffeine (% max. force)	SR Ca <sup>2+</sup> Remaining, %	<i>n</i>
<b>Type II</b>				
<i>Load level 1</i> (30-s load)	140 $\pm$ 31			
8 mM caff		0 $\pm$ 0	75 $\pm$ 16	3
8 mM caff + carnosine		0 $\pm$ 0	63 $\pm$ 19	3
<i>Load level 2</i> (60-s load)	198 $\pm$ 18			
8 mM caff		53 $\pm$ 13	30 $\pm$ 5	4
8 mM caff + carnosine		59 $\pm$ 14	25 $\pm$ 5 *	4
<b>Type I</b>				
<i>Load level 1</i> (5- to 10-s load)	29 $\pm$ 11			
8 mM caff		0 $\pm$ 0	68 $\pm$ 9	3
8 mM caff + carnosine		14 $\pm$ 5	57 $\pm$ 11	3
<i>Load level 2</i> (10- to 20-s load)	52 $\pm$ 10			
8 mM caff		39 $\pm$ 14	62 $\pm$ 7	6
8 mM caff + carnosine		59 $\pm$ 14*	51 $\pm$ 7 *	6
<i>Load level 3</i> (20- to 30-s load)	117 $\pm$ 17			
8 mM caff		55 $\pm$ 20	27 $\pm$ 24	3
8 mM caff + carnosine		63 $\pm$ 22	26 $\pm$ 23	3

Values are means  $\pm$  SE, expressed as a percentage relative to 1) the endogenous SR Ca<sup>2+</sup> content, 'Endo.' (first column) (see MATERIALS AND METHODS), 2) the maximum Ca<sup>2+</sup>-activated force, max. (second column), and 3) the total amount of releasable Ca<sup>2+</sup> initially present in sarcoplasmic reticulum (SR) for the given level of loading (third column). Force response to 8 mM caffeine (caff), in absence and presence of carnosine (8 mM), in type I and type II fibers with the SR loaded at various levels. Relative amount of Ca<sup>2+</sup> still remaining in the SR after the 15-s exposure to caffeine is also shown. \*Value found with carnosine present was significantly different from that found in same fiber in absence of carnosine (paired *t*-test; *P* < 0.05).



endogenous level. As seen in the example in the middle force traces in Fig. 4B, the 8 mM caffeine stimulus failed to elicit any force response in this type I fiber when the SR was loaded at approximately its endogenous level (20-s load; see Fig. 3B), but when 8 mM carnosine was present and the SR was loaded to the same level, the caffeine stimulus elicited a force response reaching  $\sim 50\%$  of maximum  $\text{Ca}^{2+}$ -activated force. Clearly, this marked potentiating effect of carnosine on the caffeine-induced force response could not be explained solely by the accompanying increase in contractile apparatus  $\text{Ca}^{2+}$  sensitivity (force-pCa staircases in same fiber with and without carnosine shown on far right of Fig. 4B). When the SR was loaded with  $\text{Ca}^{2+}$  above its normal endogenous level, such that the 8 mM caffeine stimulus elicited a comparatively large force response even in control conditions (e.g., with 30-s load; Fig. 4B, left), the presence of carnosine potentiated the caffeine-induced force responses by more than could be accounted for by the increased contractile sensitivity. Altogether, in five of the six type I fibers, the potentiation of the caffeine response was beyond that expected purely from the effects of carnosine on the contractile apparatus. Moreover, if carnosine was also present when exposing type I fibers to the 8 mM caffeine solution (for 15 s), the amount of  $\text{Ca}^{2+}$  remaining in the SR afterward was significantly reduced (e.g., see load level 2 in Table 2), further indicating that the caffeine-induced  $\text{Ca}^{2+}$  release had been enhanced in the presence of carnosine.

## DISCUSSION

*Carnosine increases submaximal force output by the contractile apparatus in both type I and type II human fibers.* One major finding of this study was that raised cytoplasmic carnosine concentrations increase the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in both type I and type II human fibers, with little or no change to either the Hill coefficient or maximum  $\text{Ca}^{2+}$ -activated force. The concentrations of carnosine applied to each fiber segment in these contractile apparatus experiments (8 and 16 mM) approximately spans the physiological range for both type I and type II fibers (see INTRODUCTION). Importantly, raising the concentration of carnosine from the approximate level present normally ( $\sim 8$  mM) to the level attained by supplementation ( $\sim 16$  mM) further increased the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in every fiber examined (Fig. 2 and Table 1). This increase in  $\text{Ca}^{2+}$  sensitivity equates to a substantial increase in the submaximal force for a given cytoplasmic free  $[\text{Ca}^{2+}]$  (e.g., the  $[\text{Ca}^{2+}]$  producing 50% of maximum level would instead generate  $\sim 60\%$  maximal force; see Fig. 2B). Although similar increases in  $\text{Ca}^{2+}$  sensitivity with carnosine have been documented in mechanically skinned fibers from rat EDL muscle (14) and in chemically skinned fibers from frog and other nonmammalian muscle (29), this is the first study to characterize the effect of physiological levels of carnosine in human muscle fibers. The substantial potentiating effect of carnosine on contractile  $\text{Ca}^{2+}$  sensitivity observed here seems likely to be of importance in helping to mitigate against the effects of cytoplasmic factors that cause a decrease in  $\text{Ca}^{2+}$  sensitivity, such as the progressive inorganic phosphate accumulation (2, 11, 34) that occurs with repeated contractions.

*Effect of carnosine on SR  $\text{Ca}^{2+}$  release.* In this study, we used mechanically skinned human vastus lateralis fibers be-

cause, unlike the case in chemically skinned fibers, EC coupling remains fully functional, and all key EC coupling proteins such as the RyRs and ancillary proteins are in their in situ positions, and in particular the RyRs are still coupled to the voltage-sensors/DHPRs in the t-tubules. Furthermore, the solutions used in these studies broadly mimicked the normal intracellular environment and importantly contained the physiological level of free  $[\text{Mg}^{2+}]$  (i.e., 1 mM) since  $\text{Mg}^{2+}$  is known to exert a strong inhibitory effect on the RyRs in skeletal muscle (25, 26, 30, 33). Earlier experiments on RyR activity in SR vesicles from rabbit hindlimb muscle (7) had found that carnosine (30 mM) sensitized the RyRs to  $\text{Ca}^{2+}$ , but only when the free  $[\text{Mg}^{2+}]$  was 50  $\mu\text{M}$ . Others have shown that carnosine (in the range of 0.1–20 mM) increased the frequency and duration of opening of isolated RyRs extracted from porcine gracilis muscle and incorporated into lipid bilayers (48), but this was found in the absence of any  $\text{Mg}^{2+}$ . In that same study, the authors also showed that carnosine, even at concentrations as low as 2.5 mM, induced  $\text{Ca}^{2+}$  release and contraction in glycerol-treated (chemically skinned) human vastus lateralis muscle fibers (48). However, once again, this was with the free  $[\text{Mg}^{2+}]$  well below its physiological level (at  $<0.3$  mM), and with the SR loaded with  $\text{Ca}^{2+}$  well above its endogenous level. Furthermore, each fiber in that study had been first exposed to very high intracellular  $[\text{Ca}^{2+}]$  ( $\sim 0.5$  mM) to ascertain maximal tension (48), and such exposure to high intracellular  $[\text{Ca}^{2+}]$  has since been shown to disrupt the coupling between the DHPRs and the RyRs, increasing leakage of  $\text{Ca}^{2+}$  out of the SR (21, 23).

It was found here that the force responses to 8 mM caffeine were potentiated in the presence of carnosine in both fiber types (Fig. 4 and Table 2). To determine whether the responses were potentiated solely by the effects of carnosine on the contractile apparatus or also by some additional potentiating effect on  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, it was necessary to compare its effects on both processes in the same fiber (e.g., see Fig. 4, left and right). This was performed for all fibers examined and it was concluded that the potentiation of the caffeine-induced force response in type II (fast-twitch) fibers was wholly explained by the increase in  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in every case. This is similar to our findings in type II fibers from rat muscle (14), where the potentiating effect of 8 mM carnosine on both caffeine-induced and depolarization-induced force responses in EDL muscle fibers was found to be fully accounted for by the direct effects of carnosine on the contractile apparatus, with no evidence of any increase in the  $\text{Ca}^{2+}$  sensitivity of the RyRs under physiological conditions similar to those used in the present study. Interestingly, rat EDL muscle fibers are almost exclusively type IIb and type IIx/d (see Ref. 9), whereas most if not all of the human type II fibers examined here were likely to be type IIa fibers, because even though the MHC antibody used here did not distinguish between the human fast MHC isoforms, several previous studies have found that the great majority of the type II fibers in human vastus lateralis muscle are type IIa (10, 19, 43). The earlier findings in the rat EDL fibers suggest that the effects of carnosine seen in the type II fibers here are quite probably common to all type II human fibers.

In contrast to the findings in type II fibers, the potentiating effect of carnosine on caffeine-induced force responses in human type I fibers was not explicable solely by its effects on



the contractile apparatus, with the carnosine evidently also augmenting caffeine-induced Ca<sup>2+</sup> release, particularly when the SR Ca<sup>2+</sup> load level was relatively low and showing strong threshold-like behavior in response to the caffeine stimulus (Fig. 4B and Table 2). It should be noted, however, that even though the carnosine caused additional caffeine-induced Ca<sup>2+</sup> release from the SR, the total amount of the extra Ca<sup>2+</sup> released was relatively small (compare SR Ca<sup>2+</sup> remaining after 8 mM caffeine treatment with and without carnosine in Table 2). Furthermore, it is also important to note that, even though carnosine can sensitise the RyRs to Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in type I fibers, this does not necessarily mean that such an effect would be manifested in vivo, where activation of Ca<sup>2+</sup> release through the RyRs is widely thought to be tightly controlled by the dihydropyridine receptors (DHPRs) with little if any contribution of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (15, 22, 41). Furthermore, it has been shown that, even though treatments such as oxidation can greatly increase the sensitivity of the RyRs to caffeine-induced and Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release, this does not seemingly alter the amount of Ca<sup>2+</sup> released by the normal physiological process of action-potential-induced Ca<sup>2+</sup> release mediated by DHPR activation of the RyRs (28, 38), at least in rat type II fibers. It nevertheless remains possible that CICR has some significant role in normal EC coupling in type I fibers and that the potentiating effects of carnosine on CICR seen here might add to its overall positive effects on the force response.

*Other considerations of the role of carnosine in vivo.* In the experiments here, the pH of the solutions was heavily buffered at pH 7.1 by HEPES (see MATERIALS AND METHODS), and thus the pH buffering effects of carnosine (1, 5, 8, 12) would not have influenced the findings. Acidosis of the cytoplasmic milieu occurring during strenuous exercise has been suggested to lead to a decrease in the Ca<sup>2+</sup> sensitivity of the contractile apparatus, and it is possible that the presence of carnosine in fibers in vivo would ameliorate this decrease to some extent. We note, however, that the deleterious effects of acidosis on contractility seen in in vitro experiments at room temperature are markedly smaller when the experiments are performed at closer to normal body temperatures (37, 47). In addition to acting as an in vivo pH buffer, carnosine is also a cytoplasmic antioxidant (8, 20, 36). Oxidation of the contractile apparatus can cause either an increase or decrease in the Ca<sup>2+</sup> sensitivity of the contractile apparatus depending on the particular oxidant and the glutathione levels within the fiber (28). The antioxidant action of carnosine would not have been a factor in the present experiments since few oxidants are generated in this preparation either at rest or with repeated activation (24), however, carnosine may play an important role in vivo by helping prevent or reduce oxidative changes to the contractile apparatus or SR proteins.

*Concluding remarks.* In addition to its likely important roles as a pH buffer, membrane stabilizer, osmotic shock protector, and anti-oxidant and anti-aging agent, it was found here that carnosine directly augments force production by the contractile apparatus in both type I and type II human vastus lateralis muscle fibers. Given the present interest in carnosine as an ergogenic and therapeutic aid, the finding that carnosine directly increases submaximal force output in both fiber types offers mechanistic insight into at least one major way by which carnosine loading likely confers benefits to muscle perfor-

mance under some circumstances. In addition, in type I fibers, normal Ca<sup>2+</sup> release might also be directly potentiated by carnosine, which might be of importance particularly when Ca<sup>2+</sup> release is hindered to some extent, such as during the later stages of muscle fatigue (2). Taken together, the present results suggest that the increase in muscle carnosine levels occurring with carnosine supplementation are likely to help counter the effects of factors causing decreased Ca<sup>2+</sup> sensitivity of the contractile apparatus, and in type I fibers possibly those decreasing Ca<sup>2+</sup> release, and this could be expected to help reduce or slow the decline in muscle performance occurring during fatiguing contractions.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: T.L.D., R.M.M., and G.D.L. conception and design of research; T.L.D., C.R.L., and R.M.M. performed experiments; T.L.D. and G.D.L. analyzed data; T.L.D. and G.D.L. interpreted results of experiments; T.L.D. prepared figures; T.L.D. drafted the manuscript; T.L.D., C.R.L., M.J.M., R.M.M., and G.D.L. edited and revised the manuscript; T.L.D., C.R.L., M.J.M., R.M.M., and G.D.L. approved the final version of the manuscript.

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