Effects of calcitonin gene-related peptide on rat soleus muscle excitability: mechanisms and physiological significance

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Submitted 13 December 2007; accepted in final form 20 July 2008

Macdonald WA, Nielsen OB, Clausen T. Effects of calcitonin gene-related peptide on rat soleus muscle excitability: mechanisms and physiological significance. Am J Physiol Regul Integr Comp Physiol 295: R1214–R1223, 2008. First published July 23, 2008; doi:10.1152/ajpregu.00893.2007.—Intense exercise causes a large increase in extracellular K⁺ ([K⁺]o) in contracting muscles. This triggers Na⁺ entry and loss of K⁺ from contracting muscles. The ensuing elevation of extracellular K⁺ ([K⁺]o, extracellular K⁺) has been suggested to cause fatigue by depressing muscle fiber excitability. In isolated muscles, however, repeated contractions confer some protection against this effect of elevated K⁺. We hypothesize that this excitation-induced force-recovery is related to the release of the neuropeptide calcitonin gene-related peptide (CGRP), which stimulates the muscular Na⁺-K⁺ pumps. Using the specific CGRP antagonist CGRP-(8-37), we evaluated the role of CGRP in the excitation-induced force recovery and examined possible mechanisms. Intact rat soleus muscles were stimulated to evoke short tetani at regular intervals. Increasing extracellular K⁺ ([K⁺]o) from 4 to 11 mM decreased force to ~20% of initial force (P < 0.001). Addition of exogenous CGRP (10⁻⁹ M), release of endogenous CGRP with capsaicin, or repeated electrical stimulation recovered force to 50–70% of initial force (P < 0.001). In all cases, force recovery could be almost completely suppressed by CGRP-(8-37). At 11 mM [K⁺]o, CGRP (10⁻⁸ M) did not alter resting membrane potential or conductance but significantly improved action potentials (P < 0.001) and increased the proportion of excitable fibers from 32 to 70% (P < 0.001). CGRP was shown to induce substantial force recovery with only modest Na⁺-K⁺ pump stimulation. We conclude that the excitation-induced force recovery is caused by a recovery of excitability, induced by local release of CGRP. The data suggest that the recovery of excitability partly was induced by Na⁺-K⁺ pump stimulation and partly by altering Na⁺ channel function.

Na⁺-K⁺ pump; Na⁺ channels; Cl⁻ channels; extracellular K⁺

IN SKELETAL MUSCLE, THE SIGNAL for contraction involves the propagation of action potentials from the neuromuscular junction, along the sarcolemma and down into the transverse-tubular (T system), where it activates the voltage sensor molecules, resulting in sarcoplasmic reticulum (SR) Ca²⁺ release and ultimately force production. High frequency activity causes muscles to lose K⁺, with extracellular K⁺ ([K⁺]o) reported to rise from 4 to 8 mM in the plasma (26) and higher (12–14 mM) in the interstitium (32, 29, 45). Exposure of isolated muscles to such [K⁺]o leads to a substantial reduction in tetanic force (for reviews see Refs. 7, 44). Indeed, at elevated [K⁺]o, isolated muscle fibers are depolarized (19), Na⁺ channels undergo slow inactivation (41), and action potential amplitude is reduced (39), which all contribute to a reduction in muscle excitability. Ultimately, this causes a complete loss of excitability in a number of the fibers of the muscles and consequently a reduction in maximal force production (37) and in the amplitude and area of compound action potentials (M waves; Ref. 35).

In isolated muscles, the depressing effect of elevated [K⁺]o on muscle contractility can be alleviated by the application of the neuropeptide calcitonin gene-related peptide (CGRP) (2). CGRP is a small 37 amino acid neuropeptide that is found in motor and sensory nerve terminals in skeletal muscle (46). In response to muscle activity, CGRP is released from the nerve endings in skeletal muscle (49) by a mechanism that, much like the release of ACh, is dependent on external Ca²⁺ (42). The binding of CGRP to sarcolemmal bound CGRP receptors, located primarily at the motor end plate region of the muscle (15), stimulates adenylate cyclase activity, leading to an increase in intracellular cAMP (47, 49), a potent intracellular second messenger and stimulator of the Na⁺-K⁺ pump (7). The CGRP-induced force recovery of elevated [K⁺]o-depressed muscles has therefore been suggested to be due to stimulation of the Na⁺-K⁺ pump and subsequent regaining of muscle excitability (31, 34).

Force recovery of isolated muscles depressed by elevated [K⁺]o has also been achieved by repeated stimulation of the muscles, a process termed excitation-induced force recovery (31). It was suggested that this recovery of force was due to nervous release of CGRP from the preparation during the repeated stimulation paradigm. Skeletal muscle has a limited supply of CGRP, which is replenished through axonal transport from the site of production in the cell body of the nerve (20). Indeed, muscles that have been denervated for 7 days, and therefore have a lower CGRP content, exhibit no excitation-induced force recovery (31). This suggests that CGRP is important for this force recovery. However, the exact role of CGRP in excitation-induced force recovery is not known.

Here, we investigate the mechanisms by which CGRP increases skeletal muscle excitability and test the following hypotheses: 1) excitation-induced force recovery is caused by local release of CGRP; 2) CGRP-induced force recovery is via a restoration of muscle excitability; and 3) CGRP-induced increase in excitability is via stimulation of the Na⁺-K⁺ pump.

METHODS

Animals, preparation, and incubation of muscles. All handling and use of animals complied with Danish animal welfare regulations. The University of Aarhus Laboratory conducts its animal studies under license number 1982-39911-436, and all procedures and euthanasia are supervised by an animal welfare officer. In accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
formed using either 4- or 12-wk-old female or male Wistar rats of own breed, weighing 65–75 g or ~250 g, respectively. Animals were kept in a thermostated environment at 21°C with a 12:12-h light-dark cycle and fed ad libitum. The animals were killed by cervical dislocation, followed by decapitation. Intact soleus muscles (20–25 and ~100 mg wet wt for 4- and 12-wk-old animals, respectively) were prepared and incubated in standard Krebs-Ringer bicarbonate buffer (KR) containing the following (in mM): 122.1 NaCl, 25.1 NaHCO₃, 2.8 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.3 CaCl₂, and 5.0 D-glucose. Total buffer Cl⁻ was 127.4 mM. In Cl⁻ free buffer, methanesulphonate salts replaced NaCl and KCl, and Ca(NO₃)₂ replaced CaCl₂. In buffers containing 9, 11, or 13 mM K⁺, KCl replaced part of the NaCl, thus maintaining Cl⁻ concentration, osmolarity, and ionic strength constant. In buffers containing 10 mM Mg²⁺, 1.2 mM MgSO₄, and 15 mM NaCl were replaced with 1.2 mM Na₂SO₄ and 10 mM MgCl₂, respectively. All incubations took place at 30°C under continuous gassing with a mixture of 95% O₂-5% CO₂, resulting in a buffer pH of 7.4.

Measurement of force. Muscles from 4-wk-old animals were mounted for isometric contractions in thermostatted chambers containing standard KR, adjusted to optimal length for force production, and exposed to field stimulation across the central region through platinum electrodes using 2-s trains of 12-V pulses at 60 Hz every 10 min. In most experiments, the pulse duration was 0.2 ms, but in a few control experiments pulse duration was reduced to 0.02 ms. We have previously shown that when 0.02-ms pulses are used, the activation of muscle fibers exclusively takes place via motor nerve excitation, with no direct effect on muscular voltage-gated Na⁺ channels. Stimulation with 0.2-ms pulses, however, also leads to direct activation of the muscle fibers making the force response insensitive to tubocurarine (34). To induce excitation-induced force recovery, the muscles were stimulated using 1-s trains of 0.2 ms pulses at 30 Hz every 60 s or 2-s trains of 0.2-ms pulses at 60 Hz every 60 s. Force was measured using force displacement transducers (Grass FT03, Grass-Telefactor, West Warwick, RI) and recorded with a chart recorder and/or on a computer using Chart 5.4 software (ADInstruments, Sydney, Australia). The mean absolute tetanic force produced under control conditions by using Chart 5.4 software (ADInstruments, Sydney, Australia). The mean absolute tetanic force produced under control conditions by muscles from 4-wk-old rats was 0.41 nN/mg, and fitted to a two parameter exponentially decaying function (55). For each fiber investigated, the three ΔV₉₀-to-I ratios were plotted on a log scale against the inter-electrode distance (x) on a linear scale and fitted to a two parameter exponentially decaying function (\(y(x) = y_{0 \text{ - exp}}\)) giving a straight line (see Fig. 6B). From fibers that showed an accurate fit (\(r^2 \geq 0.99\)), the ordinate intercept \((y_0)\) was taken as the input resistance \(R_i\) and the length constant \((\lambda)\) was calculated from the slope of the fitted line \((b = 1/\lambda)\). With the assumption of an internal resistivity \((R_i)\) of 180 Ω cm\((1)\), \(R_i\), and \(\lambda\) were used to calculate the fiber diameter and specific membrane conductance, as previously described in detail previously (37). Any fibers in which the RMP depolarized by > 7 mV, while the two electrodes were inserted in the fiber, were disregarded. Similarly, fibers with a RMP more positive than ~60 mV at 4 mM K⁺ or ~55 mV at 9 mM K⁺ were disregarded.

Electrophysiological measurements were performed in muscles that were initially allowed to equilibrate in standard KR for 30 min before recordings. In experiments at 9 mM K⁺, muscles were incubated in standard KR for 30 min before exposure to 9 mM K⁺ for 30 min before recordings. In experiments with CGRP, recording began 10 min after the addition of CGRP, continued for 30 min, and was time matched to corresponding controls.

Measurement of Na⁺-K⁺ pump activity and intracellular Na⁺ content. Previous studies have shown that ⁸⁶⁹Rb⁺ is a reliable tracer for determination of K⁺ transport via the Na⁺-K⁺ pump (9) and that stimulation of the Na⁺-K⁺ pump results in a reduction in intracellular Na⁺ of isolated muscles (13). To detect the effects of CGRP on the Na⁺-K⁺ pumps, muscles from 4-wk-old animals were preincubated for 30 min in standard KR, followed by the addition of CGRP for 10, 20, or 40 min. Other muscles were preincubated for 30 min in standard KR, before incubation for 60 min at 11 mM K⁺ buffer followed by the addition of CGRP for 10, 20, or 40 min. In experiments where muscles were exposed to CGRP for 10 or 20 min, ⁸⁶⁹Rb⁺ (0.2 μCi/ml) was added to the buffer during CGRP exposure. Finally, the muscles were washed for 4 × 15 min in ice-cold Na⁺-free Tris-sucrose buffer to remove extracellular ⁸⁶⁹Rb⁺ and Na⁺. The muscles were then blotted, weighed, and taken for counting of ⁸⁶⁹Rb⁺ activity by Cerenkov radiation in a β-counter. The amount of ⁸⁶⁹Rb⁺ activity retained after washout was calculated, and the uptake of K⁺ was then determined by converting the relative uptake of ⁸⁶⁹Rb⁺ to K⁺ using the concentration of K⁺ in the incubation medium (5). Muscles from the ⁸⁶⁹Rb⁺ influx experiments were retained to measure intracellular Na⁺ and soaked overnight in 0.3 M TCA, which gives complete extraction of Na⁺ from the muscle tissue (5), before Na⁺ concentrations were measured using flame photometry (FLM3, Radiometer). The intracellular contents of Na⁺ were then determined as previously described using a factor of 1.46 to correct for loss of intracellular Na⁺ during the 4 × 15 min washout (5).

Chemicals and isotopes. All chemicals were of analytical grade. Rat CGRP was used in all experiments and added from 10⁻⁶ M stock, dissolved in double distilled water. The competitive CGRP antagonist CGRP-(8-37) was dissolved directly in the buffer. Both CGRP and CGRP-(8-37) were obtained from Bachem. Capsaicin was added from 10⁻⁸ M stock dissolved in double distilled water and was obtained

AJP-Regul Integr Comp Physiol • VOL 295 • OCTOBER 2008 • www.ajpregu.org
form Sigma-Aldrich. \(^{86}\)Rb\(^+\) was from Amersham International (Aylesbury, Buckinghamshire, UK).

**Statistics.** All data are means ± SE, where \(n\) represents both the number of muscles and the number of animals, unless noted otherwise. The statistical significance of any difference between groups was accepted at \(P < 0.05\), as determined using Student’s two-tailed \(t\)-test for either paired or nonpaired observations, or one-way or two-way ANOVA, where appropriate.

**RESULTS**

**Effect of CGRP-(8-37) on force recovery induced by exogenous and endogenous CGRP.** In this study, the competitive CGRP antagonist CGRP-(8-37) was used to probe for the role of CRGP in the excitation-induced force recovery in muscles exposed to elevated \([K^+]_o\). To evaluate the potency of CGRP-(8-37), isolated 4-wk-old soleus muscles were exposed to 11 mM K\(^+\). As illustrated in Fig. 1, this clearly depressed the function of the muscles and after 60 min exposure to 11 mM K\(^+\), tetanic force was reduced to \(\sim 20\%\) of the initial tetanic force at 4 mM K\(^+\). Addition of either \(10^{-8}\) M (Fig. 1A) or \(10^{-9}\) M (Fig. 1B) CGRP caused a significant, albeit transient, recovery in force (\(P < 0.001\)). Importantly, pretreatment of muscles for 30 min with \(10^{-5}\) M CGRP-(8-37) before the addition of CGRP, completely suppressed the effect of \(10^{-9}\) M CGRP on force (\(P < 0.001\), Fig. 1B), and significantly reduced the force recovery induced by \(10^{-8}\) M CGRP (\(P < 0.001\), Fig. 1A).

Figure 2 shows that CGRP-(8-37) had a similar effect on the force recovery induced by 10\(^{-5}\) M CGRP before the addition of CGRP, where force was maintained at a steady level.

**Excitation-induced force recovery.** Force recovery in elevated \([K^+]_o\), depressed muscle can also be achieved by repeatedly stimulating the muscles (31). As shown in Fig. 3, repeated stimulation of muscles exposed to 11 mM K\(^+\) for 60 min, using 2-s trains of 0.2-ms pulses at 60 Hz, every 60 s for 30 min, induced a significant force recovery from 23±4 to 72±5% of initial force (\(P = 0.001\); Fig. 3A). A significant recovery of force (from 22±9 to 36±12% of control force in 8 min; \(n = 4\); \(P = 0.041\)) was also seen when the temperature was increased to 37°C, 30 min before the beginning of the repeated stimulation. At 30°C, excitation-induced force recovery was a graded response, as stimulating muscles with a 1-s trains of pulses at 30 Hz, every 60 s for 40 min, significantly recovered force from 18±2 to 48±5% of initial force (\(P < 0.001\); Fig. 3B). The addition of \(10^{-5}\) M CGRP-(8-37) 30 min before the start of the repeated stimulation regime significantly reduced the excitation-induced force recovery elicited by a 30-Hz stimulation (recovery to 24±5% compared with 48±5% of initial force; \(P < 0.001\); Fig. 3B), indicating that nervous release of CGRP is important for excitation-induced force recovery. However, addition of CGRP-(8-37) could not prevent excitation-induced force recovery elicited by a 60-Hz stimulation (25±2 to 59±8% of initial force; Fig. 3A). In all muscles, force was completely restored to initial levels when returned to standard KR (data not shown).

To further examine the importance of CGRP release from presynaptic nerve terminals for the excitation-induced force recovery, the release of endogenous CGRP from nerves was blocked by increasing extracellular \([Mg^{2+}]_o\) to 10 mM (23, 42). To test the efficiency of this method in blocking the motor nerve, a series of control experiments was performed in which the effect of 10 mM \(Mg^{2+}\) on the force response to motor nerve activation was examined. In these experiments, the muscle preparation was stimulated with pulses of a 0.02-ms duration, which exclusively leads to the activation of the muscle fibers via motor nerve excitation (34). In muscles incubated at 4 mM K\(^+\) and normal \(Mg^{2+}\) (1.2 mM \(Mg^{2+}\)), stimulation with 0.02-ms pulses produced a tetanic force of 94±1% of the tetanic force elicited using 0.2-ms pulses (\(n = 4\)). However, in muscles incubated at 10 mM \(Mg^{2+}\), the force produced when they were stimulated with 0.02-ms pulses was reduced to 1±1% of the tetanic force using 0.2-ms pulses (\(n = 4\)). In contrast, in muscles stimulated directly using 0.2-ms pulses, increasing the \([Mg^{2+}]_o\) from 1.2 to 10 mM only resulted in a small, although significant, reduction in tetanic force to 94±1% of initial force (\(P < 0.01\); \(n = 4\)). These
control experiments show that increasing [Mg$^{2+}$]o to 10 mM effectively blocks ACh release and presumably also CGRP release from the nerve endings, while muscle fiber excitability and function are preserved.

To examine the role of CGRP release from the presynaptic nerve terminals for the excitation-induced force recovery, the effect of 10 mM Mg$^{2+}$ on the force response in muscles at 11 mM K$^+$ and stimulated directly using 0.2-millisecond pulses was tested. As shown in Fig. 4, the force depression after incubation at 11 mM K$^+$ was the same in muscles at 10 mM Mg$^{2+}$ as in muscles at 1.2 Mg$^{2+}$. In contrast, the excitation-induced force recovery elicited by repeated direct stimulation (2-s trains of 0.2-ms pulses at 60 Hz every 60 s) was significantly reduced in the presence of 10 mM Mg$^{2+}$ (P < 0.001; Fig. 4). Returning the muscles exposed to 11 mM K$^+$ and 10 mM Mg$^{2+}$ to buffer containing 11 mM K$^+$ and only 1.2 mM Mg$^{2+}$ resulted in a significant excitation-induced force recovery to 79 ± 2% of initial force. In all muscles, force was completely restored to initial levels when the buffer was returned to standard KR (4 mM K$^+$ and 1.2 mM Mg$^{2+}$; data not shown). These experiments show that although the muscle fibers were stimulated directly, the excitation-induced force recovery was turned off if the function of the motor nerve and, therefore, its release of CGRP were blocked by elevating [Mg$^{2+}$]o.

Effect of CGRP on muscle excitability. To determine whether the CGRP-induced force recovery was associated with an improvement of muscle excitability, action potentials were measured at 4 mM K$^+$ and at elevated [K$^+$]o without and with 10$^{-8}$ M CGRP. In these experiments, with the use of soleus muscle from 12-wk-old rats, 9 mM K$^+$ was used rather than 11 mM K$^+$, as it has previously been shown that soleus muscles from 12-wk-old rats are more sensitive to the depressing effect of elevated [K$^+$]o, and are completely inexcitable at 11 mM K$^+$ (37). Representative traces are shown in Fig. 5 with summarized data in Table 1. At 4 mM K$^+$, the addition of CGRP (10$^{-8}$ M) resulted in a significant 5-mV hyperpolarization of RMP (P < 0.001), a significant 7-mV higher action potential overshoot (P < 0.001) and significantly faster maximum rates of depolarization (40%, P < 0.001) and repolarization (25%, P < 0.001). In these experiments, the action potentials were initiated in single fibers by a 400-ms constant current injection. The number of spontaneous action potentials that fired during the 400-ms current injection significantly increased from 1.2 ± 0.1 (range 1–2) in control muscles at 4 mM K$^+$ to 2.2 ± 0.2 (range 1–6, P < 0.001; Fig. 5, A and B) after the addition of 10$^{-8}$ M CGRP. In a separate set of experiments, the addition of CGRP at 4 mM K$^+$ resulted in a significant 35% reduction in rheobase current required to elicit an action potential (P < 0.001; Table 1).

Increasing [K$^+$]o from 4 to 9 mM significantly depressed muscle excitability (all action potential parameters, P < 0.001). The addition of 10$^{-8}$ M CGRP at 9 mM K$^+$ did not result in any hyperpolarization of RMP (P = 0.241); however, there was a significant 6-mV increase in action potential overshoot (P = 0.002) and significantly faster maximum rates of depolarization (35%, P = 0.039) and repolarization (50%, P = 0.001). Furthermore, the addition of CGRP at 9 mM K$^+$ resulted in a large increase in the number of excitable fibers from 32% of all fibers in muscles at 9 mM K$^+$ to 72% of all fibers in muscles at 9 mM K$^+$ with CGRP.

Fig. 2. Effect of CGRP-(8-37) on capsaicin-induced force recovery. Muscles were equilibrated in KR, before exposure to buffer containing 11 mM K$^+$, where they were kept until steady-state force responses were achieved. Force recovery induced by capsaicin (10$^{-7}$ M) was then triggered without (●) or with (○) CGRP-(8-37) (10$^{-6}$ M). Muscles were stimulated with 0.2-ms, 12-V pulses at 60 Hz for 2 s every 10 min. Data points are means ± SE; n = 4.

Fig. 3. Effect of CGRP-(8-37) on excitation-induced force recovery. Muscles were equilibrated in KR before exposure to 11 mM K$^+$ buffer, where they were kept until steady-state force responses were achieved. Excitation-induced force recovery was then elicited with either 2.0-s trains of pulses of 60 Hz every 60 s (A) or 1.0-s trains of pulses of 30 Hz every 60 s (B). Muscles were incubated without (●) or with (○) CGRP-(8-37) (10$^{-6}$ M) for 30 min before the repeated stimulation paradigm. Except where indicated in figure, muscles were stimulated with 0.2-ms, 12-V pulses at 60 Hz for 2 s every 10 min. Data points are means ± SE; n = 4.
Fig. 4. Effect of elevated extracellular [Mg²⁺] on excitation-induced force recovery. Muscles were equilibrated in KR containing 4 mM K⁺ and 1.2 mM Mg²⁺ (○) or 10 mM Mg²⁺ (□) before an increase in buffer K⁺ to 11 mM K⁺. Excitation-induced force recovery was elicited using 2.0-s trains of 60-Hz pulses every 60 s for 60 min. After 30 min of excitation-induced force recovery, the muscles exposed to 11 mM K⁺ and 10 mM Mg²⁺ were placed in 11 mM K⁺ and 1.2 mM Mg²⁺ buffer (indicated by arrow). Except where indicated in figure, muscles were stimulated with 0.2-ms, 12-V pulses at 60 Hz for 2 s every 10 min. Data points are means ± SE; n = 4.

Effect of CGRP on muscle resting membrane conductance. To determine whether the effect of CGRP on muscle excitability at elevated [K⁺]₀, could be related to an opening or closing of ion channels at RMP and therefore a change in resting membrane conductance, muscle cable parameters were measured. Figure 6A shows representative traces of membrane responses to a hyperpolarizing current of −40 nA recorded at three different locations in two fibers incubated at 9 mM K⁺ without and with 10⁻⁸ M CGRP, respectively. From these recordings, the ΔVₘ-to-I ratios were calculated and related to the inter-electrode distance as shown in Fig. 6B. From such plots, the length constant (λ), input resistance (Rᵢ), and membrane conductance (Gₐₙ) were determined in accordance with the methods of Boyd and Martin (4). Table 2 shows summarized data of these parameters and that the values of λ (P = 0.560), Rᵢ (P = 0.691), and Gₐₙ (P = 0.880) were not altered by the addition of 10⁻⁸ M CGRP, suggesting that CGRP had no effect on passive membrane properties of the muscles. As the resting membrane conductance in skeletal muscle is composed of the resting membrane conductance for K⁺ (G钾) and Cl⁻ (G chloride), CGRP-induced force recovery does not appear to be mediated via an altered function of the K⁺ or Cl⁻ channels that are conducting current at potentials around the resting membrane potential.

To further explore for an involvement of Cl⁻ channels in the CGRP-induced force recovery, the effect of 11 mM K⁺ and CGRP on force was tested in muscles incubated in Cl⁻-free buffer (Fig. 7). These experiments showed that transferring muscles from KR to Cl⁻-free buffer for 40 min resulted in a nonsignificant 10% ± 6% increase in tetanic force (P > 0.05). Muscles were then moved to Cl⁻-free buffer containing 11 mM K⁺, where the force was significantly reduced to ~65% of initial force (P > 0.001). Since Cl⁻-free conditions per se partially prevent the depression of force in muscles incubated at 11 mM K⁺, the level of force at 11 mM K⁺ in these experiments was higher than in the previous experiments (Figs. 1, 2, 3, and 4). Subsequent addition of 10⁻⁸ M CGRP resulted in a significant force recovery to 91% ± 7% of initial force (P < 0.001). After 30 min, force returned to pre-CGRP levels. A similar experiment was performed where 13 mM K⁺ was used instead of 11 mM K⁺ (Fig. 7B). In Cl⁻-free buffer containing 13 mM K⁺, the tetanic force was reduced to almost zero (1 ± 1% of initial force, P < 0.001). The addition of 10⁻⁸ M CGRP resulted in a significant force recovery to 77% ± 5% of initial force (P < 0.001), which returned to pre-CGRP levels after 40 min. In all muscles, force was completely restored to initial values when returned to standard KR. Since force recovery occurred in Cl⁻-free buffer, where G chloride is already abolished, these experiments show that Cl⁻ channels are not involved in the CGRP-induced force recovery.

Effect of CGRP on the Na⁺-K⁺ pump. The CGRP-induced force recovery of elevated [K⁺]₀, depressed muscles has been suggested to be due to the stimulation of the Na⁺-K⁺ pump and the subsequent regaining of muscle excitability (2, 31). Figure 8A shows that at 4 mM K⁺, incubation with 10⁻⁸ M CGRP for 10 min induced a significant decrease in intracellular Na⁺ content of 27%. When only 10⁻⁹ M CGRP was added, the reduction in intracellular Na⁺ content was slower. Furthermore, at this concentration pretreatment of muscles for 30 min with 10⁻⁵ M CGRP-(8-37) prevented any significant decrease in intracellular Na⁺, even after a 40-min exposure to CGRP (P = 0.940) compared with controls. Application of CGRP-(8-37) alone did not significantly alter the intracellular Na⁺ content compared with controls (P = 0.615). Exposure to CGRP also induced a significant decrease in intracellular Na⁺ content of muscles incubated at 11 mM K⁺ (Fig. 8B), although the reduction induced by the presence of 10⁻⁵ M CGRP was smaller than in muscles at 4 mM K⁺. These reductions in intracellular Na⁺ content are indicative of an acceleration in Na⁺-K⁺ pump activity. To further substantiate this effect of CGRP, the K⁺ uptake was determined using ⁸⁶Rb⁺ as a tracer for K⁺ (9). At 4 mM K⁺, the addition of 10⁻⁸ M CGRP increased the K⁺ uptake in the subsequent 10 min from 479 ± 23 to 570 ± 29 nmol·g wet wt⁻¹·min⁻¹ (n = 6 vs. 6; P = 0.033). Similar results were found at 11 mM K⁺, where the K⁺ uptake was increased from 673 ± 20 to 917 ± 21 nmol·g wet wt⁻¹·min⁻¹ (n = 6 vs. 6; P < 0.001). In contrast, the addition of only 10⁻⁹ M CGRP was without effect on the K⁺ uptake of the muscles. Thus at 11 mM K⁺ the K⁺ uptake was 709 ± 22 and 772 ± 20 nmol·g wet wt⁻¹·min⁻¹ (n = 6 vs. 6; P = 0.421) without and with CGRP for 20 min. Similarly, the K⁺ uptake at 4 mM K⁺ was unaltered by addition of 10⁻⁹ M CGRP for 10 min being 461 ± 13 and 464 ± 15 nmol·g wet wt⁻¹·min⁻¹ (n = 6 vs. 6; P = 0.904) or after 20 min being 415 ± 20 and 442 ± 13 nmol·g wet wt⁻¹·min⁻¹ (n = 6 vs. 6; P = 0.293), respectively.

DISCUSSION

Here, using the CGRP antagonist CGRP-(8-37), we show for the first time that excitation-induced force recovery of muscles with depressed excitability (exposure to elevated [K⁺]₀) was specifically due to the release of CGRP and subsequent recovery of excitability. Furthermore, we show that CGRP increases muscle excitability via a mechanism that can only be partially explained by Na⁺-K⁺ pump stimulation, that is not related to...
an alteration in resting K\(^+\) or Cl\(^-\) conductance, and that may partly be related to a change in Na\(^+\) channel function.

**CGRP and force recovery.** The addition of exogenous CGRP resulted in force recovery of isolated muscles depressed by elevated [K\(^+\)]\(_o\), which is in agreement with previous studies (2, 6, 31). At a normal [K\(^+\)]\(_o\), CGRP has also been shown to increase twitch force in the diaphragm muscle (46, 16) and to increase tension and contraction rate in guinea pig atria (33).

Based on experimental reductions in the endogenous CGRP content of muscles, either by pretreatment with capsaicin or by denervation for 7 days, which severely hampered excitation-induced force recovery, a role of CGRP release from the nerves in excitation-induced force recovery has been suggested, although not categorically established (31). Kuiack and McComas (21) suggested that the excitation-induced force recovery in anesthetized rat hindlimb was due to \(\beta_2\)-adrenoreceptor agonist stimulation of the Na\(^+\)-K\(^+\) pump, presumably elicited by circulating catecholamines, as it could be prevented by the application of the \(\beta\)-antagonist propranolol. However, in the same study, the contralateral resting leg showed no hyperpolarization, which would be expected if the plasma catecholamine level increased. The proposed role of catecholamines is also in conflict with Nielsen et al. (31), who found that excitation-induced force recovery was insensitive to propranolol.

In previous studies (31) we have shown that repeated activation of muscles via stimulation of the motor nerve using 0.02-ms pulses or stimulation of the nerve via a suction electrode (34) causes a similar excitation-induced force recovery in K\(^+\) depressed muscles as direct stimulation. In further support for a role of CGRP release from the nerves in excitation-induced force recovery, we show here that inhibition of the CGRP release via elevation of [Mg\(^{2+}\)]\(_o\) (23) significantly attenuates the force recovery in muscle fibers stimulated directly, while returning the preparation to normal [Mg\(^{2+}\)]\(_o\) allows rapid turning on of excitation-induced force recovery.

Table 1. Effect of CGRP on action potential properties of muscle fibers from 12-wk-old rats

<table>
<thead>
<tr>
<th>Muscles/Fibers (n)</th>
<th>RMP, mV</th>
<th>Rheobase, nA</th>
<th>Overshoot, mV</th>
<th>Max Rate Depol., V/s</th>
<th>Max Rate Repol., V/s</th>
<th>%Excitable</th>
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<td>59±3</td>
<td>12±1</td>
<td>314±10</td>
<td>-105±2</td>
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<td>38±2*</td>
<td>19±1*</td>
<td>449±18*</td>
<td>-131±5*</td>
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<tr>
<td>9 mM K(^+)</td>
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<td>-12±2</td>
<td>63±9</td>
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<td>-6±1†</td>
<td>86±7†</td>
<td>-56±3†</td>
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Fibers were considered to be excitable if they produced an action potential in response to a constant depolarizing current of 100 nA or less for 400 ms. Action potentials were defined as a membrane deflection in response to the depolarizing current to greater than -20 mV. RMP, resting membrane potential; Max rate depol., maximum rate of depolarization; Max rate repol., maximum rate of repolarization. Values are means ± SE; n values for rheobase determinations were 6/39 and 5/30 (muscles/fibers) for 4 mM K\(^+\) and 4 mM K\(^+\) + 10\(^{-8}\) M calcitonin gene-related peptide (CGRP), respectively. *P < 0.001, significant difference compared with 4 mM K\(^+\); †P < 0.001, significant difference compared with 9 mM K\(^+\).
The magnitude of the excitation-induced recovery of force in Fig. 3B was similar to that observed upon addition of $10^{-9}$ M CGRP (Fig. 1B). Moreover, CGRP-(8-37) ($10^{-5}$ M) could almost block both the excitation-induced force recovery obtained in Fig. 3B and the force recovery induced by $10^{-9}$ M CGRP but only partially inhibit the recovery induced by $10^{-8}$ M CGRP. This suggests that the concentration of CGRP at the sarcolemma, due to the repeated stimulation paradigm used in Fig. 3B, must be $10^{-9}$ M. By the same token, the extracellular CGRP concentration, when the muscles underwent excitation-induced force recovery with more intense stimulation (Fig. 3A), where presumably more CGRP would be released, is likely to be closer to $10^{-8}$ M.

**CGRP increases muscle excitability.** At 4 mM K$^+$, $10^{-8}$ M CGRP led to an increase in muscle excitability (Table 1; Fig. 5B). In muscles at elevated [K$^+$], the determination of rheobase current and action potentials was encumbered by a complete loss of excitability in a large proportion of the tested fibers. Importantly, however, $10^{-8}$ M CGRP increased the

![Fig. 6. Representative trace illustrating the effect of CGRP on the passive membrane properties. Two intracellular microelectrodes were inserted into the same fiber, 1 electrode was used to pass hyperpolarizing constant current pulses and the other electrode (recording electrode) was used to measure the membrane potential responses at 3 different locations along the fiber. A: representative traces showing the response of the membrane potential to injection of $-40$ nA hyperpolarizing constant current pulses recorded by the recording electrode at the indicated inter-electrode distances in 2 fibers from a muscle incubated at 9 mM K$^+$ and 9 mM K$^+$ + 10$^{-8}$ M CGRP, respectively. Vertical and horizontal calibration bars show membrane potential and time, respectively. B: plots of the ratios between the change in steady-state membrane potential and the amount of current injected from the representative trace in A, as a function of the inter-electrode distance in the single fibers from the 9 mM K$^+$ (●) and 9 mM K$^+$ + 10$^{-8}$ M CGRP (○) muscles. See Table 2 for summarized data.

![Fig. 7. Effect of Cl$^-$ free buffer on CGRP-induced force recovery. A: control muscles (●) were equilibrated in KR before exposure to 4 mM K$^+$-Cl$^-$ free buffer, where they were kept until steady-state force responses were achieved. Muscles were then exposed to 11 mM K$^+$-Cl$^-$ free buffer for 100 min. CGRP muscles (○) were treated the same as controls except that $10^{-8}$ M CGRP was added after 60 min in 11 mM K$^+$-Cl$^-$ free buffer. B: muscles were treated exactly the same as in A, except that experiments were performed at 13 mM K$^+$. Muscles were stimulated with 0.2-ms, 12-V pulses at 60 Hz for 2 s every 10 min. Values are means with bars denoting SE (n = 4).

Table 2. Effect of CGRP on electrical properties of muscle fibers from 12-wk-old rats

<table>
<thead>
<tr>
<th>[K$^+$], mM</th>
<th>[Cl$^-$], mM</th>
<th>Muscles/Fibers (n)</th>
<th>RMP, mV</th>
<th>$\lambda$, mm</th>
<th>$R_m$, MΩ</th>
<th>Diameter, μm</th>
<th>$G_m$, μS/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>127.4</td>
<td>6/24</td>
<td>$-70 \pm 1$</td>
<td>$0.75 \pm 0.03$</td>
<td>$0.37 \pm 0.02$</td>
<td>$51 \pm 2$</td>
<td>$1,259 \pm 63$</td>
</tr>
<tr>
<td>9</td>
<td>127.4</td>
<td>10/31</td>
<td>$-62 \pm 1$</td>
<td>$0.69 \pm 0.02$</td>
<td>$0.23 \pm 0.01$</td>
<td>$58 \pm 1$</td>
<td>$1,788 \pm 65$</td>
</tr>
<tr>
<td>9 + CGRP</td>
<td>127.4</td>
<td>7/21</td>
<td>$-62 \pm 1$</td>
<td>$0.70 \pm 0.02$</td>
<td>$0.22 \pm 0.01$</td>
<td>$60 \pm 2$</td>
<td>$1,772 \pm 82$</td>
</tr>
</tbody>
</table>

Hyperpolarizing current pulses of 150-ms duration were injected through current electrode and recording electrode recorded membrane voltage responses at 3 locations in each fiber. AY-to-I ratios were plotted on a log scale against interelectrode distance and fitted to a 2-parameter exponentially decaying function giving a straight line (Fig. 6B). Slope of the line was used to calculate the length constant ($\lambda$) and the ordinate intercept gave the input resistance ($R_m$). Conductance was calculated from $\lambda$ and $R_m$ according to Boyd and Martin (Ref. 4) using an assumed internal resistivity ($R_i$) of 180 Ωcm. [K$^+$], extracellular K$^+$: $G_m$: membrane conductance. Muscles were allowed to incubate at 9 mM K$^+$ for 30 min before recordings, while 10$^{-8}$ M CGRP was added 10 min before recordings. Values are means ± SE, with n representing number of muscles/fibers used in each group. No statistical significance was found between 9 mM K$^+$ and 9 mM K$^+$ + CGRP for any parameter (all $P > 0.34$).
fraction of excitable fibers (Table 1). In addition, a comparison of the action potentials in those fibers that were excitable at elevated [K⁺]₀ showed that CGRP increased the action potential overshoot and maximum rates of depolarization and repolarization. These effects were, however, rather small and the potential overshoot and maximum rates of depolarization and repolarization have been observed after excitation-induced force recovery of elevated [K⁺]₀, depressed muscles (31, 34). These mechanisms are likely to be essential for the large force recovery induced by 10⁻⁸ M CGRP or by excitation with a more intense stimulation paradigm as in Fig. 3A.

However, in the present study, the addition of 10⁻⁹ M CGRP to muscles at elevated [K⁺]₀ only induced a small decrease in intracellular Na⁺ (Fig. 8B) and no significant increase in ⁸⁶Rb⁺ uptake, suggesting that at 11 mM K⁺, there is, at most, a modest stimulation of the Na⁺-K⁺ pump. Moreover, no hyperpolarization, even with 10⁻⁸ M CGRP, was observed at elevated [K⁺]₀, which was also observed by Cairns et al. (6) at a 10 times higher CGRP concentration. Instead, those authors attributed the CGRP-induced force recovery to an increase in the sarcomemmal Na⁺ gradient. In the present study, the reduction in intracellular Na⁺ after the addition of 10⁻⁹ M CGRP was much smaller and it is unlikely that the ensuing increase in sarcomemmal Na⁺ gradient is large enough to induce the substantial force recovery shown in Fig. 1B. These findings suggest, therefore, that other mechanisms apart from Na⁺-K⁺-pump stimulation contribute to the restoration of force induced by addition of CGRP or by excitation.

K⁺ channels? In vascular smooth muscle cells, CGRP has been shown to alter K⁺ channel function (38, 28). In skeletal muscle, this could lead to a restoration or maintenance of muscle excitability by hyperpolarizing the membrane. However, no significant alteration in Gₖ was observed upon addition of CGRP suggesting that there was no major alteration to K⁺ conductance (Table 2). A similar lack of effect of CGRP on input resistance has been observed in segmentated diaphragm muscle (48). In addition, Clausen and Overgaard (10), using Ba²⁺ to inhibit a significant proportion of K⁺ channels inducing reduced muscle excitability, observed a large CGRP-induced force recovery, suggesting that CGRP did not act via the K⁺ channels responsible for the RMP. Geukes Foppen and Siegenbeek van Heukelom (18) using diaphragm muscle, described a cAMP dependent membrane hyperpolarization that was due to Ca²⁺-activated K⁺ channels. However, they could not find such a mechanism in rat soleus muscles, suggesting that this effect may be tissue specific. Furthermore, fibers rather than a change in the action potential parameters of those fibers still excitable at elevated [K⁺]₀.

Mechanisms of CGRP-induced restoration of excitability. The binding of CGRP to CGRP receptors on the sarcolemma leads to an increase in intracellular cAMP (47, 49), which is a potent second messenger and can potentially alter the function of a number of membrane proteins of importance for muscle excitability.

Na⁺-K⁺ pumps? There is substantial evidence indicating that at 4 mM K⁺ concentrations down to 10⁻⁹ M CGRP stimulate the Na⁺-K⁺-pump (2, 7). It was therefore suggested that CGRP-induced force recovery in depolarized muscle occurs via a Na⁺-K⁺ pump mediated increase in the sarcolemmal Na⁺ gradient (6) and/or Na⁺-K⁺ pump mediated membrane hyperpolarization (2). In cardiac muscle, cAMP stimulation of the Na⁺-K⁺ pump is thought to occur via PKA phosphorylation of phospholemman (12), and a similar mechanism may exist in skeletal muscle. Indeed, at 4 mM K⁺, CGRP resulted in Na⁺-K⁺ pump stimulation, which most likely explains the CGRP-induced membrane hyperpolarization (2). Likewise, a reduction in intracellular Na⁺ content and membrane hyperpolarization have been observed after excitation-induced force recovery of elevated [K⁺]₀, depressed muscles (31, 34). These mechanisms are likely to be essential for the large force recovery induced by 10⁻⁸ M CGRP or by excitation with a more intense stimulation paradigm as in Fig. 3A.

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at elevated $[K^+]_o$ the RMP and equilibrium potential for $K^+$ are very close together and therefore there is almost no driving force for $K^+$. Thus at elevated $[K^+]_o$, the opening of $K^+$ channels would not actually cause much hyperpolarization (50). Therefore, it is unlikely that CGRP restores muscle excitability via the opening of $K^+$ channels responsible for the resting $K^+$ conductance.

**Cl^- channels?** In skeletal muscle, $G_{Cl^-}$ is responsible for $\sim80\%$ of $G_m$ and therefore plays a major role in muscle excitability (3). It has recently been shown that closure of $Cl^-$ channels causes a restoration of force in elevated $[K^+]_o$ depressed muscles (50, 37). As CGRP does not alter $G_m$ (Table 2), however, it is unlikely that there is any alteration in $G_{Cl^-}$. Furthermore, Fig. 7 shows that even in $Cl^-$-free buffer, where $G_{Cl^-}$ is already reduced to zero, there is a large CGRP-induced force recovery. CGRP must therefore act via a $Cl^-$ channel independent mechanism.

**Na^+ channels?** Voltage-gated $Na^+$ channels are responsible for the depolarizing phase and the propagation of action potentials in skeletal muscle. It is possible that CGRP may alter the function of $Na^+$ channels, which could increase muscle excitability by allowing action potentials to trigger more easily. Indeed, in neural cells, CGRP has been shown to increase the $Na^+$ current through the $Na_{v,1.9} Na^+$ channels (30). In the present study, at 4 mM $K^+$, membrane hyperpolarization with CGRP would remove some of the slow inactivation of $Na^+$ channels. At normal RMP, a population of $Na^+$ channels is in the slow inactivated state and this proportion drastically increases with prolonged membrane depolarization as experienced with elevated $[K^+]_o$ (41). The rheobase current required to trigger action potentials was also substantially reduced upon addition of CGRP at 4 mM $K^+$. As there is a hyperpolarization of the RMP and no change in $G_m$, a greater number of $Na^+$ channels must be available. Furthermore, the depolarizing phase of the action potentials was faster upon addition of CGRP, indicating a larger $Na^+$ current. At elevated $[K^+]_o$, $10^{-8}$ M CGRP did not lead to membrane hyperpolarization, so it is unlikely that $Na^+$ channels are recovering from slow inactivation simply from a change in RMP. Alternatively, CGRP may induce a shift in the slow inactivation curve of the $Na^+$ channels to more positive membrane potentials, resulting in more $Na^+$ channels being available for action potential initiation. It is clear that the action potentials are greatly improved after the addition of CGRP (Table 2) suggesting that more $Na^+$ channels are activated and that the $Na^+$ channels are also more easily activated in the presence of CGRP. A shift in the slow inactivation curve of the $Na^+$ channels to more positive membrane potentials has also been implicated in the increased tolerance of muscles to elevated $[K^+]_o$, that is induced when the temperature is increased (40, 36). Since excitation-induced force recovery was also seen at 37°C, however, the effect of CGRP seems to be additive to the effects of temperature on the function of the $Na^+$ channels.

CGRP has also been shown to modulate muscle motor end plate excitability through both increased acetylcholine receptor (AChR) phosphorylation (27) and prolonged mean open time (22). Furthermore, CGRP modifies the amount of acetylcholine esterase (ACHE) at the motor end plate, specifically the $G_4$ isoform of ACHE (14). Together the increase in AChR and the decrease in ACHE enhance the ability of the muscle to respond to neuronal stimulation, (i.e., to maintain excitability).

**Perspectives**

During intense exercise, there is a large loss of $K^+$ from the contracting muscles, which elevates plasma $K^+$ and depresses skeletal muscle function in not only the actively contracting muscles but also in the resting muscles in the body. Exercise also induces a release of CGRP from nerve endings. The present results indicate that by counterbalancing the inhibitory effects of elevated $[K^+]_o$, CGRP is important for the maintenance of excitability and contractility in skeletal muscle. The local release of CGRP from nerve endings in the muscle may also explain the observations that, in patients suffering from hyperkalemic periodic paralysis, continued local muscle activity provides resistance to paralysis, while all other muscles become paralyzed (25, 11). Local CGRP release in the diaphragm may also explain why these patients can continue to breathe, while all other muscles are paralyzed. Repeated exhausting muscle work may lead to the depletion of CGRP stores in nerves and muscle. In this case, the capacity to perform more work may depend on a resting period to allow replenishment of these CGRP stores.

**ACKNOWLEDGMENTS**

The technical assistance of Ann-Charlotte Andersen, Marianne Sturup Johansen, Tove Lindahl Andersen, and Vibeke Uhre is gratefully acknowledged. We also thank Dr. John A. Flatman for discussions and technical assistance.

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**GRANTS**

This study was supported by grants from Aarhus Universitets Forskningsfond, the Danish Medical Research Council (j.nr. 9802488 and j.nr. 271-05-0304), the Danish Biomembrane Research Center, and the Lundbeck Foundation.

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