β-alanine supplementation enhances human skeletal muscle relaxation speed but not force production capacity

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Running head: β-alanine supplementation and muscle contractile properties

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ABSTRACT

PURPOSE: β-alanine (BA) supplementation improves human exercise performance. One possible explanation for this is an enhancement of muscle contractile properties, occurring via elevated intramuscular carnosine resulting in improved calcium sensitivity and handling. This study investigated the effect of BA supplementation on *in vivo* contractile properties and voluntary neuromuscular performance. **METHODS:** Twenty-three men completed two experimental sessions, pre- and post-28 days supplementation with 6.4 $g \cdot d^{-1}$ of BA (n = 12) or placebo (PLA; n = 11). During each session, force was recorded during a series of knee extensor contractions: resting and potentiated twitches and octet (8 pulses, 300 Hz) contractions elicited via femoral nerve stimulation; tetanic contractions (1 s, 1 – 100 Hz) via superficial muscle stimulation; and maximum and explosive voluntary contractions. **RESULTS:** BA supplementation had no effect on the force-frequency relationship, or the force responses (force at 25 ms and 50 ms from onset, peak force) of resting or potentiated twitches, and octet contractions ($P > 0.05$). Resting and potentiated twitch electromechanical delay and time-to-peak tension were unaffected by BA supplementation ($P > 0.05$), although half-relaxation time declined by 7-12% ($P < 0.05$). Maximum and explosive voluntary forces were unchanged after BA supplementation. **CONCLUSION:** BA supplementation had no effect on evoked force responses, implying that altered calcium sensitivity and/or release are not the mechanisms by which BA supplementation influences exercise performance. The reduced half-relaxation time with BA supplementation might, however, be explained by enhanced reuptake of calcium, which has implications for the efficiency of muscle contraction following BA supplementation.

Key words: beta-alanine; muscle contractile properties; electrical stimulation; force-frequency relationship

INTRODUCTION

Carnosine (β-alanyl-L-histidine) is a cytoplasmic dipeptide synthesised from β-alanine (BA) and histidine, and is found in high concentrations within mammalian skeletal muscle. Carnosine is formed, primarily in skeletal and brain tissue, by bonding histidine and BA in a reaction catalysed by carnosine synthase (23; 40). The availability of BA in the human diet is the rate-limiting factor for carnosine synthesis in human skeletal muscle [for a brief review see (20)]. Long-term (4-10 weeks) dietary supplementation with BA significantly increases human skeletal muscle carnosine content (19; 21; 24). Interest in elevating carnosine levels through BA supplementation has dramatically increased since it was first shown that doing so increased high-intensity cycling capacity (21). Since then, it has been well established that BA supplementation can improve high-intensity exercise performance (e.g. 2000 m rowing performance and 100-200 m swimming performance) and capacity during exercise of ~1-6 minutes [see reviews: (22; 34)]. However, the physiological mechanisms for these ergogenic effects remain poorly understood.

Carnosine is suggested to have several physiological roles in muscle, which are pertinent to muscle function and performance. For example, its molecular structure makes it well suited to act as a pH buffer (36). The pKa of its imidazole ring is 6.83, placing it right in the middle of the pH transit range of exercising muscle. This means that an increase in carnosine content within the skeletal muscles also results in an expansion of the imidazole ring content, concomitantly increasing the muscle buffering capacity. As a result, performance improvements in high-intensity exercise (particularly when hydrogen cation accumulation is likely to limit performance) have largely been ascribed to increases in intracellular buffering [see review: (34; 35)].

Alternative mechanisms for the enhancement of exercise performance following BA supplementation have been proposed. For example, previous work in rat skeletal muscle suggested a role for carnosine in increasing the sensitivity of the contractile apparatus to calcium ions (Ca^{2+}) (10). More recent work in skinned human *m. vastus lateralis* fibre preparations showed a similar increase in Ca^{2+} sensitivity (11). Although only slight changes were shown in the maximum Ca^{2+} activated force (≤3%), a significant leftward shift in the

force-calcium concentration relationship was shown, indicating that force for a given submaximal Ca^{2+} concentration was increased in the presence of higher carnosine levels in both fibre types. Elevated carnosine levels also increased Ca^{2+} release from the sarcoplasmic reticulum of type I fibres, whereby carnosine appeared to enhance the Ca^{2+} sensitivity of ryanodine receptors and potentiated Ca^{2+} induced Ca^{2+} release (11). Thus, it was suggested that elevated carnosine after BA supplementation could alleviate the decline in contractile performance during fatiguing contractions by countering factors that might cause reduced calcium sensitivity and release (11).

A recent study provided the first evidence that dietary BA supplementation may influence the muscle contractile properties of mice (13), potentially via elevated intramuscular carnosine and its effect on calcium sensitivity and handling (11). BA supplementation was associated with a leftward shift in the electrically-evoked force-frequency relationship of excised muscle, which is analogous to the force-calcium concentration relationship (5; 27), eliciting a 10-30% increase in the force produced at low stimulation frequencies (13). However, the possibility that dietary BA supplementation might change *in vivo* human muscle contractile properties, and thus voluntary muscle performance, has not been investigated. There is a need to examine this possibility given that we would expect a wider range of performance effects of carnosine than have currently been shown if improved calcium handling were the major physiological role of carnosine in human skeletal muscle (34).

As such, we examined the effects of 28 d BA supplementation on the intrinsic contractile properties of human skeletal muscle *in vivo*, as well as on voluntary muscle function. Intrinsic contractile properties were assessed via the force-frequency relationship in response to muscle stimulation, and the evoked twitch and octet [8 pulses at 300 Hz, which drives the muscle at its maximum capacity for rapid or "explosive" force production; (8)] responses to supramaximal nerve stimulation. We hypothesised that BA supplementation would enhance intrinsic contractile properties; producing a left-ward shift in the force-frequency relationship; increasing the peak and explosive force responses to twitch and octet stimulation and increasing explosive voluntary force production. In addition, we hypothesised that the altered contractile properties would lead to changes in motor control, reflected as a shift in the force-electromyography (EMG) relationship towards lower EMG levels for a given level of force.

METHODS

Participants

Twenty-six participants were recruited to the study and were stratified and allocated to the two supplement groups [placebo (PLA) or β-alanine (BA)] on the basis of maximum knee extensor strength (maximum voluntary force, MVF; see below) values recorded during the familiarisation session, such that the two groups were matched for knee extensor strength. However, three participants withdrew from the study (two from PLA and one from BA), one during familiarisation due to a lack of tolerance of electrical stimulation and two following baseline testing with no reason provided. As such, twenty-three participants completed all aspects of the study (PLA group: n = 11; age, 25.6 ± 5.6 y; body mass, 79.1 ± 13.0 kg; height, 1.80 ± 0.07 m; BA group: n = 12; age, 26.1 ± 7.4 y; body mass, 90.1 ± 32.1 kg; height, 1.79 ± 0.06 m). All participants provided written informed consent, and completed this study, which was approved by the Institutional Human Ethical Review Committee. None of the participants had taken any nutritional supplements in the previous 6 months. Participants had no injuries to the lower limbs, were not involved in any systematic physical training, and were categorised as having moderate habitual levels of physical activity using the International Physical Activity Questionnaire Short Format [http://www.ipaq.ki.se/ipaq.htm; (7)]. Throughout the study participants were requested to maintain similar levels of physical activity and dietary intake; this was verbally confirmed at the start of each session. None of the subjects were vegetarian or vegan, and therefore they would likely have encountered small amounts of BA in their diet (1).

Study design

This was a double-blind placebo controlled experiment. Participants completed three experimental sessions over a five week period: a familiarisation session, which preceded a baseline session by at least 7 days, and a follow-up session after 28 days of supplementation with either BA or PLA. Participants were instructed to abstain from alcohol and strenuous/unaccustomed exercise for 36 hours prior to measurement sessions, with caffeine prohibited on the day of measurement sessions. Compliance with these requests was confirmed verbally with each participant prior to them commencing each session. Measurement sessions were completed at a consistent time of day, with recordings of force

and surface EMG during a series of voluntary and involuntary (electrically evoked) isometric contractions of the knee extensors of the dominant leg. The familiarisation session involved all the voluntary and evoked contractions, except the evoked octet contractions. The baseline and follow-up sessions involved an identical protocol performed according to a strict schedule. All raw data, exclusions and statistical analyses were completed blind to supplement group.

Supplementation

Participants received 6.4 $g \cdot d^{-1}$ of either BA (sustained-release Carnosyn^{TM}) or a matched placebo (PLA, maltodextrin) for 28 days (2 × 800mg tablets, ingested 4 times per day). The sustained-release formulation used in this study has been shown to reduce or remove the paraesthesia often experienced by participants following doses of free BA powder (9). We would expect the increase in muscle carnosine content to be close to 15 $mmol.kg^{-1}$ dry muscle (an increase of circa 65% in a participant eating a mixed diet), given that Harris et al. (2006) reported this level of increase following a similar but slightly lower total dose of BA. None of the participants reported any feelings of paraesthesia during the study. Throughout supplementation participants completed a log to verify supplement compliance, with similar compliance reported at 91 ± 7% in the BA group and 88 ± 10% in the PLA group (independent sample *t*-test, $P = 0.60$).

Supplements were provided to each participant in identical white tubs by an individual not directly involved in testing or data analysis, in order to maintain the double-blind. BA tablets were tested by the manufacturer prior to release for the study and conformed to the label claim for BA content. In addition, BA and PLA supplements were independently tested by HFL Sports Science, UK, prior to use to ensure no contamination with steroids or stimulants according to ISO 17025 accredited tests.

Experimental set-up

Knee extension force

Participants were seated in a rigid, custom-built dynamometer with hip and knee joint angles of approximately 95° and 100° (180° = full extension), as adapted from previous studies (17; 18). Adjustable strapping across the pelvis and shoulders prevented extraneous movement during muscle activation. An ankle cuff was attached to the dominant leg of the participant ~2 cm proximal to the medial malleolus and was in series with a linear strain gauge (615, Tedea-Huntleigh, Herzliya, Israel) oriented perpendicular to the tibia. Dynamometer configuration was established during the familiarisation session and replicated thereafter. The force signal was amplified (×1000) in the frequency range of 0 – 500 Hz, and sampled at 2000 Hz using an external A/D converter (1401; CED, Cambridge, UK), interfaced with a personal computer (PC) using Spike 2 software (CED, Cambridge, UK). Force data were low-pass filtered in both directions at 450 Hz using a fourth-order zero-lag Butterworth filter prior to analysis. Baseline resting force was subtracted from all force recordings to correct for the effects of gravity.

Electromyography (EMG)

EMG signals were recorded from the superficial quadriceps: *m. rectus femoris* (RF), *m. vastus medialis* (VM) and *m. vastus lateralis* (VL). After preparation of the skin by shaving, light abrasion and cleaning with alcohol, bipolar surface electrodes (2.5 cm inter-electrode distance; silver/silver chloride, 95 mm^2 area, Ambu Blue Sensor, Ambu, Ballerup, Denmark) were attached over each muscle at standardised percentages of thigh length, as measured from the knee joint space to the greater trochanter: RF, 55%; VM, 25% and VL, 45%. These sites were selected to avoid the innervation zones of each of the assessed muscles (32). A reference electrode was placed on the patella of the same limb. EMG signals were pre-amplified by active EMG leads (input impedance 100 MΩ, CMMR > 100 dB, base gain 500, 1st order high pass filter set to 10 Hz; Noraxon, Scottsdale, U.S.A) connected in series to a custom-built junction box and subsequently to the same A/D converter and PC software that enabled synchronisation with the force data. The signals were sampled at 2000 Hz. EMG data were band-pass filtered in both directions between 20 and 450 Hz using a fourth-order zero-lag Butterworth filter prior to analysis.

Electrical stimulation

A constant current variable voltage stimulator (DS7AH, Digitimer Ltd, Welwyn Garden City, UK) was used to assess knee extensor contractile properties whilst the participant was voluntarily passive. Square-wave pulses (0.2 ms duration) were delivered via: (i) supramaximal femoral nerve stimulation to evoke maximal resting twitch, potentiated twitch and octet contractions; (ii) percutaneous sub-maximal muscle stimulation to evoke contractions at a range of frequencies (1 to 100 Hz) to assess the force-frequency relationship. Femoral nerve stimulation involved a cathode stimulation probe (1 cm diameter, Electro-Medical Supplies Ltd, Wantage, UK) firmly pressed into the skin over the femoral nerve in the femoral triangle, and an anode (7×10 cm carbon rubber electrode; Electro-Medical Supplies Ltd, Wantage, UK) coated with electrode gel and taped to the skin over the greater trochanter. The precise location of the cathode was determined as the position that evoked the greatest twitch response for a particular submaximal electrical current (typically 30–50 mA). For percutaneous stimulation, the surfaces of two carbon rubber electrodes (14 × 10 cm; Electro-Medical Supplies Ltd, Wantage, UK) were coated with electrode gel and secured over the proximal and distal surface of quadriceps at standardised percentages of thigh length, as measured from the patella to the anterior superior iliac spine (ASIS): proximal electrode placed 20% distal to the ASIS; distal electrode placed 10% proximal to the patella.

Protocol and measurements

Measurements were completed in the following order, according to a consistent time schedule including ≥ 3 minutes rest between successive measurements.

Force and EMG onsets for all evoked and voluntary contractions were identified manually using visual identification by the same investigator, in accordance with a previously published method (16; 37). This approach is considered more valid than the use of automated methods of identification (38).

Resting twitches

Resting twitches were evoked following ≥15 min passive sitting, in order to remove any lingering potentiation, which incorporated the time for securing the participant in the

dynamometer, and preparing them for EMG and electrical stimulation. Single electrical impulses were delivered with stepwise increments in the current, separated by 10 s to allow for neuromuscular recovery, until a plateau in the amplitude of twitch force and compound muscle action potentials (M-waves) were reached. The stimulus intensity was then increased by 25% above the value required to elicit a plateau to ensure supramaximal stimulation, and three discrete supramaximal stimuli separated by 10 s were then delivered to elicit maximal twitch responses and M-waves.

The time difference between M-wave onset (first electrode site to be activated) and twitch force onset was defined as the electromechanical delay (EMD). Twitch force was measured at 25 and 50 ms from onset, as markers of the explosive force production during the rising slope, and at the peak of the force response. The time-to-peak tension (TPT) and half-relaxation time (HRT) were also recorded. All measurements were averaged across the three maximal twitch contractions. The M-wave response for the three quadriceps electrodes was measured for M-wave area, from EMG onset to the point where the signal returned to baseline, and averaged across the three sites. The mean M-wave area of the three supramaximal stimuli was defined as the maximal M-wave area (M_{max}) and was used for normalisation of voluntary quadriceps EMG (6).

Maximum voluntary contractions and potentiated twitches

A brief warm-up of 3 sub-maximal knee extension contractions at 50%, 75% and 90% of the participants' perceived maximal force were performed; contractions lasted ~3 s each and were separated by ~20 s. Participants then completed 4 maximum voluntary contractions (MVCs) of the knee extensors ≥60 s apart, during which they were instructed to contract "as hard as possible" for 3-4 s. During and after each contraction they received strong verbal encouragement reiterating the instructions, together with online feedback of the force signal and a marker of their maximum force during that session displayed onscreen. Supramaximal stimulation of the femoral nerve, using the same configuration and stimulus intensity as for resting twitches, was used to elicit a maximal potentiated twitch ~1 s after each of the MVCs. The greatest instantaneous force during either the knee extensor MVCs or explosive voluntary contractions (see below) of that trial was defined as MVF. The root mean square

(RMS) of the EMG signal for each muscle (RF, VM, VL) was calculated over a 500 ms epoch surrounding MVF (250 ms either side) and normalised to the corresponding M_{max} (6), before averaging across all 3 sites to calculate a mean quadriceps value. The EMD, force at 25 and 50 ms from onset, peak twitch force, TPT and HRT were averaged across the four maximal potentiated twitch contractions.

Explosive voluntary contractions

The protocol followed previously published procedures (6; 16). Participants completed ≥10 isometric explosive voluntary knee extensions, each separated by ~20 s. Starting from a completely relaxed state, they were instructed to respond to an auditory signal by extending their knee "as fast and hard as possible" for ~1 s, with an emphasis on "fast". An on-screen cursor was used to provide online feedback on their explosive performance, displaying the maximum rate of force development (2 ms time constant) of their best attempt. Strong verbal encouragement was provided to participants to exceed this target during each subsequent contraction. A second visual marker on the screen depicted 80% of the peak force recorded during MVCs, which participants were expected to achieve or exceed during each explosive contraction. Resting force was also displayed on a sensitive scale during all explosive contractions to aid the detection of pretension or countermovement. The explosive contractions were performed until 10 contractions, with no prior countermovement or pre-tension, been had been recorded.

The three contractions with the greatest maximum rate of force development, meeting the following criteria, were used for analysis: (i) no prior countermovement or pre-tension, and (ii) peak force ≥80% MVF. Analyses involved measurement of the force–time and EMG–time traces in short periods after their onsets. Explosive force was measured at 25 ms intervals up to 150 ms after force onset. The RMS of the EMG signal from each muscle was measured over three consecutive 50 ms time periods from EMG onset of the first agonist muscle to be activated (*i.e.*, 0-50, 50-100 and 100-150 ms). Thereafter, RMS EMG at each EMG site was normalized to M_{max} and averaged to provide a mean quadriceps value. All measurements were averaged across the three selected contractions.

Force-EMG relationship (via voluntary incremental knee extension contractions)

A series of submaximal knee extension contractions were performed at 15% increments of MVF, in ascending order, up to 90%. Horizontal cursors on the screen in front of participants depicted the target levels of force. Participants were instructed to reach the target quickly and maintain the level of force as accurately as possible for ~3 s. Contractions were separated by ~20 s. The RMS of the EMG and average force over a stable 500 ms part of the force trace (minimal standard deviation of the force trace for that contraction) were analysed at each of the contraction intensities. The EMG RMS values were normalised to M_{max} and plotted against the respective force values. Linear regression was used to evaluate the slope and intercept of the force-EMG relationship incorporating all data between 15 – 90% MVF.

Octet contractions

Octet contractions [8 impulses at 300 Hz; (8)] were evoked via supramaximal stimulation of the femoral nerve. First, a brief series of single stimuli were administered, and twitch force and M-wave amplitudes were monitored to confirm that the stimuli were supramaximal. The current was increased if necessary to ensure supramaximal stimulation. Then 3 discrete pulse trains (≥15 s apart) were delivered with a supramaximal current (+25%) to evoke maximal octet contractions. The current increased by ~5% after each pulse train in order to confirm a plateau in both the peak force and maximum rate of force development. On some occasions, where the first pulse train elicited a submaximal response, a 4^{th} pulse train was delivered to ensure 3 maximal responses. The octet force response was measured at 25 and 50 ms from force onset, as well as at the peak. All measurements were averaged across the 3 analysed contractions.

Force-frequency relationship

Surface EMG electrodes were removed and carbon rubber electrodes attached over the quadriceps, taking ~5 minutes. The force-frequency relationship was then evaluated during tetanic contractions elicited via submaximal percutaneous electrical stimulation (3; 15).

Initially, 100 Hz contractions were evoked at increasing current intensities, ≥30 s apart, to determine the current that elicited 50% of baseline MVF. This current (typically 110 – 200

mA) was then used for the following force-frequency measurements. The final calibration contraction at 100 Hz and the subsequent measured contractions were separated by ≥60 s. The force-frequency relationship contractions consisted of two twitch contractions (1 Hz), followed by single contractions of 1 s duration at each of 9 different frequencies (5, 10, 15, 20, 30, 40, 50, 80, 100 Hz) performed in ascending order with ~30 s between contractions. Peak force was defined as the greatest instantaneous force. Thereafter, the force values at each stimulation frequency were normalized to 100 Hz force. The force-frequency relationship was fitted with a Hill curve and evaluated for frequency at 50% of the maximum force response (11).

Statistical Analysis

Dependent variables measured over several time points/periods (force and EMG during explosive voluntary contractions, evoked twitch and octet force) were analysed using a three-way (group × session × time point) analysis of variance (ANOVA). Similarly, the force-frequency relationship was assessed by a three-way (group × session × frequency) ANOVA. Other dependent variables (MVF, HRT, TPT, slope and intercept of force-EMG relationship, frequency at 50% of force response for the force-frequency relationship) were evaluated using two-way ANOVA (group × session). A Greenhouse-Geisser correction was applied when the ANOVA assumption of sphericity was violated, and significant interaction effects were followed-up by independent sample *t*-tests on the individual percentage change values for each condition. The change in group mean values was used to calculate the percentage change values presented. Intra-individual variability was assessed using the mean intra-individual coefficient of variation (CV) across the two measurement session for the PLA group [(mean ÷ standard deviation) × 100]. Statistical analyses were completed using SPSS version 21 (SPSS Inc, Chicago, USA) and statistical significance was accepted at $P \leq 0.05$. Data are presented as mean ± one standard deviation (1SD).

RESULTS

Electrically-evoked contractile properties

Resting twitches

There was no influence of supplementation on resting twitch force (P = 0.46 and 0.70, Figure 1A), EMD (P = 0.63, Fig 2A) or TPT (P = 0.29; Fig 2B), although there was a group × session interaction for HRT (P = 0.018; Fig 2C). *Post hoc* analysis showed that the change in HRT was greater for the BA group (-12 ± 10 %) compared to the PLA group (+2 ± 11%; P < 0.01). Mean CV values for the PLA group were: force at 25, 50 ms and peak were 14%, 9% and 8%; EMD 7%; TPT 3%; HRT 7%.

Potentiated twitches

There was no influence of supplementation on potentiated twitch force (P = 0.44 and 0.52, Figure 1B), EMD (P = 0.48, Fig 2D) or TPT (P = 0.32; Fig 2E). However, there was a group × session interaction for HRT (P = 0.041; Fig 2F) and *post hoc* analysis showed that the change in HRT was greater for the BA group (-7 ± 11 %) compared to the PLA group (+1 ± 8 %; P = 0.050). Mean CV values for the PLA group were: force at 25, 50 ms and peak were 6%, 3% and 3%; EMD 6%; TPT 3%; HRT 4%.

Octet contractions

Supplementation did not influence resting octet force at any time point (Figure 1C). Mean CV values for the PLA group were: force at 25, 50 ms and peak were 10%, 3% and 4%.

Force-frequency relationship

The peak force at each frequency of stimulation (Fig 3) and the frequency at 50% of the force response (Table 1) were both unaffected by supplementation. Mean CV values for relative force (% maximum at 100 Hz) in PLA group were 6-8% at 1 - 10 Hz, 1-3% at 15 - 80 Hz, and 6% for the frequency at 50% of force response.

388

389 **Maximum and explosive voluntary force production**

390 There was affect of supplementation on MVF (Fig 4). The mean CV for MVF in the PLA
391 group was 3%. Similarly, there was no influence of supplementation on force measured at 25
392 ms intervals during explosive voluntary contractions (Fig 4). The mean CV values for
393 voluntary force production in the PLA group were: 13-17% at 25 – 50 ms, 4-7% from 75 –
394 150 ms and 3% at MVF.

395

396 **Neuromuscular activation**

397 *Agonist neuromuscular activation during maximum voluntary and explosive voluntary*
398 *contractions*

399 Agonist EMG normalised to M_{max} during MVCs and explosive contractions was not affected
400 by supplementation (Fig 5), indicating that neuromuscular activation was consistent across
401 measurement sessions. The mean CV values for agonist EMG in the PLA group were: 26%,
402 23% and 9% in the 0-50, 50-100 and 100-150 ms time windows, and 13% at MVF.

403

404 *Force-EMG relationship*

405 The slope and y-intercept of the force-EMG relationship were unaffected by supplementation
406 (Fig 5, Table 1). The mean CV value for slope of the force-EMG relationship in the PLA
407 group was 15%. Although the CV was very high for the intercept of the relationship (80%) as
408 a consequence of intercept values being close to zero, the mean difference between sessions
409 was actually very low when expressed as a percentage of maximal EMG at MVF (4%).

410

411 **DISCUSSION**

412 The present study is the first to comprehensively examine the influence of BA
413 supplementation on the electrically-evoked contractile properties of human skeletal muscle *in*
414 *vivo*. BA supplementation had no effect on the force-frequency relationship, evaluated during
415 submaximal muscle stimulation. Similarly, BA did not influence the EMD, explosive force

(at 25 and 50 ms), peak force or TPT of resting twitch, potentiated twitch or octet contractions elicited by supramaximal stimulation of the femoral nerve. In line with these findings, there were no changes in maximum or explosive voluntary force production following BA supplementation. The only significant effect of BA was a 12% and 7% reduction in HRT during resting and potentiated twitch contractions.

Knee extensor intrinsic contractile properties

The force-frequency relationship of the knee extensors was evaluated during submaximal muscle stimulation at a range of frequencies (1-100 Hz) in order to evaluate potential effects of BA supplementation on calcium handling and sensitivity, since an association between intracellular calcium levels and force production in response to different stimulation frequencies has previously been shown (5). BA supplementation, however, had no effect on knee extensor force production at relatively low (1-15 Hz) or high (20-80 Hz) frequencies of muscle stimulation, corresponding to relatively low (19 – 53% force at 100 Hz) and high (63 – 95% force at 100 Hz) levels of force. Previous *in vitro* research showed that increasing cytoplasmic carnosine levels from those normally present to levels approaching those attained after supplementation produced a marked enhancement in Ca^{2+} sensitivity (*i.e.*, an increased force response to submaximal Ca^{2+} levels) of fibres from human *m. vastus lateralis*, as well as enhanced Ca^{2+} release in type I fibres (11). Thus the present data showing no effect of BA supplementation on the force-frequency relationship, the *in vivo* analogue of the force-calcium concentration relationship (5; 27), responses might therefore be taken to imply that supplementation did not grossly influence Ca^{2+} sensitivity (10; 11) or Ca^{2+} release (11; 33).

The present force-frequency data are supported by the findings that force and contraction time responses to supramaximal nerve stimulation at low- (resting and potentiated twitch) and high-frequencies (300 Hz, octet) were not affected by BA supplementation. Improved Ca^{2+} sensitivity or release would be expected to be particularly beneficial in situations where calcium saturation is submaximal; during resting twitch contractions evoked by a single nerve impulse, for example. Combined evaluation of resting and potentiated twitch responses might have been expected to reveal any influence of BA supplementation on these processes, since the mechanisms for potentiation include the phosphorylation of myosin, which increases the

447 sensitivity of the contractile elements to Ca^{2+}, as well as altered Ca^{2+} handling (39). However,
448 neither peak force, TPT nor the rate of force development (force at 25 and 50 ms) of resting
449 and potentiated twitches were affected by BA supplementation. Similarly, the resting and
450 potentiated twitch EMD, which reflects the time for excitation-contraction coupling processes
451 and for muscle shortening to remove slack from the muscle tendon unit (29; 31), was
452 unaltered following BA supplementation. These data further imply that BA supplementation
453 had little influence on Ca^{2+} sensitivity or release.

454

455 The current data appear at odds with the human single fibre data mentioned above (11), and
456 recent findings in mouse muscle where 10-31% increases in force were shown at frequencies
457 between 25-125 Hz, but not at 1 Hz, following BA supplementation (13). Several factors
458 could explain the present results and apparent contrast with the previous data. Firstly, there
459 are obvious differences with the study of Dutka et al. (2012), including the manner by which
460 they increased carnosine levels (acute exposure to a carnosine containing solution), the
461 conditions of the muscle (skinned fibres devoid of connective tissue and not attached to bone)
462 and the manner in which it was activated (exposure to Ca^{2+} buffered solutions), all of which
463 bear little resemblance to the present *in vivo* study. Secondly, although enhanced Ca^{2+}-
464 induced Ca^{2+} release was observed following exposure to carnosine in single fibre
465 preparations (11), the authors concede that this may not occur *in vivo*, since this mechanism
466 might have limited relevance to the control of Ca^{2+} release through ryanodine receptors by
467 the dihydropyridine receptors (12; 25). Thirdly, species differences in carnosine metabolism
468 and histidine-containing dipeptide content (4; 13) could explain the discrepancy between the
469 data of Everaert et al. (2013) in mice and the data from the present study. The potential for
470 inter-species differences is suggested by the fact that previous human data showed no fibre-
471 type differences in the carnosine-related changes in Ca^{2+} sensitivity (11), whilst there was
472 some suggestion of fibre type differences in mice (*i.e.*, differences in the response of "slow"
473 *soleus* versus "fast" *extensor digitorum longus* muscles to BA supplementation) (13). It
474 should be noted that we did not measure muscle carnosine content in the present study and so
475 we cannot confirm the actual change due to BA supplementation or whether this directly
476 relates to the individual responses in muscle contractile properties. It is likely, given the
477 previous data on the topic [e.g. (19)], that the increase in muscle carnosine would be around
478 15 $mmol.kg^{-1}$ dry muscle or +65% in these particpants, with this supplementation regimen.

Whilst the majority of the evoked contractile properties showed no change in response to BA supplementation, HRT decreased by 7-12% during resting and potentiated twitch contractions. Muscle relaxation is initiated by a reduction in sarcoplasmic reticulum Ca^{2+} concentration. The rate of relaxation may be influenced by: (i) the rate of dissociation of Ca^{2+} from troponin (26); (ii) the rate of translocation of Ca^{2+} to a site close to the sarcoplasmic reticulum (28); and (iii) the rate of re-uptake of Ca^{2+} into the sarcoplasmic reticulum by ATPase driven Ca^{2+} pumps (30). At present, there do not appear to be any reports of carnosine influencing these aspects of excitation-contraction coupling. Interestingly, however, Everearts et al. (2013) reported an attenuation of the fatigue-related increases in relaxation times after BA supplementation in murine soleus muscle. Whilst their finding in this case could be a consequence of enhanced buffering capacity, during the repeated contractions, since BA supplementation had no influence on resting rate of relaxation, their report further highlight the functional implications of the present data. During fatigue, the rate of muscle relaxation slows as a consequence of a reduced rate of cross-bridge dissociation or impaired Ca^{2+} pumping into the sarcoplasmic reticulum (2). The latter is energetically costly (30) and, as such, any improvements in Ca^{2+} handling with BA supplementation could reduce the total energy expenditure during high-intensity cyclic joint movements by reducing that energy cost, and also by improving the efficiency of joint movements by reducing co-contraction. Future research should attempt to confirm the present findings and extend them by investigating the changes in evoked contractile properties during fatigue in order to better understand the influence of BA supplementation on muscle contractility and implications for metabolic and movement efficiency during exercise.

Voluntary force production and motor control

BA supplementation had no effect on MVF, a finding consistent with the lack of changes in electrically-evoked twitch or tetanic (octet) peak force in the present study. Maximum isometric force is not affected by either increased Ca^{2+} sensitivity or increased myoplasmic Ca^{2+} concentration (27), and previous studies reported minimal effects of carnosine on maximum calcium activated force (0-3% increase) (11) and of BA supplementation on maximal twitch and tetanic force (13). Improved Ca^{2+} sensitivity or release would be

expected to be beneficial for force production in situations where calcium saturation is submaximal [*e.g.*, during the rising phase of voluntary force production where neuromuscular activation is submaximal, (14)] and during sustained submaximal contractions. Thus, one might have expected improvements in explosive voluntary force and/or alterations in the force-EMG relationship, indicative of the change in neuromuscular activation required to produce a given change in force, if BA supplementation had influenced these Ca^{2+} related functions. However, in accordance with the lack of changes in the force-frequency relationship, as well as the force responses during twitch and octet contractions, BA supplementation did not influence voluntary explosive force or the force-EMG relationship. The similar neural drive during both the maximum voluntary contractions and explosive voluntary contractions confirm that the force results were not confounded by changes in neuromuscular activation over time.

Conclusions

The results of the present study showed that BA supplementation had no effect on the force-frequency relationship, implying a lack of any effect on muscle Ca^{2+} sensitivity or release. In support of these data, there was no effect of BA supplementation on force responses to resting and potentiated twitches and octet contractions. As such, the study findings do not support the idea that exercise performance and capacity improvements after BA supplementation are due to enhanced Ca^{2+} sensitivity or release. We do, however, show a reduction in HRT with BA supplementation, which might possibly be explained by enhanced reuptake of Ca^{2+} into the sarcoplasmic reticulum. This has potentially important implications for the efficiency of muscle contraction following BA that should be explored in future studies, since this could conceivably contribute to the ergogenic potential of BA supplementation during high-intensity exercise involving rapid muscle contractions.

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Figure legends

Figure 1. Electrically-evoked force of BA and PLA groups pre- and post-supplementation: (A), resting twitch force; (B), potentiated twitch force; (C), octet force. Data are mean ± 1SD.

Figure 2. Contraction times during twitch contractions evoked via femoral nerve stimulation for BA and PLA groups pre- and post-supplementation. Resting twitch: EMD (A); TPT (B); and HRT (C). Potentiated twitch: EMD (D); TPT (E); and HRT (F). Data are mean ± 1SD. $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ for *post-hoc* independent *t*-test on % change values in BA and PLA groups.

Figure 3. Force-frequency relationship assessed during submaximal percutaneous stimulation for BA and PLA groups pre- and post-supplementation. Data are mean ± 1SD.

Figure 4. Explosive and maximum voluntary force of BA and PLA groups pre- and post-supplementation (A). Agonist EMG normalised to M_{max} during explosive contractions (0-50, 50-100 and 100-150 ms from onset) and at MVF for the BA and PLA groups pre- and post-supplementation (B). Data are mean ± 1SD.

Figure 5. Force-EMG relationship measured during submaximal voluntary contractions (15 – 90% MVF) for BA and PLA groups pre- and post-supplementation. Data are mean ± 1SD.

678 **Tables**

679

Table 1. Characteristics of the force-frequency and force-EMG relationships of BA and PLA groups pre- and post-supplementation. Data are mean ± 1SD.

	Pre		**Post**	
	BA	**PLA**	**BA**	**PLA**
Force-frequency relationship				
Frequency at 50% of force response (Hz)	17.3 ± 2.4	18.8 ± 1.5	16.8 ± 1.7	18.0 ± 1.9
Force-EMG relationship				
Intercept (RMS:M_{max})	-0.49 ± 0.74	-0.70 ± 0.47	-0.53 ± 0.91	-0.56 ± 0.43
Slope (RMS:$M_{max} \cdot N^{-1}$)	0.0175 ± 0.0054	0.0180 ± 0.0035	0.0174 ± 0.0037	0.0170 ± 0.0037

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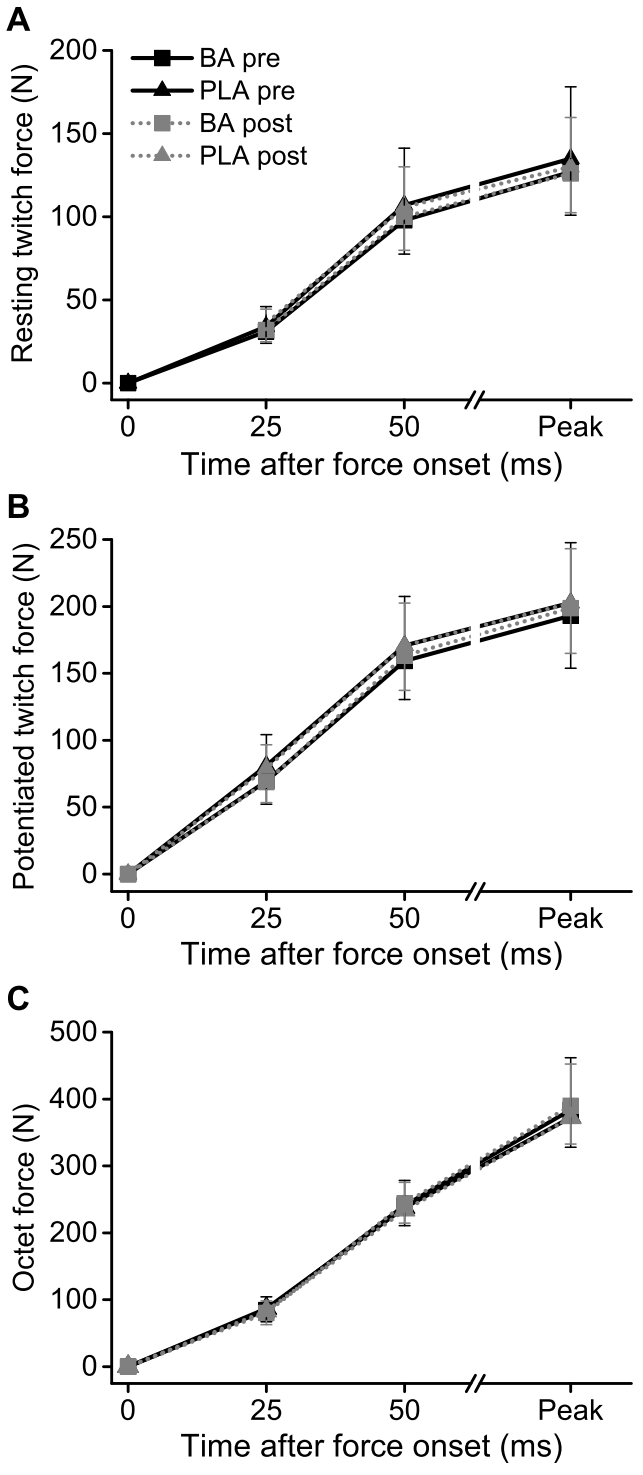
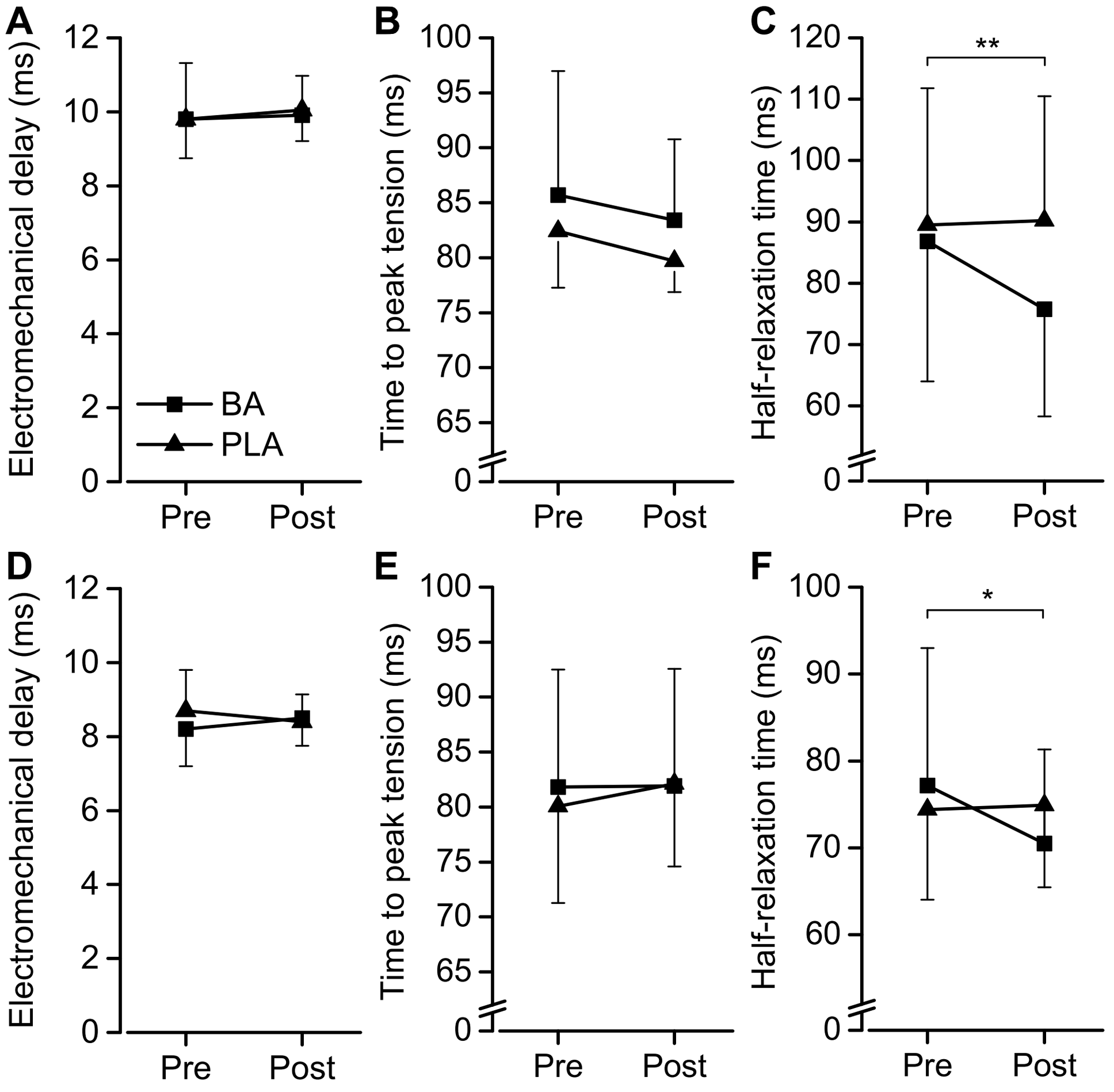
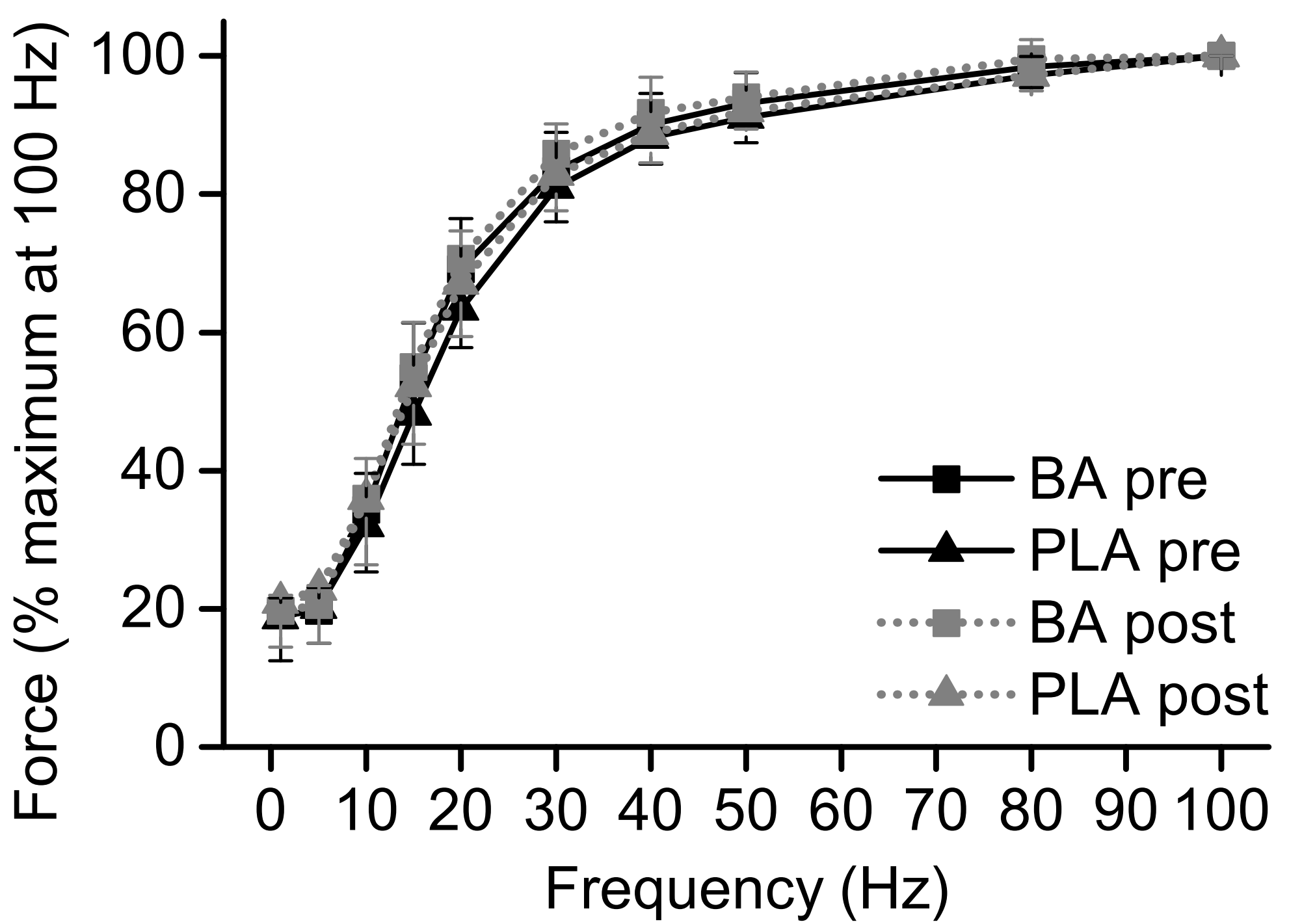
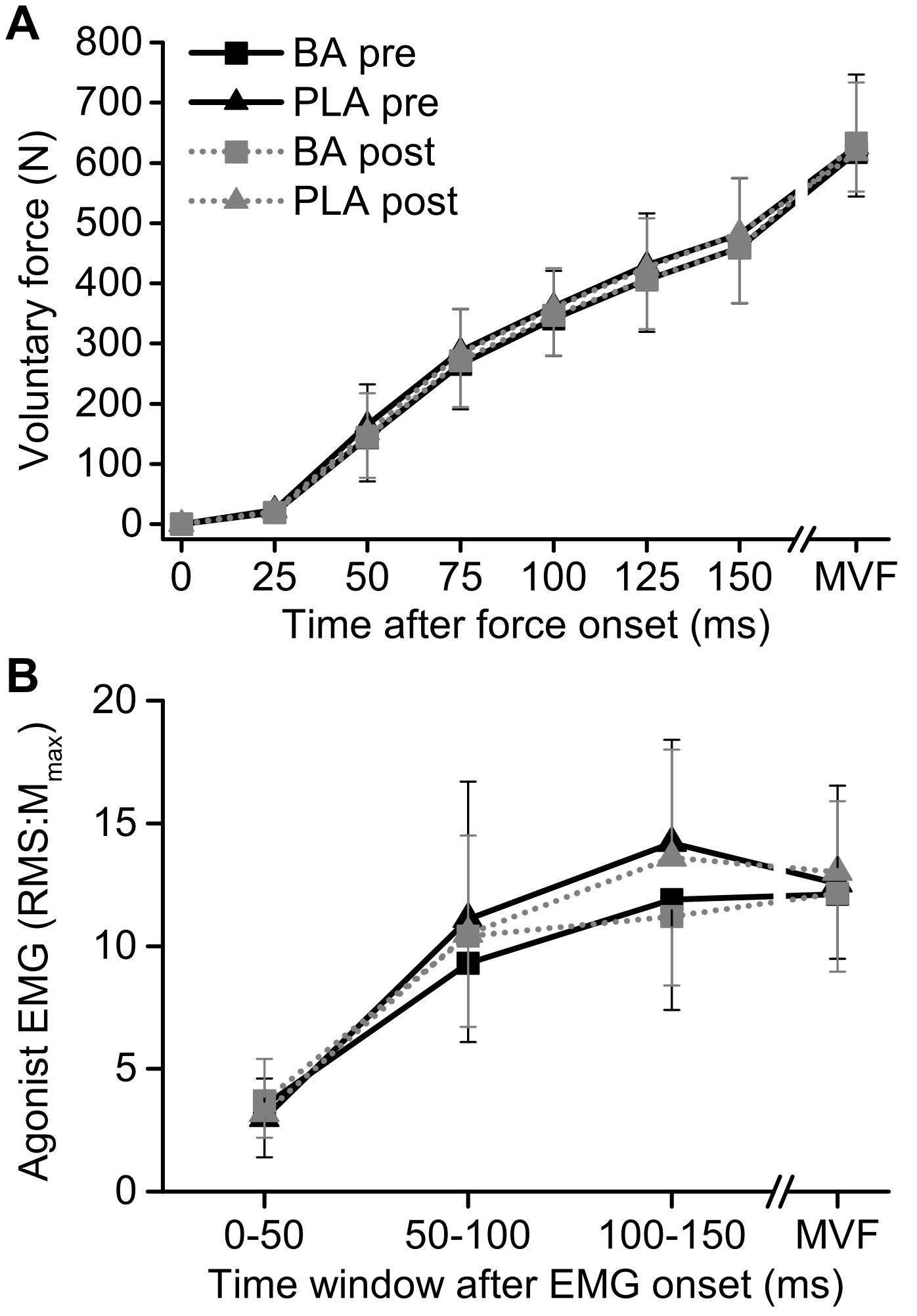

A
BA pre
PLA pre
BA post
PLA post
Resting twitch force (N)
200
150
100
50
0
0
25
50
Peak
Time after force onset (ms)
B
Potentiated twitch force (N)
250
200
150
100
50
0
0
25
50
Peak
Time after force onset (ms)
C
Octet force (N)
500
400
300
200
100
0
0
25
50
Peak
Time after force onset (ms)


A
B
C
D
E
F
Electromechanical delay (ms)
Time to peak tension (ms)
Half-relaxation time (ms)
BA
PLA
Pre
Post
**
*


Force (% maximum at 100 Hz)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
Frequency (Hz)
BA pre
PLA pre
BA post
PLA post


A
800
700
600
500
400
300
200
100
0
Voluntary force (N)
BA pre
PLA pre
BA post
PLA post
0
25
50
75
100
125
150
MVF
Time after force onset (ms)
B
20
15
10
5
0
Agonist EMG (RMS:M$_{max}$)
0-50
50-100
100-150
MVF
Time window after EMG onset (ms)

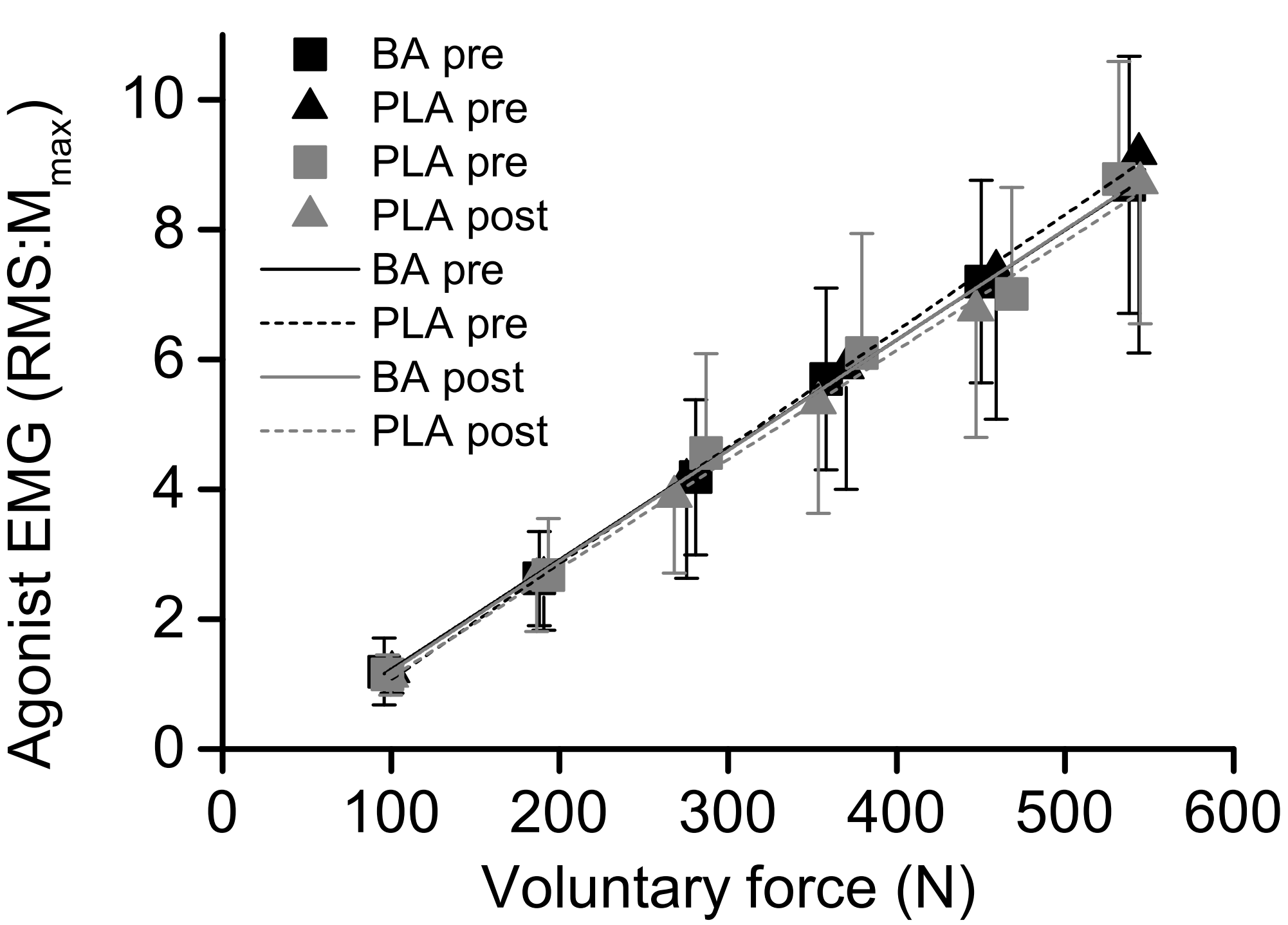

BA pre
PLA pre
PLA pre
PLA post
BA pre
PLA pre
BA post
PLA post
Agonist EMG (RMS:M$_{max}$)
0
2
4
6
8
10
0
100
200
300
400
500
600
Voluntary force (N)

Table 1.

	Pre		Post	
	BA	**PLA**	**BA**	**PLA**
Force-frequency relationship				
Frequency at 50% of force response (Hz)	17.3 ± 2.4	18.8 ± 1.5	16.8 ± 1.7	18.0 ± 1.9
Force-EMG relationship				
Intercept (RMS:M_{max})	-0.49 ± 0.74	-0.70 ± 0.47	-0.53 ± 0.91	-0.56 ± 0.43
Slope (RMS:$M_{max} \cdot N^{-1}$)	0.0175 ± 0.0054	0.0180 ± 0.0035	0.0174 ± 0.0037	0.0170 ± 0.0037