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POTASSIUM AND CAFFEINE CONTRACTURES IN FAST AND SLOW MUSCLES OF THE CHICKEN

BY M. HUERTA AND E. STEFANI*

From the Department of Physiology and Biophysics, Centro de Investigación del IPN, Apartado Postal 14-740, México 14, D.F.

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SUMMARY

1. K^+ contractures, caffeine contractures and electrical properties were studied in slow (posterior latissimus dorsi; p.l.d.) and fast (anterior latissimus dorsi; a.l.d.) chicken muscles.

2. P.l.d. K^+ contractures show a transient increase of tension that relaxes spontaneously. Contractures in a.l.d. show an initial component followed by a maintained tension.

3. A.l.d. K^+ contractures of similar amplitude and time course were reproduced at 4 min intervals. In p.l.d., the interval needed for full recovery is about 30 min. In *Cl-free* saline p.l.d. and a.l.d. K^+ contractures can be reproduced at 4 min intervals.

4. The time course of repolarization after a short exposure to 160 mM-KCl was much slower in p.l.d. than in a.l.d. In *Cl-free* saline the time course of repolarization becomes faster in p.l.d.

5. The membrane resistance was not modified in a.l.d. and was increased in p.l.d. by *Cl-free* saline. The calculated Cl^- conductance in p.l.d. was about 70 % of the total membrane conductance.

6. In a.l.d., Mn^{2+}, D600 and external Ca^{2+} reduction greatly diminishes the maintained phase of the K^+ contracture leaving the initial phase almost unmodified. Under similar conditions p.l.d. K^+ contractures were slightly reduced.

7. P.l.d. caffeine contractures (10–40 mM) were not maintained and they were not modified by *Ca-free* saline, Cd^{2+}, Co^{2+}, Mn^{2+} and D600.

8. A.l.d. caffeine contractures (2–15 mM) were maintained and were highly dependent on external Ca^{2+}. In addition they were greatly reduced by Cd^{2+}, Co^{2+}, Mn^{2+} and D600.

9. It is suggested that caffeine contractures of a.l.d. are elicited by a Ca^{2+} entry into the muscle from the external fluid.

INTRODUCTION

There is good evidence that most of the Ca^{2+} that activates contractile proteins in twitch muscle fibres of the frog is released from the sarcoplasmic reticulum upon membrane depolarization (Ebashi, 1976; Lüttgau & Glitsch, 1976; Endo, 1977).

* To whom requests for reprints should be addressed.

However, external Ca^{2+} plays a supporting role in maintaining the tension during K^+ contractures (Stefani & Chiarandini, 1973; Lüttgau & Spiecker, 1979; Cota & Stefani, 1981). On the other hand, K^+ contractures of slow muscle fibres of the chicken clearly depend on external Ca^{2+} (Page, 1969). To further investigate the role of extracellular Ca^{2+} on contractile activation, we have made a comparative study of the electrical properties and the ionic dependence of K^+ and caffeine contractures in fast (posterior latissimus dorsi; p.l.d.) and slow (anterior latissimus dorsi; a.l.d.) muscle fibres of the chicken.

METHODS

Experiments were performed on a.l.d. and p.l.d. muscles of chickens up to 3 weeks old.

Mechanical recording. Small fibre bundles were dissected from a.l.d. and p.l.d. The diameters of the bundles were generally of 0·5 mm. To avoid additional diffusional delays, bundles with smaller diameters (0·1–0·3 mm) were selected when the effect of removing external Ca^{2+} was studied. The bundles were left resting for about 1 hr in saline and then mounted horizontally in the experimental chamber and stretched to 1·1–1·2 of their slack length. The distal end was fixed to the bottom of the chamber, while the proximal one was attached to the lever of an isometric mechanoelectric transducer (Grass FT10C or an RCA 5734 tube) wired to a rectilinear and/or a curvilinear pen recorder. The experimental chamber had a capacity of 0·5 ml. The fluid could be fully changed in 2–3 sec by means of a rapid flow of new solution through the inlet in one extreme of the chamber. The bundles were equilibrated for 5 min with continuous flow (30 ml./min) of the test solution before applying the contracture fluid. In contractures performed in the absence of Cl^- the bundles were previously equilibrated for 1 hr in *Cl-free* saline. Submaximal (80 mM-k^+) and maximal (160 mM-K^+) K^+ contractures were elicited at 4–30 min intervals. Caffeine contractures were explored on the 2–40 mM range. The observed effects were reproduced in five to eight bundles and were reversible.

Electrical recording and membrane constants. Fibres from a.l.d. and p.l.d. muscles were impaled with 50–90 MΩ micropipettes filled with 3 M-KCl. The E_{RP} corresponds to the values measured immediately after the insertion of the micropipette (*ca.* 10–100 msec) since the fibres depolarize after the impalement (Stefani & Steinbach, 1969). Square pulses of current (I_o, increasing steps of 1 or −2 nA) were injected intracellularly via a second micropipette at a distance smaller than 50 μm from the voltage-recording (V_o) micro-electrode. V_o/I_o is the effective resistance (R_{eff}). The fibres were maintained at −80 mV with d.c. current (< −10 nA). The membrane constants were calculated for −2 nA pulses. The space constant (λ) was obtained from $\lambda = V_o \pi d^2/2\, I_o R_i$ where R_i (Ωcm) is the specific resistance of the myoplasm and d is the fibre diameter. R_i was assumed following Hartree & Hill (1921) and Fedde (1969). From the difference in ionic concentration between frog and chicken salines and from the frog muscle R_i value of 163 Ωcm (21·5 °C, Hodgkin & Nakajima, 1972), the R_i for chicken muscle fibres was calculated as 112 Ωcm (22 °C). To measure d, the muscles were initially frozen in 2-methylbutane cooled at −125 °C in liquid nitrogen, thereafter 10 μm thick transverse sections were obtained on a cryostat at −15 ° to −20 °C. The sections were placed on glass slides, mounted in neutral glycerine jelly and photographed at ×480. The mean value between the greater and smaller diameter was taken as d. The membrane resistance times unit area was calculated according to $R_m = \lambda^2 4 R_i/d$. The membrane time constant (τ_m) was obtained by measuring the time needed for the voltage deflexion to reach half of its final value ($t_{\frac{1}{2}}$) since $\tau_m = 4{\cdot}398\, t_{\frac{1}{2}}$ Gage & Eisenberg, 1969). The membrane capacity per unit area (C_m) was derived from $C_m = \tau_m/R_m$.

Solutions. The *normal* saline contained (mM): NaCl 167, KCl 5, $CaCl_2$ 5, $MgCl_2$ 2 and glucose 2 g/l. Solutions with high K^+ were obtained by isotonic replacement of NaCl for KCl. Solutions with high concentrations of Ca^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+}, were prepared by adding appropriate volumes of 1 M-$CaCl_2$, 1 M-$CoCl_2$, 0·1 M-$MnCl_2$ or 0·1 M-$CdCl_2$ stock solutions. To reduce possible changes of the surface potential Hille, Woodhull & Shapiro, 1975; Chiarandini, Sánchez & Stefani, 1980), $CaCl_2$ was equimolarly replaced by $MgCl_2$. Solutions with Ca^{2+} omitted (*low-Ca* saline), had a Ca^{2+} contamination of 3–6 μM which was measured by using the pH-metric method of Moisescu & Pusch (1975). When required, the ionized Ca^{2+} in this solution was drastically reduced by adding 5 mM-Na_2EGTA (*Ca-free* saline). *Cl-free* salines were prepared by replacing all Cl^- by methanesulph-

onate ($CH_3SO_3^-$) (methanesulphonic acid from Eastman Organic Chemicals, Rochester, N.Y.). All solutions were buffered to pH 7·4 with Tris chloride or with imidazole-chloride. D600; (methoxy-derivative of iproveratril, Knoll Pharmaceuticals), and caffeine (Baker) were added from concentrated aqueous solutions.

Experiments were performed at room temperature (22–25 °C). Values are expressed as mean ± S.E. of mean; the number of observations is in parentheses.

RESULTS

Repriming of K^+ contractures and membrane properties in Cl and Cl-free salines

As previously reported by Page (1969), K^+ contractures in a.l.d. show an initial phase followed by a maintained tension (Fig. 1 *A*; Fig. 3 *A* and *D*). On the other hand, p.l.d. K^+ contractures show a transient increase of tension that relaxes spontaneously (Fig. 1 *B* and *C*). To test the action of Ca^{2+} channels blocking agents and of external

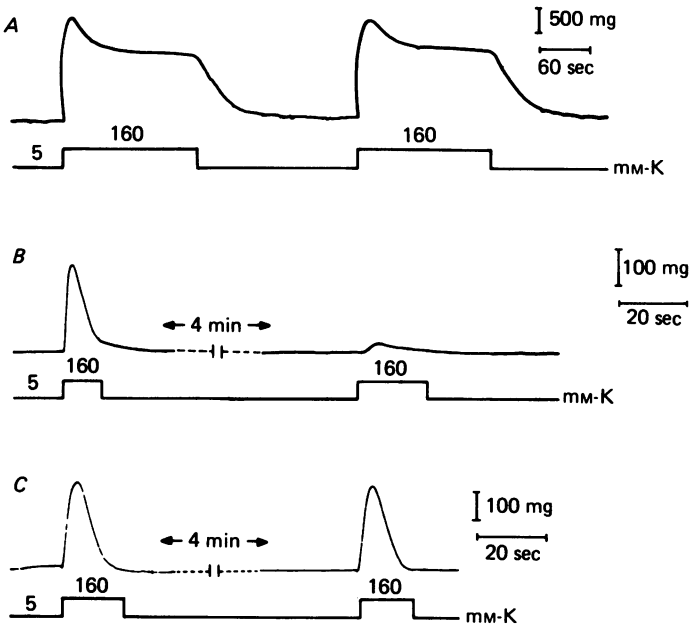

Fig. 1. Repriming of K^+ contracture in a.l.d. and p.l.d. muscles. In *A* (a.l.d.), two subsequent K^+ contractures (4 min interval) of similar size and time course are shown. In *B* (p.l.d.) the second K^+ contracture elicited after 4 min interval is greatly diminished. When the p.l.d. muscle is equilibrated in *Cl-free* saline (for 1 hr), the second K^+ contracture performed 4 min after is almost identical to the first one.

Ca^{2+} reduction, it is necessary to study initially the reproducibility of K^+ contractures. In a.l.d. muscle K^+ contractures (160 mM-K) of similar amplitude and time course can be reproduced at 4 min intervals (Fig. 1 *A*). In contrast, after a similar interval, p.l.d. muscles almost become unresponsive to a second K^+ stimulation (Fig. 1 *B*). The interval needed for full recovery between contractures is about 30 min. This finding could be explained by a larger Cl^- permeability in p.l.d. muscle, resulting in a longer time needed to repolarize after exposure to high KCl (Hodgkin & Horowicz, 1959).

In agreement with this hypothesis, p.l.d. K^+ contractures elicited in *Cl-free* saline can be reproduced at relatively short intervals (4 min) (Fig. 1*C*). *Cl-free* saline did not modify the repriming of a.l.d. K^+ contractures.

A series of experiments were carried out in a.l.d. and p.l.d. to measure their resting Cl^- conductance. The time course of repolarization after 5 min exposure to 160 mM-K^+ in *Cl* and *Cl-free* salines was measured initially. In a.l.d. the E_{RP} was $-68{\cdot}5 \pm 1{\cdot}8$ mV

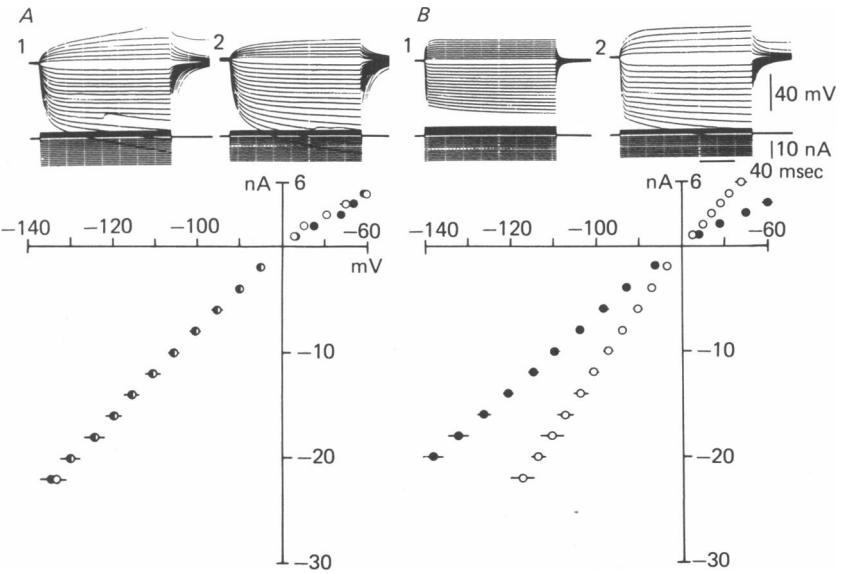

Fig. 2. Current–voltage relation in a.l.d. (*A*) and p.l.d. (*B*) muscles in *control* saline (1, ○) and in *Cl-free* saline (2, ●). The membrane potential was maintained at −80 mV with a constant d.c. current. Upper records are voltage deflexions and lower records are current steps. Downward deflexions are hyperpolarizing voltages and inward current steps. Increasing steps of −2 and 1 nA were injected. Note spontaneous miniature potential in *A*, 1 and 2. The voltage was measured at the end of 150 msec pulses. Symbols are the mean ± S.E. (n = 18 in *A* and 8 in *B*).

(13) in *normal* saline, $-9{\cdot}3 \pm 1{\cdot}4$ mV (6) in 160 mM-KCl, and $-66{\cdot}0 \pm 2{\cdot}5$ mV (7) 3–6 min after washing in normal saline. In p.l.d. the E_{RP} was $-69{\cdot}7 \pm 2{\cdot}6$ mV (10) in *normal* saline and $-20{\cdot}7 \pm 1{\cdot}6$ mV (6) in 160 mM-KCl. At different times after washing in *normal* saline the E_{RP} was: $-41{\cdot}0 \pm 2{\cdot}0$ mV (7) after 8–12 min, $-55{\cdot}2 \pm 2{\cdot}1$ mV (6) after 18–20 min and $-66{\cdot}0 \pm 2{\cdot}0$ mV (4) after 25–30 min. The slower time course of repolarization of p.l.d. can be tentatively explained by KCl loading and accounts for the slower repriming of K^+ contractures. As expected, in *Cl-free* saline the repolarization of p.l.d. is faster. The E_{RP} in *Cl-free* saline (5 mM-K^+) was $-71{\cdot}8 \pm 1{\cdot}4$ mV (8), $+3{\cdot}8 \pm 1{\cdot}0$ mV (5) during 5–10 min exposure to 160 mM-K^+ (Cl-free) and $-75{\cdot}0 \pm 1{\cdot}0$ (7) 5–10 min after washing in *Cl-free* saline (5 mM-K^+).

The Cl^- permeability was further analysed by studying the membrane resistance in *Cl* and *Cl-free* salines. Fig. 2 shows voltage deflexions obtained by injecting current steps of increasing amplitude (1 nA and −2 nA) and the corresponding I_o/V_o relation in a.l.d. (*A*) and p.l.d. (*B*), in *control* saline (1, ○) and *Cl-free* saline (2, ●). It can be seen that in *Cl-free* saline the membrane conductance is decreased in p.l.d. and

is not modified in a.l.d. Note that in the I/V curve both muscles show anomalous rectification (Katz, 1949). Table 1 shows the membrane properties calculated from -2 nA steps (see *Methods*). As expected from the above results, in a.l.d. the R_{eff} and R_m were not modified in *Cl-free* saline. On the other hand, in p.l.d., R_m is 850 ± 20 Ωcm^2 in *normal* saline and 2900 ± 80 Ωcm^2 in *Cl-free* saline, and the calculated Cl^- conductance is about 70% of the total membrane conductance. A similar value was reported by Lebeda & Alburquerque (1975). These observations indicate that the Cl^- permeability in p.l.d., is large and that it is negligible in a.l.d. However, a small Cl^- conductance would have remained undetected in a.l.d. due to the method.

TABLE 1. Membrane constants of a.l.d. and p.l.d. muscle fibres

Muscle	Solution	R_{eff} (MΩ)	d (μm)	τ_m (msec)	λ (mm)	R_m (Ωcm^2)	C_m ($\mu F/cm^2$)
A.l.d.	Normal	$2{\cdot}6 \pm 0{\cdot}5$	$22{\cdot}2 \pm 0{\cdot}6$	12 ± 1	$1{\cdot}8 \pm 0{\cdot}6$	5800 ± 140	$2{\cdot}1 \pm 0{\cdot}2$
	Cl-free	$2{\cdot}5 \pm 0{\cdot}6$	—	14 ± 2	$1{\cdot}8 \pm 0{\cdot}5$	5800 ± 140	$2{\cdot}4 \pm 0{\cdot}4$
P.l.d.	Normal	$1{\cdot}7 \pm 0{\cdot}1$	$15{\cdot}4 \pm 0{\cdot}4$	$1{\cdot}3 \pm 0{\cdot}1$	$0{\cdot}53 \pm 0{\cdot}03$	850 ± 20	$1{\cdot}50 \pm 0{\cdot}04$
	Cl-free	$3{\cdot}2 \pm 0{\cdot}1$	—	$3{\cdot}7 \pm 0{\cdot}7$	$0{\cdot}99 \pm 0{\cdot}03$	2900 ± 80	$1{\cdot}30 \pm 0{\cdot}05$

Values are mean ± S.E. The measured capacity is in agreement with the expected value for twitch muscle fibres of the frog with comparable diameters (Hodgkin & Nakajima, 1972). ($n = 18$ in a.l.d. and 8 in p.l.d.). d was measured as described in Methods.

Effects of D600, Mn^{2+} and external Ca^{2+} on K^+ contractures

The possible role of Ca^{2+} inflow during K^+ contractures in a.l.d. was tested by studying the effects of blockers of Ca^{2+} channels such as D600 and Mn^{2+} (Hagiwara & Nakajima, 1966; Kohlhardt, Bauer, Krause & Fleckenstein, 1972). Fig. 3*A* shows a control K^+ contracture of a.l.d.; in *B*, the same bundle was pre-incubated with 3×10^{-5} M-D600 for 5 min before the contracture. The initial phase of the contracture remained unchanged, but the sustained phase of the tension was greatly reduced in such a way that the muscle fibres relaxed spontaneously. This effect was reversible (Fig. 3*C*). A similar observation was found when 10 mM-Mn^{2+} was added to the solutions (Fig. 3*E*). The reduction of the sustained phase by Mn^{2+} and D600 was equally observed on contractures elicited by 80 mM- and 160 mM-K^+. P.l.d. K^+ contractures (160 mM-K^+) were slightly diminished by 10 mM-Mn^{2+} and 3×10^{-5} M-D600. This reduction was much more pronounced for 80 mM-K^+ contracture. As reported by Page (1969), in *low-Ca* saline, K^+ contractures in p.l.d. were practically unaffected, while in a.l.d. the initial component became smaller and the sustained phase was practically abolished.

Effects of external Ca^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+} and D600 on caffeine contractures

Fig. 4*A–C* shows caffeine contractures in p.l.d. muscle. The threshold concentration to elicit tension ranged from 10 to 40 mM in different bundles. With these concentrations, tension developed slowly and was followed by a very slow relaxation phase (*A*). With larger concentrations of caffeine (20–40 mM), tension clearly rose to a peak and then relaxed to a low sustained level (Fig. 4*B* and *C*). Caffeine contractures in p.l.d. muscles were reproduceable when elicited at 40 min intervals and they were practically unmodified in *Ca-free* saline (Fig. 4*E* and *D*), and after the addition of

blockers of Ca^{2+} channels such as 1 mM-Cd^{2+}, 1–5 mM-Co^{2+}, 5–10 mM-Mn^{2+} and 3×10^{-5} M-D600 (Hagiwara & Nakajima, 1966; Kohlhardt *et al.* 1972; Baker, Meves & Ridgway, 1973; Kostyuk & Krishtal, 1977).

A.l.d. muscles were more sensitive to caffeine. Tension could be elicited with 2–3 mM-caffeine. Fig. 5*A–C* shows contractures elicited when caffeine was added to the

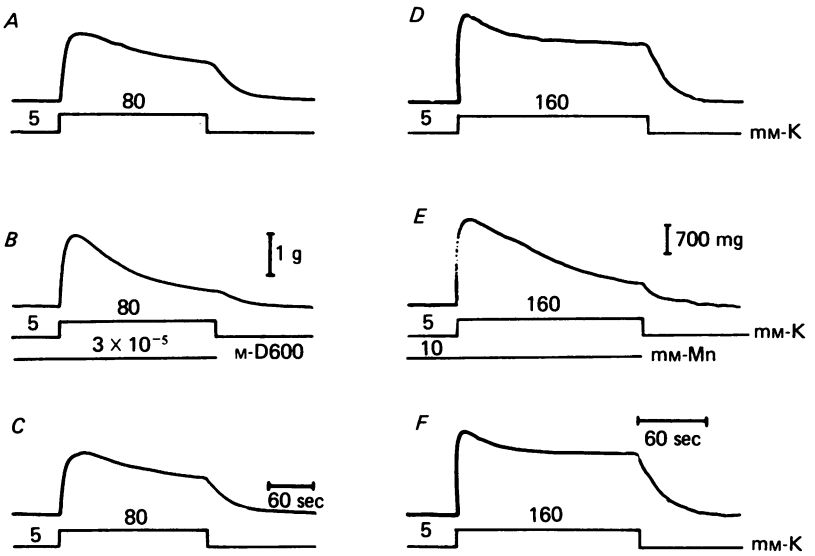

Fig. 3. Effect of D600 and Mn^{2+} on K^+ contractures. *A*, contracture in *control* saline; *B*, the bundle was equilibrated in *control* saline with 3×10^{-5} M-D600 added for 5 min before the K^+ contracture; *C*, recovery in *control* saline. Effect of Mn^{2+}: *D*, *control* saline; *E*, the bundle was equilibrated in *control* saline with 10 mM-Mn^{2+} added for 5 min before the contracture; *F*, recovery in *control* saline.

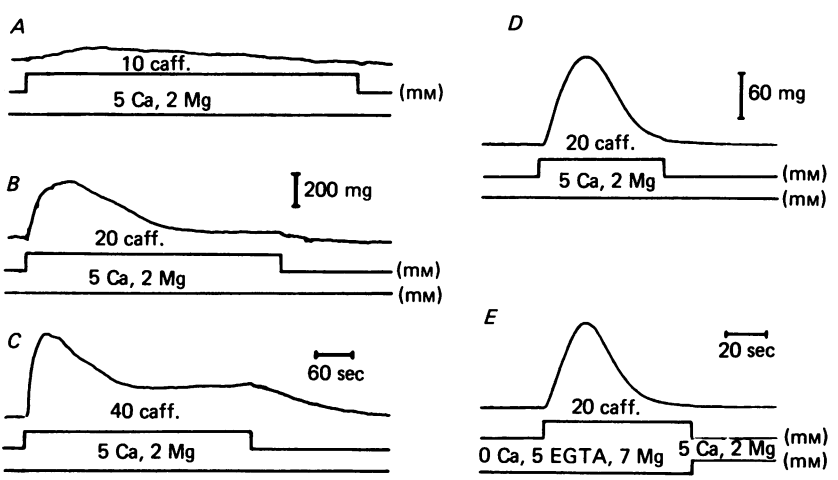

Fig. 4. Caffeine contractures in p.l.d. muscle bundle: *A*, *B* and *C*, contractures elicited with 10, 20 and 40 mM-caffeine respectively. Effect of Ca^{2+} removal on caffeine contracture: *D*, contracture in *control* saline; *E*, the bundle was exposed for 5 min before the contracture in *Ca-free* saline.

normal saline. The tension was sustained and the muscle fibres relaxed rapidly after caffeine withdrawal. Contractures with similar amplitude and time course could be elicited every 40 min. In contrast to p.l.d. muscle, caffeine contractures of a.l.d. muscles were highly Ca^{2+}-dependent. Fig. 5*D* shows that external Ca^{2+} has to be continuously present to maintain the tension during caffeine contracture. When external Ca^{2+} was omitted from the contracture fluid the tension declined with a

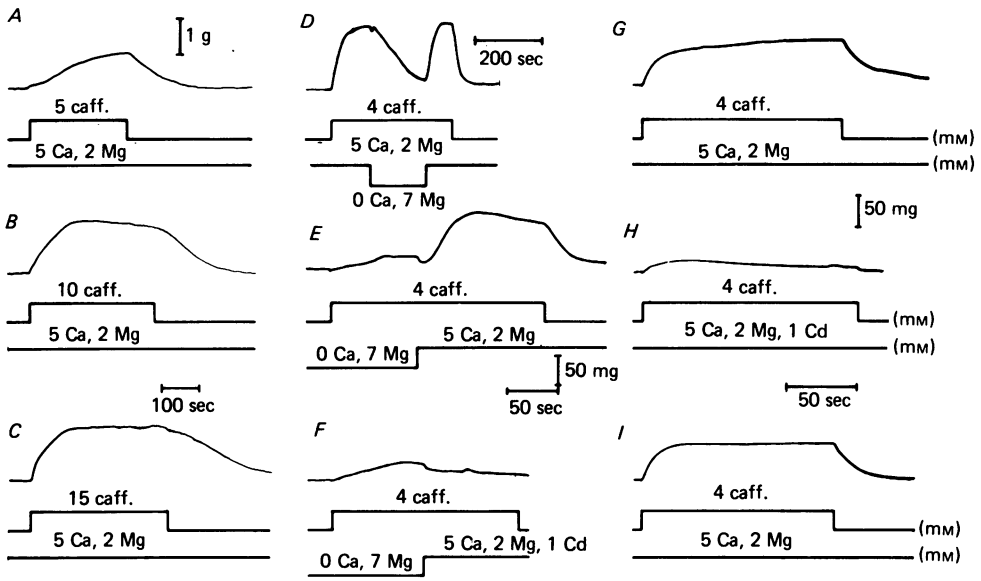

Fig. 5. Caffeine contracture in a.l.d. muscle; *A*, *B* and *C*, contractures elicited with 5, 10 and 15 mM-caffeine respectively. Calcium dependence of caffeine contractures: *D*, tension decline when external Ca^{2+} was removed from the contracture fluid, and tension development when 5 mM-Ca^{2+} was added. *E*, the bundle was exposed to *low-Ca* saline 5 min before the application of caffeine, during the caffeine perfusion 5 mM-Ca^{2+} was added to the contracture fluid. *F*, in the same bundle, same procedure as in *D*, but Ca^{2+} and Cd^{2+} were added simultaneously. Effect of 1 mM-Cd^{2+}: *G*, control contracture; *H*, the bundle was equilibrated with a *normal* saline with 1 mM-Cd^{2+} added during 5 min before the contracture; *I*, recovery.

half-time of 100 sec and was re-established when external Ca^{2+} was restored. In addition, Fig. 5*E* shows that caffeine elicited a very small tension when the bundle was flushed in *low-Ca* saline 5 min prior to the contracture and that tension rapidly developed when 5 mM-Ca^{2+} was added to the saline in the presence of 4 mM-caffeine.

The dependence on external Ca^{2+} was further confirmed by studying the effects of the blockers of Ca^{2+} channels. The experiment in Fig. 5*F* was made under the same conditions as in *E*, but 5 mM-Ca^{2+} and 1 mM-Cd^{2+} were added simultaneously. The addition of Cd^{2+} prevented tension development, indicating that caffeine induces a Ca^{2+} entry.

To rule out depletion of intracellular Ca^{2+} stores in *low-Ca* salines, the effect of blocking agents of Ca^{2+} channels was further tested. Fig. 5*G–I* shows the blocking effect of 1 mM-Cd^{2+}. In *G*, a control caffeine contracture can be seen. In *H*, the same bundle was pre-incubated in the *normal* saline with 1 mM-Cd^{2+} added for 5 min prior

to the contracture. In the presence of Cd^{2+} the tension was greatly reduced and the muscle relaxed spontaneously. This effect was reversible (Fig. 5*I*). Similar observations were obtained with 1–5 mM-Co^{2+}, 5–10 mM-Mn^{2+} and 3×10^{-5} M-D600. In conclusion, it can be postulated that caffeine induces Ca^{2+} entry which has a major role in tension development, ruling out Ca^{2+} depletion as an important factor.

K^+ and caffeine contractures in a.l.d. muscles were equally affected by external Ca^{2+} reduction and by blockers of Ca^{2+} channels. It was therefore important to study whether caffeine contractures were associated with membrane depolarizations. In agreement with the data on frog muscle (Lüttgau & Oetliker, 1968) we found that the E_{RP} of a.l.d. muscle fibres was not modified by 4 mM-caffeine. The E_{RP} in *control* saline was $-73{\cdot}2 \pm 1{\cdot}6$ mV (11) and with 4 mM-caffeine added it was $-73{\cdot}9 \pm 1{\cdot}7$ mV (11).

DISCUSSION

Slow muscle fibres of chicken, a.l.d. (Ginsborg, 1960; Fedde, 1969; Cullen, Harris, Marshall & Ward, 1975; Lebeda & Albuquerque, 1975) resemble slow (tonic) muscle fibres of the frog (Stefani & Steinbach, 1969; Gilly & Hui, 1980) and of extraocular rat muscle (Chiarandini & Stefani, 1979; Bondi & Chiarandini, 1979). These fibres do not have a detectable chloride conductance and they show a relatively larger R_m and τ_m. Only chicken slow fibres have sodium-dependent action potentials and, contrary to frog slow fibres, chicken and rat slow fibres show anomalous rectification. The difference in Cl^- resting conductance explains the different repriming between p.l.d. and a.l.d. K^+ contractures.

The present results confirm the observations of Page (1969) i.e. that the sustained phase of tension in a.l.d. K^+ contractures depend on the presence of Ca^{2+} in the external solution. This conclusion is further supported by the fact that the sustained phase was reduced by blocking agents of Ca^{2+} channels, such as Mn^{2+} and D600. The lack of effect of Mn^{2+} on the initial phase suggests that its blocking action on the sustained phase was mainly due to a reduction of Ca^{2+} entry, instead of a general indirect effect due to screening negative surface charges (Hille *et al.* 1975; Chiarandini *et al.* 1980).

The dependence of tension on external Ca^{2+} in a.l.d. was further confirmed by the action of caffeine. This alkaloid elicits tension in skeletal muscle fibres by releasing Ca^{2+} from the sarcoplasmic reticulum without changes in the E_{RP} (Axelsson & Thesleff, 1958; Lüttgau & Oetliker, 1968; Caputo, 1966; Endo, 1977). As expected, the membrane potential in a.l.d. was not altered by caffeine. In agreement with the observations on frog twitch muscle fibres (Chiarandini *et al.* 1980), caffeine contractures in p.l.d. muscles were not modified when external Ca^{2+} was omitted, indicating that caffeine is mainly acting by releasing Ca^{2+} from the sarcoplasmic reticulum. In contrast, in slow muscles (a.l.d.), caffeine contractures were reduced by the absence of external Ca^{2+} and by the addition of blocking agents of Ca^{2+} channels. External Ca^{2+} is needed for eliciting as well as for maintaining caffeine contractures. These results suggest that during caffeine contracture in a.l.d. the external fluid is the main Ca^{2+} source.

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