J. Physiol. (1965), **179**, *pp.* 456–478
With 7 text-figures
Printed in Great Britain

STUDIES ON THE ADENOSINE TRIPHOSPHATASE, CALCIUM UPTAKE AND RELAXING ACTIVITY OF THE MICROSOMAL GRANULES FROM SKELETAL MUSCLE

BY K. S. LEE, K. TANAKA* AND D. H. YU†

From the Department of Pharmacology, Downstate Medical Center, State University of New York, Brooklyn, New York, U.S.A.

(*Received* 14 *December* 1964)

Since the original works by Marsh (1951) and Bendall (1952*a*, *b*) the so-called 'relaxing factor' has attracted attention in research on muscle contraction. Subsequent works by numerous investigators have shown that the 'relaxing factor' is located in the microsomal fraction from homogenized muscle cells and indicated that the source of this fraction is the endoplasmic reticulum of intact muscle cells (Porter & Palade, 1957; Nagai, Makinose & Hasselbach, 1960; Porter, 1961; Ebashi & Lipmann, 1962; Muscatello, Anderson-Cedergren & Azzone, 1962). Furthermore, the demonstration of active uptake of Ca^{2+} by the microsomal fraction led to the hypothesis that the endoplasmic reticulum causes relaxation of contracted myofibrils by removing Ca^{2+} from the environment of the myofibrils (Ebashi, 1961*a*, *b*; Ebashi & Lipmann, 1962; Hasselbach & Makinose, 1961, 1962, 1963; Weber, Herz & Reiss, 1963). In the meantime, beginning with the observation of Skou (1957) on fractionated crab nerve, the microsomal fraction from a variety of tissues, including muscle, was found to have another interesting biochemical property, namely Na^+–K^+-activated ATPase activity (Skou, 1962; Lee & Yu, 1963; and others), and this ATPase activity appeared to be intimately linked to Na^+–K^+ exchange at the cellular membrane (Post, Meritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; and others).

In the present work, we have investigated the ATPase activity, the relaxing activity (on myofibrils), and the calcium uptake activity of the microsomal fraction (or 'relaxing factor') from skeletal muscle under a variety of experimental conditions. In particular, we have investigated the effects of various cations on these activities, as well as on the contraction (syneresis) of myofibrils in the absence of the microsomal fraction. Very good correlation between relaxing activity and calcium uptake

* Present address: Public Health Service, Yamaguchi Prefecture, Japan.

† Post-doctoral fellow on Pharmacology Training Grant 5TIGM163 of the United States Public Health Service.

activity has been demonstrated in the presence of various cations. On the basis of our results, it is proposed that the maximum syneresis of myofibrils occurs when one Ca^{2+} and one Mg^{2+} are associated with a unit of contractile protein and ATP. In addition evidence is presented which indicates that electrical stimulation may modify the uptake of Ca^{2+} by the endoplasmic reticulum. An attempt has been made to relate our experimental findings on isolated microsomes and myofibrils to events occurring in living muscle during contraction and relaxation.

METHODS

Preparation of the 'relaxing factor' and myofibrils

The 'relaxing factor' was prepared, with a slight modification, by the method of Weber *et al.* (1963). The skeletal muscle from rabbit was quickly sliced with a sharp stainless knife and was homogenized in 4 volumes of 120 mM-KCl/5 mM-histidine buffer (pH 6·5) at 0° C in a Waring Blendor for total of 40 sec with a pause of 20 sec after each 10 sec of homogenization. The homogenates were centrifuged for 30 min each at 5000 and 10,000 *g* for the removal of myofibrils and mitochondria, respectively. The supernatant was then centrifuged at 26,000 *g* for 1 hr in a Spinco centrifuge Model L and the resulting precipitate was washed twice with an approximately 40-fold volume of the histidine-buffer solution, followed by centrifugation at 56,000 *g*. During the washing procedure, the bottom layer of the precipitate, which had a darker colour, was discarded. The final precipitate was then suspended in the same histidine buffer, whose volume ratio to the original muscle was approximately 1:10. This suspension will be called the 'relaxing factor' or RF. The mean protein content of RF was $8{\cdot}36 \pm 2{\cdot}5$ mg (S.E.) per ml. of suspension.

Myofibrils were prepared according to the method described by Weber (1959), and the final precipitate of myofibrils was suspended in 100 mM-KCl/1 mM histidine buffer (pH 6·5) whose volume was approximately equal to that of muscle used. To this suspension an equal volume of glycerol was added and the mixture was stored at 0° C as a stock suspension. Just before an experiment the myofibrillar stock suspension was diluted with 10 volumes of 100 mM-KCl, and centrifuged for 15–20 min at 3000 *g* to remove most of the glycerol. The precipitate was then suspended in 100 mM-KCl solution to give a final volume equal to one-half of that of the stock myofibrillar suspension. The mean protein content of the final suspension was 17 ± 4 mg ($\pm$S.E.)/ml.

Measurement of syneresis of myofibrils

First, 1–1·5 ml. of myofibrils, tris–maleate buffer (pH 6·5) and appropriate amounts of alkali metal salts and other agents, as indicated in tables or figures, were mixed in a total volume of 4 ml. The reaction was initiated by adding 1·0 ml. of Mg-ATP (equimolar concentrations of tris-ATP and $MgCl_2$) and the mixture vigorously stirred by a magnetic stirrer. The mixture was immediately transferred to graduated tubes with narrowed tips and centrifuged for 2 min in a clinical centrifuge, and the volume of the centrifuged precipitate was read. Then the inorganic phosphate and protein content of the supernatant were determined.

In experiments on the effect of electrical 'stimulation' on syneresis, the myofibrillar mixture was placed in a container in which was mounted a pair of platinum electrodes as illustrated in Fig. 1. Square wave pulses, at frequencies and durations given in the text, were passed through the suspension during the 2 min period of stirring. A Grass stimulator (Model 4) was used. Other procedures remained identical with those in the experiments in

which no electrical stimulation was used. All syneresis experiments were performed at 25° C.

Assay of calcium uptake by RF

Each tube contained 0·01 M-tris–maleate buffer (pH 6·5), 10 μM total $CaCl_2$ (8 μM-$CaCl_2$ + 2 μM-$^{45}CaCl_2$), and 3 mM-$MgCl_2$. To these tubes the specified amount of RF and various alkali metal salts, as indicated in Tables, were added to make a final volume of 10 ml. At the beginning of an experiment the mixtures were transferred to Spinco tubes, and the reaction was initiated by the addition of ATP to give a concentration of 3 mM just before inserting the tubes into a Spinco centrifuge (Model L with No. 40 rotor). The mixture was immediately spun at 25° C for 15 min at 100,000 *g*. The radioactivity of both precipitate and supernatant was then analysed.

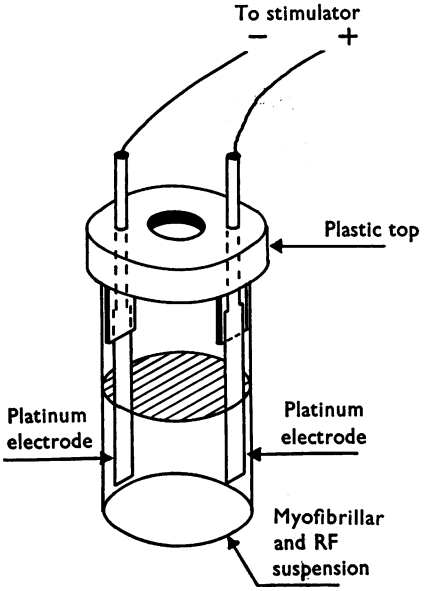

Fig. 1. Stimulating electrode.

Assay of ^{45}Ca release from RF

Approximately 0·4 mg protein of RF per ml. of medium was incubated for 10 min at 25° C in a medium containing 0·01 M-tris–maleate buffer (pH 6·5), 20 μM-$^{45}CaCl_2$ and 3 mM-Mg-ATP. Following the incubation, the mixture was immediately centrifuged at 25° C in the Spinco centrifuge at 100,000 *g*. The resulting precipitate, which was ^{45}Ca-labelled RF, was then resuspended in tris–maleate buffer containing 3 mM-Mg-ATP, whose volume was half that of the original incubation medium. Each 5 ml. of this RF suspension was then quickly placed in tubes which already contained 5 ml. of different alkali metal salt solutions of compositions appropriate to yield final compositions of resuspending media as those indicated in Table 3. These tubes were then immediately centrifuged at 25° C for 15 min at 100,000 *g*. The radioactivity of both precipitate and supernatant was analysed in the same manner as that in the ^{45}Ca uptake study.

Measurement of ATPase activity

The original RF was diluted 50-fold with 5 mM histidine buffer, pH 6·5. This diluted solution (50 μl.) was used in 1 ml. of reaction mixture whose composition was 0·125 M-tris–

maleate buffer (pH 6·5), 3 mM-tris–ATP, and varying amounts of Mg^{2+}, Ca^{2+} and alkali metal salts, as indicated in tables or figures. The reaction was initiated by the addition of the tris–ATP and the incubation was carried out at 37° C for 10 min. The reaction was terminated by the addition of cold 10% trichloroacetic acid and the determination of inorganic phosphate of the supernatant following centrifugation was carried out by the method of Fiske & Subbarrow (1925).

Other methods

Protein was determined by the biuret method. Tris–ATP (with a little sodium contamination) was purchased from Sigma Chemical Co. or prepared from disodium ATP (Sigma) with Dowex-50 resin in the H^+ form according to the procedure of Järnfelt (1962). Triple-distilled water was used throughout experiments. Acid-washed Pyrex glassware was used in all experiments using ^{45}Ca to avoid contamination by extraneous Ca^{2+} as much as possible.

RESULTS

The effect of alkali metals on ATPase activity of RF

RF in the tris–maleate buffer alone had very little ATPase activity (about 2–3 μmole/mg protein/hr). The addition of Mg^{2+} in a concentration of 3 mM raised ATPase activity (expressed as inorganic phosphate liberated) to the level of 40 μmole ($\pm$ 3·5, S.E.)/mg protein/hr. The presence of various alkali metal ions further increased the Mg^{2+}-ATPase and the extent of this increase was dependent on both the alkali metals used and their concentrations. As shown in Fig. 2, the ATPase activity increased as the concentration of these alkali metal ions increased and the order of their activating potency (at 100 mM) was $Rb^+ > K^+ > Na^+ > Li^+$. Thus, Rb^+ (100 mM) increased Mg^{2+}-ATPase activity by more than 200%, whereas Li^+ had no significant effect up to 200 mM. Potassium alone had somewhat more stimulating effect than Na^+ alone. In the presence of 100 mM-Na^+, K^+ had further stimulating effect; however, in the presence of 100 mM-K^+, Na^+ exerted no additional significant effect on the Mg^{2+}-ATPase activity.

The effect of Ca^{2+} on the ATPase activity of RF

It was found that varying Ca^{2+} concentration in the medium had a diphasic effect on ATPase activities. This is shown in Fig. 3. Calcium in concentrations higher than 10^{-4} M was a very potent inhibitor of the Na^+- or K^+-activated ATPase. However, when the Ca^{2+} concentration was around 10^{-5} M, it stimulated both the Mg^{2+}-ATPase and Na^+- or K^+-activated ATPase.

The effect of alkali metals on ^{45}Ca uptake by RF

The fresh RF was found to take up Ca^{2+} very actively from the medium containing tris–maleate buffer, Mg^{2+}, ATP, and 10 μM of total added Ca^{2+}. The results are shown in Table 1. As can be seen in this table, the

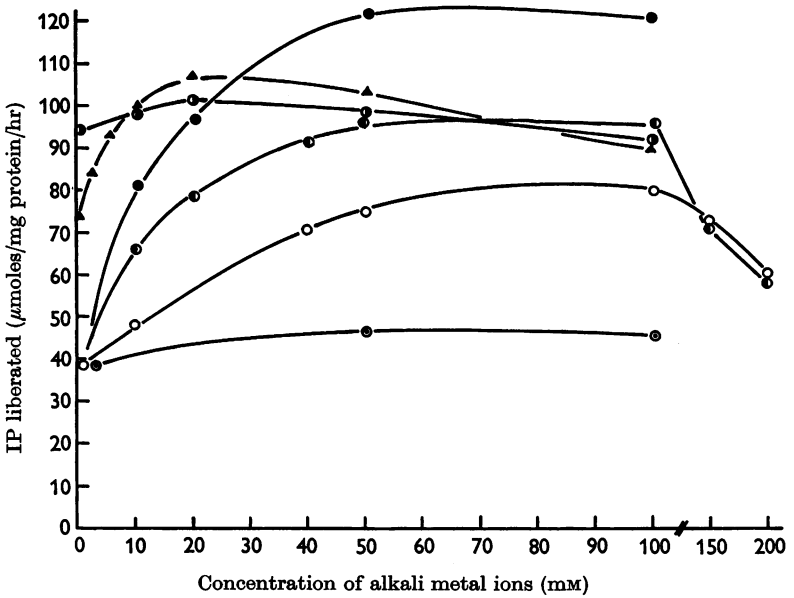

Fig. 2. Effect of alkali metal ions on Mg^{2+}-ATPase of RF (microsomal granules). All tubes contained 0·125M-tris–maleate buffer pH 6·5, 3 mM-$MgCl_2$, 3 mM-tris-ATP and enzyme. Each point represents the average of 12 experiments. Incubation, 10 min at 37° C. ◐, K^+; ○, Na^+; ●, Rb^+; ⊙, Li^+; ▲, $K^+ + Na^+$ 100 mM; ◑, $Na^+ + K^+$ 100 mM.

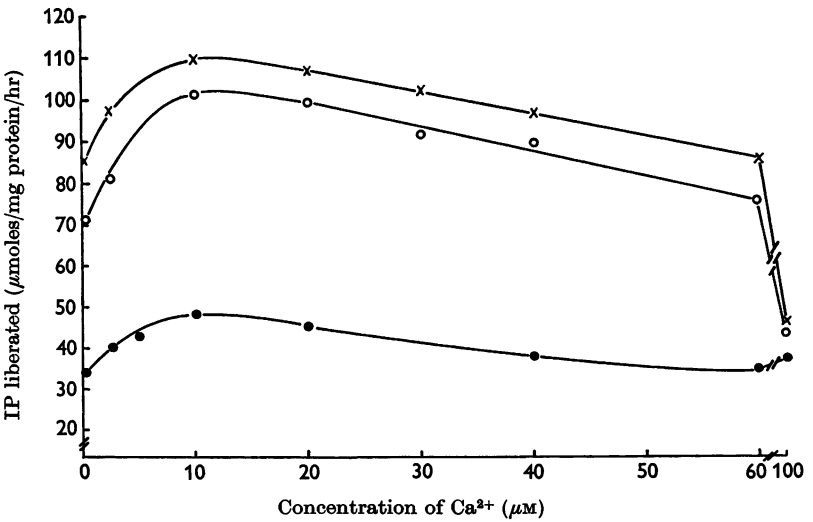

Fig. 3. Effect of Ca^{2+} on ATPase of RF. Each point represents the average of six experiments. All tubes contained 3 mM-ATP, 3 mM-$MgCl_2$, 0·125M-tris–maleate buffer, pH 6·5, and the same amount of RF as that in Fig. 2. Total volume, 1 ml. Incubation, 10 min at 37° C. ●, Mg^{2+}; ×, K^+ (100 mM); ○, Na^+ (100 mM).

TABLE 1. The effect of alkali metal ions on ^{45}Ca uptake by RF

Incubation mixture	Radioactivity remaining in supernatant relative to that in supernatant without RF						Average	Ca^{2+} concn. in supernatant* (μM)	Fraction of Ca^{2+} removed
BM without RF	1·00	1·00	1·00	1·00	1·00	1·00	1·00	10	0
BM without ATP	0·61	0·75	0·82	0·9	1·00	0·41	0·75	7·5	0·25
BM without Mg^{2+}	0·60	0·69	0·83	0·87	0·69	0·64	0·72	7·2	0·28
BM	0·073	0·044	0·037	0·033	0·05	0·051	0·048	0·48	0·95
BM+NaCl	0·18	0·13	0·11	0·092	0·077	0·088	0·113	1·13	0·87
BM+KCl	0·137	0·092	0·081	0·075	0·069	0·092	0·091	0·9	0·909
BM+NaCl+KCl	0·12	0·12	0·14	0·14	—	0·19	0·14	1·43	0·86
BM+LiCl	0·071	0·063	0·036	0·03	0·038	—	0·048	0·48	0·952
BM+RbCl	—	0·17	0·14	—	0·088	—	0·133	1·33	0·867

* Calculated on basis of an initial concentration of 10 mM-$CaCl_2$.

The composition of the basal medium (BM) was: 0·01 M-tris–maleate buffer (pH 6·5), 3 mM-$MgCl_2$, 3 mM-ATP, 10 μM total $CaCl_2$ ($^{45}CaCl_2+CaCl_2$), and RF. RbCl, NaCl, KCl, and LiCl were each at a concentration of 100 mM. Total volume, 10 ml. Average protein content of RF, 0·48 mg protein/ml.

presence of both ATP and Mg^{2+} was absolutely necessary for Ca^{2+} uptake. (The relatively small removal of Ca^{2+} in the absence of ATP or Mg^{2+} is probably adsorption rather than active uptake by RF.) Also Ca^{2+} uptake by RF was negligible when the Mg^{2+} concentration was 10^{-4}M. In the basal medium without any alkali metal ions, more than 95% of Ca^{2+}, on the average, was found in the RF precipitate after the centrifugation, leaving only 4–5% in the supernatant. The effect of alkali metal ions on Ca^{2+} uptake by RF was found to be different among the various ions. Thus, Rb^+ in a concentration of 100 mM inhibited considerably the Ca^{2+} uptake by RF so that only about 87% of Ca^{2+} added to the medium was accumulated in RF fraction. On the other hand Li^+ had no effect whatsoever. The inhibitory effect of Na^+ and K^+ appeared to fall in between that of Rb^+ and Li^+, with K^+ usually somewhat less effective than Na^+. A combination of Na^+ (100 mM) and K^+ (100 mM) was about equally effective as Rb^+ (100 mM) alone.

It should be emphasized that the Ca^{2+} concentrations shown in Table 1 represent total rather than free Ca^{2+} in the medium. Since the medium contained ATP as well as other components, the concentration of free Ca^{2+} must be considerably lower than that of total Ca^{2+}. It is theoretically possible to calculate the concentration of free Ca^{2+} ions in the presence of ATP, Mg^{2+} and other cations if (*a*) the affinity constants of the various cations for ATP (actually the ATP^{4-} anion) are known; (*b*) the concentrations of all free cations other than calcium are known; and (*c*) the total concentration of ATP is known.

Affinity constants for MgATP (K_{Mg}) and CaATP (K_{Ca}) are given by Martel & Schwarzenbach (1956), Walaas (1958), Burton (1959) and Nanninga (1961). Although Burton's values are higher than others, those values given by other three authors agree well. For the purpose of our calculation we have used a value of $2{\cdot}2 \times 10^4$ for K_{Mg} and of $1{\cdot}18 \times 10^4$ for K_{Ca} (Walaas, 1958).

The affinity constant for HATP (K_H) was taken as $10^{6{\cdot}92}$ (Melchior, 1954); and those for NaATP, KATP and LiATP were taken as 11, 8·5 and 33·5, respectively (Smith & Alberty, 1956). At equilibrium the following equation would apply:

$$\frac{[ATP]_{free}}{[ATP]_{total}} = \frac{1}{1+K_{Mg}[Mg^{2+}]+K_{Ca}[Ca^{2+}]+K_H[H^+]+K_A[A^+]}, \quad (1)$$

where $[ATP]_{free}$ is $[ATP^{4-}]$, A^+ is Na^+, K^+ or Li^+, and all concentration terms on the right of the equation represent concentrations of free ions. In addition the following equations would also apply

$$[Mg^{2+}]_{total} = [Mg\ ATP]+[Mg^{2+}] = 3\times 10^{-3}\text{M}, \quad (2)$$

$$[ATP]_{total} = 3\times 10^{-3}\text{M}, \quad (3)$$

$$\frac{[\text{MgATP}]}{[\text{Mg}^{2+}]\,[\text{ATP}]_{\text{free}}} = K_{\text{Mg}}, \tag{4}$$

$$\frac{[\text{CaATP}]}{[\text{Ca}^{2+}]\,[\text{ATP}]_{\text{free}}} = K_{\text{Ca}}, \tag{5}$$

$$[\text{Ca}^{2+}]_{\text{total}} = [\text{CaATP}] + [\text{Ca}^{2+}]. \tag{6}$$

In eqn. (1), the term $K_{\text{Ca}}[\text{Ca}^{2+}]$ is very small compared with all other terms in the denominator (because even the $[\text{Ca}^{2+}]_{\text{total}}$ without RF is only 10^{-5} M), and therefore was ignored when simultaneous equations (1), (2), (3) and (4) were used for the calculation of $[\text{ATP}]_{\text{free}}$. The value obtained for $[\text{ATP}]_{\text{free}}$ was then substituted into eqn. (3) in order to obtain $[\text{Ca}^{2+}]$ (free calcium) from eqns. (5) and (6). The results of these calculations are given in Table 2. Since the affinity constant for RbATP is not known, that for KATP was used for the calculation of BM + RbCl.

TABLE 2

Incubation mixture	Total Ca^{2+} remaining in supernatant, molar concn. (average)	Free Ca^{2+} ion concn.*
BM without RF	10^{-5}	$3{\cdot}29 \times 10^{-6}$
BM	$4{\cdot}8 \times 10^{-7}$	$1{\cdot}57 \times 10^{-7}$
BM + NaCl	$1{\cdot}1 \times 10^{-6}$	$3{\cdot}93 \times 10^{-7}$
BM + KCl	$9{\cdot}1 \times 10^{-7}$	$3{\cdot}22 \times 10^{-7}$
BM + LiCl	$4{\cdot}8 \times 10^{-7}$	$1{\cdot}99 \times 10^{-7}$
BM + RbCl	$1{\cdot}3 \times 10^{-6}$	$4{\cdot}6 \times 10^{-7}$

* For calculation of free Ca^{2+} concentration see text.

The effect of alkali metals on ^{45}Ca release from RF

As described in the section on methods, RF was preloaded with ^{45}Ca and re-suspended in media with and without alkali metal ions. The release of ^{45}Ca into the supernatant occurring during the centrifugation was measured by the analysis of radioactivity of the final supernatant and results are shown in Table 3. One millilitre of the final suspension contained the same amount of RF as that of the suspension of the first incubation as can be seen in the section on Methods. As can be seen in Table 3, about 80 % of the ^{45}Ca was concentrated in the RF precipitate during the initial incubation in these experiments. During the resuspension of this RF and recentrifugation, some ^{45}Ca in RF was released into the medium and the amount of this release was greater when alkali metal ions were present in the final suspension medium. On the average, the release was greatest with Rb^+. The presence of Na^+ or K^+ in the resuspending medium also increased the release but Li^+ had no significant effect. Thus the order

of potency of these ions in facilitating the release of Ca^{2+} from RF into the supernatant was similar to that of their inhibitory effect on Ca^{2+} uptake by RF.

TABLE 3. The effect of alkali metal ions on ^{45}Ca release

First incubation, for 'loading' RF with ^{45}Ca

Samples	Total radioactivity (counts/min)
Incubation medium, 1 ml.	160,000 ± 11,000
Supernatant after the first centrifugation, 1 ml.	28,000 ± 3,200
Precipitate (from 1 ml. incubation medium) after the first centrifugation	126,000 ± 10,500

Second incubation, for release of ^{45}Ca from RF obtained from first incubation and suspended in different media

Components of suspending medium	Radioactivity of supernatant, 1 ml. (counts/min)	% of ^{45}Ca released from RF
Buffer + MgATP	17,860 ± 1,900	14
Buffer + MgATP + NaCl	38,000 ± 3,200	30
Buffer + MgATP + KCl	33,000 ± 3,300	26
Buffer + MgATP + NaCl + KCl	41,000 ± 4,900	33
Buffer + MgATP + RbCl	43,000 ± 5,000	35
Buffer + MgATP + LiCl	21,040 ± 2,400	16

Average of 6 experiments ± standard error. Tris–maleate buffer, 0·01 M, pH 6·5; Mg-ATP 3 mM; all alkali metal ions, 100 mM.

TABLE 4. Effect of RF on syneresis under various conditions

Conditions of medium (all media contain standard components)	Volume of precipitate (ml.)	Contraction* (%)	Relaxation (%)
Standard components	0·25	100	0
Standard components + NaCl	0·23	100 (or 108)	0
Standard components + KCl	0·25	100	0
Standard components + EGTA 1 mM	0·71	35	65
Standard components + EGTA + KCl	0·96	0	100
Standard components + RF	0·45	72	28
Standard components + RF + NaCl	0·88	11	89
Standard components + RF + KCl	0·90	8	92
Standard components + RF + LiCl	0·41	77	23
Standard components + RF + RbCl	0·60	50	50
Standard components + RF + KCl + $CaCl_2$ 0·5 mM	0·26	96	4

Average of determinations in triplicate. All alkali metal ions, 100 mM.

Standard components: 0·01 M-tris–maleate buffer, pH 6·5; 3 mM MgATP; myofibrils, 4·6–5·2 mg protein/ml.; RF, 0·4–0·48 mg protein/ml.

EGTA: ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetra-acetic acid.

* A volume of 0·96 ml. was assumed to represent 0 % contraction (100 % relaxation) and a volume of 0·25 ml. to represent 100 % contraction (0 % relaxation).

The effect of alkali metal ions on the syneresis of myofibrils in the presence of RF

Since it has been postulated that the relaxing activity of the RF in muscular contraction is largely attributable to its function of Ca^{2+} uptake

from the surrounding medium (Ebashi, 1961*a*, *b*; Weber *et al.* 1963; Hasselbach & Makinose, 1963) and, since alkali metal ions have significant effects on ^{45}Ca uptake by RF, the influence of those alkali metal ions on the inhibitory effect of RF on the syneresis of myofibrils was studied. The results are shown in Table 4. In the presence of the buffer and Mg-ATP only, EGTA (ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid) and RF inhibit the syneresis to the extent of 65 and 28 % respectively. However, in the presence of K^+ 100 mM, both EGTA and RF inhibit the syneresis about 90 %. The influence of different alkali metals on the relaxing potency of RF varies. Thus, as can be seen in this Table, the syneresis is most inhibited by RF when Na^+ or K^+ is present. The presence of Li^+ has not much effect on the inhibition of syneresis and the influence of Rb^+ is between Na^+ or K^+ and Li^+.

The effect of alkali metals on syneresis of myofibrils in the absence of RF and at fixed Ca^{2+} concentrations

If the relaxing activity of RF is dependent on its function of Ca^{2+} uptake it might be expected that a good correlation would exist between the inhibitory influence of alkali metal ions on the Ca^{2+} uptake of RF and their inhibitory influence on the relaxing activity of RF on the syneresis of myofibrils. However, the results in Tables 1 and 4 show that the effects of alkali metal ions on these two functions of RF are contrary to the above expectation. For example, Na^+ or K^+ inhibits the Ca^{2+} uptake by RF (Table 1) and yet the presence of these cations facilitates the relaxation of myofibrils caused by RF (Table 4). In view of these unexpected results, it appeared likely that there may be direct effects of alkali metal ions on the requirement of Ca^{2+} by myofibrils for syneresis. To investigate this possibility, the influence of alkali metal ions on syneresis in the absence of RF was studied. For this purpose, the free Ca^{2+} ion concentration of the medium was varied by the use of the EGTA–CaEGTA buffer system (Weber & Winicur, 1961) and the syneresis of myofibrils in media containing various Ca^{2+} concentrations was observed in the presence and absence of these alkali metals. CaEGTA used here was accurately titrated so as not to contain an excess of Ca^{2+}. In experiments shown in Fig. 4, the sum of [EGTA] and [CaEGTA] was 1·0 mM and the ratio EGTA : CaEGTA was varied to give free Ca^{2+} concentrations ranging from 10^{-8} M to 10^{-5} M. It is clear from this figure that Rb^+, K^+ and Na^+, in the concentration of 100 mM each, shifted the syneresis curve to the right, so that higher concentrations of Ca^{2+} were required for the syneresis of myofibrils; whereas Li^+ at the same concentration or even at 200 mM (not shown in Fig. 4) had no significant effect on the syneresis. Rb^+ appeared to have an effect greater than that of Na^+ or K^+; however, the difference was not statistically

significant. The addition of Na^+ (100 mM) plus K^+ (100 mM) shifted the curve to still higher concentrations of Ca^{2+}.

The effect of ageing on the relaxing and ATPase activities of RF

It is known that the relaxing activity of RF is lost during storage (Ebashi & Lipmann, 1962). To compare the effect of ageing on relaxing activity with that on Na^+–K^+-activated ATPase, periodic measurements of these two functions of RF were made in the same preparation at

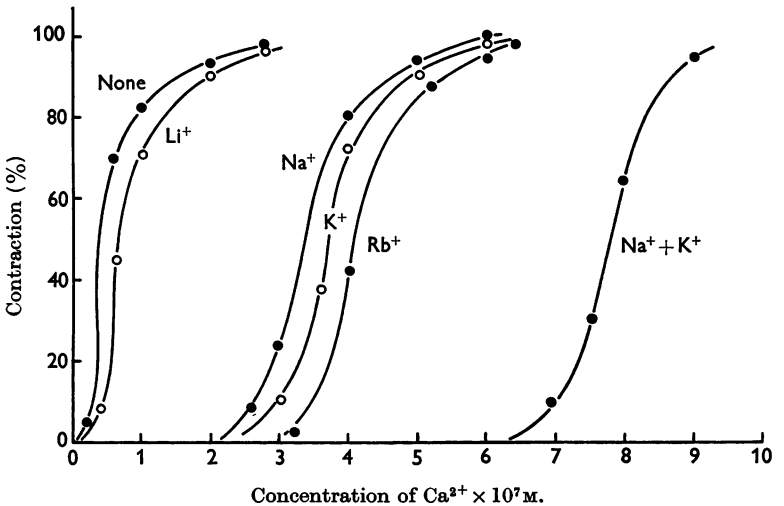

Fig. 4. Effect of alkali metal ions on syneresis of myofibrils in the absence of RF. All tubes contained 0·01 M-tris–maleate buffer, pH 6·5; 3 mM-Mg-ATP; myofibrils, 4·5–5·3 mg protein/ml.; the concentration of total [EGTA]+[CaEGTA], 1 mM. All alkali metals, 100 mM. Each point represents the average of seven experiments. Percentage of contraction expressed in the same manner as that in Table 4.

different times after storage at 0° C. The results are shown in Fig. 5. As can be seen, the relaxing activity was lost rapidly during the first day after preparation; however, the K^+-activated ATPase activity was maintained for many days. In fact, on a percentage basis, the Mg^{2+}-ATPase activity of RF fell faster than the K^+-activated portion of ATPase during the storage, thus resulting in a greater activation, on a percentage basis, by K^+ as the duration of storage increased. With Na^+ similar results to those with K^+ were obtained. In other experiments it was found that rapid deterioration of the ability of RF to accumulate Ca^{2+} occurred during storage, in parallel with the loss of the inhibitory effect of RF on syneresis of myofibrils.

The effect of deoxycholate and cardiac glycosides on the relaxing and ATPase activities of RF

Deoxycholate was found to inhibit profoundly both the Na^+- or K^+-activated Mg^{2+}-ATPase and the relaxing activity of RF, and concentrations required for this inhibition were almost identical in both cases as shown in Fig. 6. It should be noted that this agent in a concentration of 4×10^{-6} M stimulated somewhat the Na^+- or K^+-activated ATPase, but the relaxing activity was not influenced by this concentration of deoxycholate. Mg^{2+}-ATPase activity (no K^+ or Na^+ present) of RF was not at all influenced by this agent up to a concentration of 10^{-3} M.

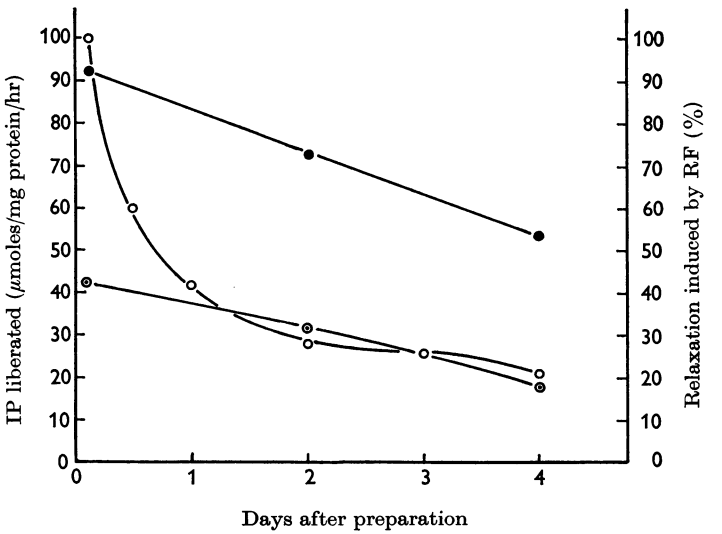

Fig. 5. Effect of ageing on the relaxing activity and K^+-activated ATPase of RF. Relaxing activity was measured under the same condition as 'standard compoment + RF + KCl' in Table 4. Incubation medium for ATPase measurement contained 100 mM-KCl, 3 mM-$MgCl_2$ and other components described in Methods. Each point represents average of six experiments. ○, Relaxing activity; ⊙, Mg^{2+}-ATPase; ●, K^+-activated ATPase.

Ouabain, in a concentration of 10^{-5} M, was also found to inhibit markedly Na^+- or K^+-activated ATPase (about 85 %), but the relaxing activity and Ca^{2+} uptake of RF was not significantly influenced by this concentration of the cardiac glycoside. Similar results were obtained with another cardiac glycoside, strophanthin-K. Neither glycoside inhibited the Mg^{2+}-ATPase activity (no K^+ or Na^+ present) of RF.

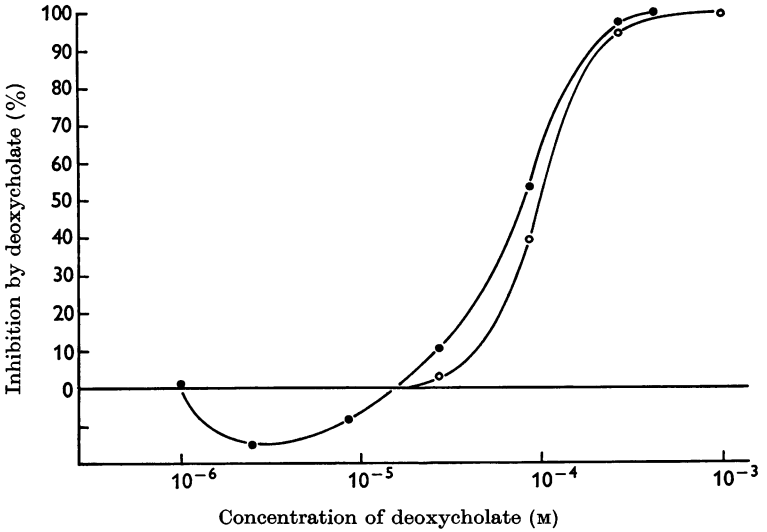

Fig. 6. Effect of deoxycholate on relaxing activity and K^+-activated ATPase. Each point represents the average of 6 experiments. Experimental conditions for ATPase and relaxing activity were the same as those in Fig. 2 with 100 mM-NaCl and in Table 4 with RF+NaCl, respectively. ●, Na^+-activated ATPase; ○, relaxing activity.

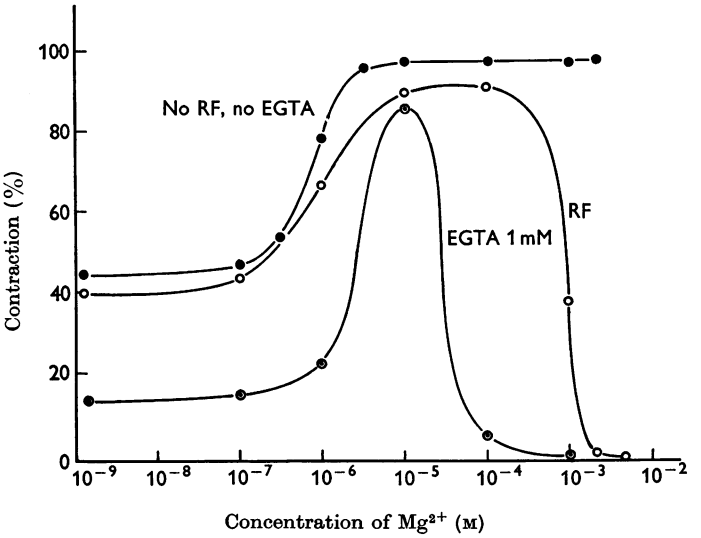

Fig. 7. Effect of Mg^{2+} concentration on syneresis of myfibrils. Each point represents the average of seven experiments. All tubes contained 0·01 M-tris–maleate buffer (pH 6·5), 3 mM-tris–ATP, 100 mM-KCl and myofibrils. Myofibrillar concentration, 4·9–5·8 mg protein/ml. RF concentration, 0·41–0·44 mg protein/ml.

The effect of Mg^{2+} concentration on syneresis of myofibrils

The concentration of Mg^{2+} affected the syneresis of myofibrils in the presence of the buffer, 100 mM-KCl and 3 mM-ATP. As shown in Fig. 7, in the absence of RF and EGTA, the syneresis of myofibrils began to be inhibited when the Mg^{2+} concentration fell below 10^{-6}M. In the presence of RF almost complete syneresis was observed with Mg^{2+} concentrations between 10^{-5} and 10^{-4}M. On either side of this range of Mg^{2+} concentration, inhibition of syneresis occurred, with the extent of inhibition increasing as the Mg^{2+} concentration was shifted further away from this range. However, it should be noted that whereas almost complete inhibition occurred at the higher concentrations of Mg^{2+} ($> 10^{-4}$M) inhibition at the lower concentrations ($< 10^{-5}$M) was never more than about 60%. In the presence of EGTA (1 mM), fairly complete syneresis occurred only in a narrow range around 10^{-5}M of Mg^{2+}, and at both 10^{-6} and 10^{-4}M-Mg^{2+} no significant syneresis was observed.

The effect of electrical stimulation of myofibrillar solution on syneresis

As mentioned in the methods section, some syneresis experiments were carried out in a chamber containing a pair of platinum electrodes. During the 2 min of the incubation period, a series of electrical pulses of 10 msec duration were passed through the electrodes. All other aspects of the procedure remained identical with those in the previous syneresis experiments. In control experiments, the same platinum electrodes were placed in the solution in the same manner as above during the incubation period but no electrical current was applied. The passage of electrical pulses was found to influence profoundly the syneresis of myofibrils, as shown in Table 5. The electrical stimulation caused the syneresis of myofibrils in the presence of RF in an amount which prevented the syneresis of myofibrils in the absence of electrical stimulation. The degree of reversal of the inhibitory effect of RF on the syneresis by the electrical stimulation appeared to be dependent on both the intensity of voltage and the frequency of electrical stimulation. Thus at 1 or 2 V and a frequency of 60/min, the stimulation caused good syneresis, while at 1 V and a frequency of 10/min, only partial syneresis occurred. However, it is important to note that the effect of electrical stimulation (causing the syneresis) was observed only when the amount of RF was not excessive. Thus in the experiment shown in Table 5, when the electrical pulses (1 V, 60/min) were passed through the medium containing 0·45 mg protein/ml. of RF, an amount sufficient to inhibit syneresis in the absence of stimulation, good syneresis occurred. However, when 0·9 mg protein/ml. of RF was present,

the same or stronger electrical stimulation was almost ineffective in reversing the inhibitory effect of RF. Thus, for the best demonstration of the effect of electrical impulses, it was necessary to use the minimal effective amount of RF.

TABLE 5. The effect of electrical stimulation on syneresis

Basic components	+	+	+	+	+	+	+
RF (mg protein per ml.)	—	0·3	0·45	0·45	0·45	0·45	0·45
Volt	—	—	—	2	1	1	1
Frequency per min	—	—	—	60	60	30	10
Precipitate (ml.)	0·21	0·45	0·82	0·29	0·38	0·50	0·56
IP (μM)	4·3	4·0	2·1	5·2	4·7	4·2	4·2
Basic components	+	+	+	+	+	+	+
RF (mg protein per ml.)	0·45	0·9	0·9	0·45	0·45	0·45	0·45
Volt	—	2	5	1	1	2	2
Frequency per min	—	60	90	60	60	60	60
				RF only	Myof only	RF only	Myof only
Precipitate (ml.)	0·80	0·78	0·68	0·40	0·69	0·38	0·64
IP (μM)	2·4	2·9	3·3	3·6	2·3	5·0	2·6

Electrical pulse is square wave of 10 msec.

All tubes contained the basic components: 0·01 M-tris–maleate, 3 mM-MgATP, 5·6 mg protein/ml. myofibril and 100 mM-KCl. Total volume 5 ml.

IP is the amount of inorganic phosphate liberated during the experimental period.

To study further the nature of this electrical stimulation, separate stimulation of myofibrils or RF was performed. In case of stimulation of RF only, myofibrils were omitted from the syneresis medium during the 2 min period when the medium was stimulated electrically and, just before the centrifugation, myofibrils were added to the medium. In case of stimulation of myofibrils only, RF was omitted from the syneresis medium during the 2 min period of electrical stimulation and RF was added to the medium just before the centrifugation. The results are shown in the last four columns in Table 5, indicated as stimulation of myofibrils only or RF only. When myofibrils only were exposed to the electrical stimulation, there was still a good inhibition of syneresis by RF; whereas when RF only was stimulated there was much less inhibition of syneresis. These results indicate that the electrical stimulation of RF and not that of myofibrils was mainly responsible for the reversal of the RF inhibition of syneresis. It should be noted that whenever syneresis was observed there was a concomitant increase in the amount of IP liberated during the experimental period (last row of Table 5). Presumably this was due to an increase in myofibrillar ATPase associated with syneresis.

In other experiments the concentration of free Ca^{2+} in the medium containing myofibrils, the buffer, Mg-ATP and KCl (100 mM) was varied using EGTA–CaEGTA system, and the syneresis of myofibrils without RF was studied with and without electrical stimulation. In these experiments

the syneresis curves (with regard to varying concentrations of Ca^{2+}) were exactly the same with and without stimulation. This indicates that the electrical stimulation has no influence on the syneresis of myofibrils when RF is not present and the concentration of free Ca^{2+} is kept constant.

DISCUSSION

The influence of alkali metal ions on ATPase and Ca^{2+} uptake of RF

The Mg^{2+}-ATPase found in RF, a microsomal fraction from skeletal muscle obtained by the modified procedure of Weber *et al.* (1963), is activated further by alkali metal ions. The order of potency of activation is $Rb^+ > K^+ > Na^+ > Li^+$, with Rb^+ increasing Mg^{2+}-ATPase activity by more than 200 %, and with Li^+ having almost no effect. The characteristics of this Na^+- or K^+-activated ATPase appear to be somewhat different from those of the so-called 'membrane ATPase' found in red blood cells, nerve, muscle and other tissues (Skou, 1957, 1962; Post *et al.* 1960; Dunham & Glynn, 1961; Lee & Yu, 1963; and others). The 'membrane ATPase' requires the presence of both Na^+ and K^+ for the activation. Furthermore, higher concentrations of Na^+ than of K^+ are needed for maximal activation. However, with the present Mg^{2+}-ATPase either Na^+ or K^+ alone can activate, and K^+ alone has a greater effect than Na^+ alone. Although the nature of the present Na^+ or K^+-activated ATPase is not known, it is probable that this ATPase is in some way related to so-called Na^+–K^+-activated 'membrane ATPase', since both ATPases reside in the microsomal fraction and since both are inhibited by cardiac glycosides and by deoxycholate.

RF used in the present work was found to take up Ca^{2+} very actively from the medium. Under our experimental conditions, when no alkali metal ion was present, RF removed over 90 % of Ca^{2+} in the medium. As shown previously (Ebashi & Lipmann, 1962; Hasselbach & Makinose, 1961, 1962, 1963), the Ca^{2+} uptake is an energy-requiring process dependent on Mg-ATP, since no significant Ca^{2+} uptake is observed when either ATP or Mg^{2+} is omitted from the medium. It is reasonably clear that the Ca^{2+} uptake is an enzymic process. The alkali metals are found to inhibit considerably the Ca^{2+} uptake of RF and the order of potency for inhibition is $Rb^+ > Na^+, K^+ > Li^+$. There is no significant difference statistically between effects of Na^+ and K^+. Also these ions promote the release of Ca^{2+} previously taken up by RF in the absence of such ions and the order of potency for facilitating release is also similar to that for inhibiting uptake (compare Tables 1 and 3). Previously, the similar inhibition of Ca^{2+} uptake of sarcoplasmic reticulum by Na^+ or K^+ was reported by Martonosi & Feretos (1964).

It should be noted that the order of potency of alkali metal ions on inhibition of Ca^{2+} uptake by RF is similar to that for the stimulatory effects of alkali metals on Mg^{2+}-ATPase of RF. In both cases Li^+ has almost no effect and Na^+ and K^+ fall between Rb^+ and Li^+. Both the alkali-metal-ion-activated ATPase and the system for Ca^{2+} uptake require Mg-ATP since the omission of either Mg^{2+} or ATP brings both of these processes to a standstill. In addition, both systems are sensitive to deoxycholate to the same extent, as can be seen in Fig. 6. Although the Na^+–K^+-activated ATPase and the Ca^{2+} uptake system have in common the above characteristics, they are not the same but distinctly different systems as shown by the difference in the influence of ageing and cardiac glycosides on them. The ability of RF to take up ^{45}Ca from the medium decreases rapidly during the course of one day after preparation; whereas the activation of Mg^{2+}-ATPase by alkali metal ions falls off very slowly over the course of several days (Fig. 5). The cardiac glycosides ouabain and strophanthin inhibit Na^+- or K^+-activated ATPase but have no effect on the Ca^{2+} uptake or relaxing activity of RF. It is interesting to note that each of these two processes is antagonized by a 'substrate' of the other process. Thus, alkali-metal-ion-activated ATPase of RF is antagonized by high concentrations of Ca^{2+} (Fig. 3) and the Ca^{2+} uptake by RF is antagonized by alkali metal ions (Table 1).

Comparison of Ca^{2+} accumulating activity and syneresis-inhibiting activity of RF in presence of alkali metal ions

Before further discussion of the Ca^{2+}-accumulating activity of RF, it is appropriate to consider the free Ca^{2+} concentrations attained after uptake by RF in the presence of various alkali metal ions. The calculated values for free Ca^{2+} concentrations given in Table 2 involve some uncertainties. First, there may have been a very small amount of 'contaminating' Ca^{2+} in the medium, so that the total Ca^{2+} initially may have exceeded somewhat the $10\,\mu M$ concentration of added radioactive Ca^{2+}. Secondly, ATP is continuously split in the presence of RF and the affinity constants of ADP and other products have to be considered. Thirdly, since the Ca^{2+} accumulation by RF is heavily dependent on the concentration of ATP, some Ca^{2+} taken up by RF is undoubtedly released during the process of centrifugation, as RF starts being packed together in the lower portion of the tube. However, in the presence of RF and $10\,\mu M$ added Ca^{2+}, the first factor would not interfere too much with calculated values. The second factor is minimized by centrifuging the mixture at the moment of ATP addition. The third factor is difficult to evaluate; however, the influence of this factor is to increase the Ca^{2+} level in the supernatant over that which would be obtained in the

medium in the presence of RF without centrifugation. In spite of these uncertainties, the calculated free Ca^{2+} ion concentrations given in Table 2 may serve as approximations of the actual concentrations. It should be noted that the calculated values of free Ca^{2+} concentrations would be considerably smaller if, as in case of previous works (Weber *et al.* 1963; Hasselbach & Makinose, 1963), the affinity constants for CaATP and Mg-ATP determined by Burton (1959) were used for calculation.

As can be seen in Table 1, alkali metal ions were found to inhibit considerably the Ca^{2+} uptake of RF. If RF causes the inhibition of syneresis by removing Ca^{2+} from the medium as postulated previously (Ebashi, 1961*b*; Weber *et al.* 1963; Hasselbach & Makinose, 1963), then it would be expected from the Ca^{2+} uptake study that alkali metal ions should antagonize the relaxing activity of RF. However, data in Table 4 show that, with the exception of Li^+, alkali metal ions potentiate the relaxing activity of the RF instead of antagonizing it. These apparently contradictory results can be clarified when effects of alkali metal ions on the syneresis of myofibrils in the absence of RF are considered. As shown in Fig. 4, alkali metal ions except Li^+ have a rather profound, direct effect on the syneresis of myofibrils in the absence of RF, and Na^+, K^+, and Rb^+ shift the syneresis curve markedly to the right so that the myofibrils require more Ca^{2+} for the syneresis. The fact that even at 200 mM Li^+ does not have any significant effect on the syneresis curve indicates that the effect of other alkali metal ions cannot be simply attributed to the increase in the ionic strength of medium.

If we now consider the calculated free Ca^{2+} concentrations given in Table 2 and the curves for syneresis as a function of free Ca^{2+} concentration in the absence of RF (Fig. 4), it is possible to account in large part for the influence of RF on the syneresis, shown in Table 4. On the reasonable assumption that the concentration of free Ca^{2+} for a given medium was essentially the same in the experiments shown in Table 2 and in those shown in Table 4 (since the amount of RF was essentially the same in both types of experiments) it is possible to estimate the degree of syneresis in experiments shown in Table 4 from the data in Table 2 and Fig. 4 if the effect of RF on syneresis is due solely to its removal of Ca^{2+} from the medium.

A comparison of estimated and actual syneresis is presented in the following table.

Component of medium	Free Ca^{2+} concentration in Table 2	Estimated % of contraction calculated from Fig. 4	% of contraction actually observed in Table 4
RF alone	$1{\cdot}57 \times 10^{-7}$	85	72
RF + NaCl	$3{\cdot}93 \times 10^{-7}$	74	11
RF + KCl	$3{\cdot}22 \times 10^{-7}$	17	8
RF + LiCl	$1{\cdot}99 \times 10^{-7}$	89	77
RF + RbCl	$4{\cdot}6 \times 10^{-7}$	72	50

Except in the case of RF + NaCl, the values calculated from Table 2 and Fig. 4 are in fair agreement with those actually observed in experiments shown in Table 4. The calculated values are somewhat higher than those observed. However, it should be pointed out that the calculated free Ca^{2+} concentrations shown in Table 2 are probably somewhat higher than the actual ones because of the release of Ca^{2+} from RF during the process of centrifugation (see above). Thus it appears that the inhibitory effects of RF on the syneresis of myofibrils can be attributed to its function of Ca^{2+} uptake from medium in the presence of a number of different monovalent cations which influence both Ca^{2+} uptake by RF and the syneresis of myofibrils directly.

It is interesting to speculate on the physiological significance of effects of alkali metal ions on syneresis of myofibrils in the presence of RF. As can be seen in Fig. 4, the syneresis curve of myofibrils in the presence of Na^+ and K^+ (each 100 mM) is further shifted to the right from that of K^+ (100 mM) alone. It is difficult to attach any physiological significance to this phenomenon since it is impossible that the change of alkali metal ion concentrations in whole muscle can occur *in vivo* to an extent of this magnitude during the excitation–contraction processes. However, the smaller Ca^{2+} uptake and greater Ca^{2+} release in the presence of $Na^+ + K^+$ than in the presence of K^+ alone (in Tables 1 and 3) may have some physiological significance. If the Na^+ influx during the excitation appreciably increases the total concentration of $Na^+ + K^+$ at the membrane site it is possible that Ca^{2+} release from the endoplasmic reticular system may be facilitated immediately following excitation.

The influence of Mg^{2+} concentration on various activities of RF and on myofibril syneresis

The Mg^{2+} concentration had a marked influence on syneresis (Fig. 7). When neither RF nor EGTA (1 mM) was present, syneresis began to be inhibited when the Mg^{2+} concentration was reduced below 10^{-6} M. However, above this concentration of Mg^{2+}, complete syneresis was observed in all experiments. When RF was present, it was found that almost complete inhibition of syneresis occurred with Mg^{2+} concentrations of 3×10^{-3} M and higher. The decrease of Mg^{2+} concentration from 3×10^{-3} to 10^{-3} M was followed by syneresis, which became almost maximal in the range from 10^{-4} to 10^{-5} M, and further decrease to 10^{-6} M or less was accompanied by a considerable inhibition of syneresis, similar to that observed in the absence of RF. When EGTA was present, good syneresis was observed only at around 10^{-5} M-Mg^{2+}.

These findings on syneresis can be best explained if the assumption is made that the contraction and relaxation of actomyosin require the com-

plexes actomyosin–ATP$^{Mg^{2+}(1)}_{Ca^{2+}(2)}$ and actomyosin–ATP$^{Mg^{2+}(1)}_{Mg^{2+}(2)}$ respectively. The (1) position of this complex combines only with Mg^{2+} but competition between Mg^{2+} and Ca^{2+} exists for the (2) position, for which it is assumed that the affinity of Ca^{2+} is very much greater than that of Mg^{2+}. In case of no RF and no EDTA, the contaminating amount of free Ca^{2+} is considered to be great enough to compete successfully with the added Mg^{2+} for position (2) and syneresis is observed as long as Mg^{2+} is present in high enough concentration (10^{-6} M or higher) to occupy position (1). In the presence of RF, the free Ca^{2+} concentration is around 3×10^{-7} M at 3 mM-Mg^{2+} and the syneresis is inhibited because most of the (2) positions are occupied by Mg^{2+} in the face of an extremely high Mg^{2+}:Ca^{2+} ratio (about 10^4:1). However, when the Mg^{2+} concentration falls below 10^{-4} M, markedly diminished Ca^{2+} uptake by RF leads to the same situation as that of no RF (or no EGTA) in spite of the presence of RF. Thus, the syneresis curve of RF becomes essentially the same as that of no RF or no EGTA below a Mg^{2+} concentration of 10^{-4} M. When EGTA 1 mM is present, the free Ca^{2+} concentration of medium is always at the level of about 10^{-8} M or less at all concentrations of Mg^{2+}. At the Mg^{2+} concentration of 10^{-4} M or higher, position (2) is occupied by Mg^{2+} since the extremely high ratio of Mg^{2+}:Ca^{2+} favours the formation of actomyosin–ATP$^{Mg^{2+}}_{Mg^{2+}}$ complex. When the Mg^{2+} concentration is reduced to 10^{-5}, the ratio of Mg^{2+}:Ca^{2+} apparently becomes low enough for Ca^{2+} to occupy position (2) and syneresis occurs due to actomyosin–ATP$^{Mg^{2+}}_{Ca^{2+}}$ complex formation. However, at a Mg^{2+} concentration of 10^{-6} or less, again the lack of Mg^{2+} for position (1) causes the inhibition of syneresis. The more complete inhibition of syneresis at a Mg^{2+} concentration of less than 10^{-6} observed with EGTA as compared with RF or no RF and no EGTA may be due to a greater lack of Mg^{2+} as well as Ca^{2+} in the EGTA system.

Previously, a somewhat similar model for two classes of sites for metal binding in the actomyosin system was proposed by Maruyama & Watanabe (1962) from their studies on the superprecipitation of myosin B. However, these authors considered that the contraction required only the binding of Mg^{2+} but not that of Ca^{2+}. Similarly, Weber & Herz (1963) suggested that the replacement of Mg^{2+} by Ca^{2+} caused superprecipitation of myofibrils under certain experimental conditions but Ca^{2+} is not required for superprecipitation.

Influence of electrical stimulation on inhibition of syneresis by RF

It is rather surprising to find that a series of electrical pulses of 10 msec duration and 1–2 V delivered at the frequency of 60/min during 2 min of incubation period caused the syneresis of myofibrils in the presence of a concentration of RF sufficient to inhibit almost completely syneresis of

myofibrils in the absence of electrical pulses. The degree of electrically induced syneresis appears to be dependent on the voltage and the frequency of electrical stimulation. Thus, as the voltage or the frequency of stimulation was decreased, a lesser degree of syneresis was observed. With regard to the mode of inducing the syneresis, the effect of electrical stimulation directly on myofibrils seems to be unlikely for the following reasons. First, no effect of electrical stimulation was found when myofibrils alone were stimulated. Secondly, in syneresis experiments in the absence of RF where the free Ca^{2+} concentration was varied using the EGTA–CaEGTA system, the electrical stimulation of this myofibrillar medium had no effect on the degree of syneresis obtained at various concentrations of free Ca^{2+} in the medium, indicating that, so long as RF is absent from the system, no effect of electrical stimulation is evident. In contrast to those negative results obtained with myofibrils in the absence of RF, the stimulation of RF alone definitely reduced the capacity of the RF to inhibit syneresis. These results support strongly the possibility that the electrical stimulation results in the syneresis of myofibrils through its influence on RF. Thus, it appears that the electrical stimulation of RF inhibits the Ca^{2+} uptake by RF and leads to the syneresis of myofibrils.

The evidence presented here directly suggests that the electrical stimulation may indirectly facilitate the release of Ca^{2+} from the endoplasmic reticular system of muscle through the inhibition of Ca^{2+} uptake by RF, and has an important implication from the physiological standpoint.

On the basis of the above results obtained in the 'stimulation' experiments, the proposal is advanced that during the excitation of muscle membrane the passage of electrical current releases Ca^{2+} from the endoplasmic reticular system and this released Ca^{2+} in turn activates myofibrils to contract. In addition, this Ca^{2+} release from the reticulo-endoplasmic system at the time of excitation may be further facilitated by the Na^+ influx accompanying the membrane excitation. This possibility is mentioned here on the basis of results given in Table 3 which show that the release of Ca^{2+} from RF is more in the presence of Na^+ and K^+ than in the presence of K^+ alone in the medium.

SUMMARY

1. The Mg^{2+}-activated ATPase of microsomal granules (RF) isolated from skeletal muscle was further stimulated in the presence of alkali metal ions. The order of potency of stimulating effect was $Rb^+ > K^+ > Na^+$ and Li^+ had no significant effect.

2. RF was found to take up Ca^{2+} very actively from the surrounding

medium. The presence of Rb^+, Na^+ and K^+ in the medium inhibited this Ca^{2+} accumulation by RF.

3. RF inhibited the syneresis of myofibrils. The quantitative analysis of data suggested that the inhibition of syneresis by RF could be attributed to lowering of Ca^{2+} concentration in media by RF.

4. Alkali metal ions except Li^+ increased the myofibrillar requirement of Ca^{2+} for syneresis in the absence of RF.

5. The effect of Rb^+, Na^+ and K^+ on syneresis of myofibrils in the presence of RF could be accounted for by their effect on myofibrils on the one hand and on RF on the other, namely the increased myofibrillar requirement of Ca^{2+} for syneresis and the decreased uptake of Ca^{2+} by RF in the presence of alkali metal ions.

6. If an assumption is made that alkali metal ions and Ca^{2+} are substrates of alkali-metal-ion-activated ATPase and Ca^{2+} uptake process of RF, respectively, then the mutual inhibition of these enzymic processes by the substrate of the other exists. This may have some physiological significance.

7. The electrical stimulation of myofibrillar solution containing RF reversed the relaxing effect of RF and induced the syneresis of myofibrils. Results obtained from the separate stimulation of either myofibrils or RF suggested that electrical stimulation caused the syneresis through its effect on RF and not on myofibrils.

8. The proposal is advanced that the electrical excitation of membrane releases Ca^{2+} from the endoplasmic reticular system and this release of Ca^{2+} may be further facilitated by Na^+ influx accompanying the excitation of membrane.

The authors wish to express their sincere thanks to Professor Robert F. Furchgott for his valuable criticism of this work and helpful suggestions for the preparation of this manuscript. This work was supported by grants (HE–03751 and HE–02875) from the National Institute of Health, Public Health Service, U.S.A.

REFERENCES

BENDALL, J. R. (1952*a*). Contribution to discussion on the thermodynamics of elasticity in biological tissues. *Proc. Roy. Soc.* B, **139**, 523–525.

BENDALL, J. R. (1952*b*). Effect of the 'Marsh factor' on the shortening of muscle fibre models in the presence of ATP. *Nature, Lond.*, **170**, 1058–1060.

BURTON, K. (1959). Formation constants for the complexes of adenosine di- or triphosphate with magnetism or calcium ions. *Biochem. J.* **71**, 388–395.

DUNHAM, E. T. C. & GLYNN, I. M. (1961). Adenosinetriphosphatase activity and the active movement of alkali metal ions. *J. Physiol.* **156**, 274–293.

EBASHI, S. (1961*a*). Calcium binding activity of vesicular relaxing factor. *J. Biochem.* **50**, 236–244.

EBASHI, S. (1961*b*). The role of 'Relaxing Factor' in contraction–relaxation cycle of muscle. *Progress of Theoretical Physics.* Supplement 17, pp. 35–40.

EBASHI, S. & LIPMANN, F. (1962). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. cell. Biol.* **14**, 389–400.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375–400.

HASSELBACH, W. & MAKINOSE, M. (1961). Die Calciumpumpe der 'Erschlaffungsgrana' des Muskels and ihre Abhängigkeit von der ATP-Spaltung. *Biochem. Z.* **333**, 518–528.

HASSELBACH, W. & MAKINOSE, M. (1962). ATP and active transport. *Biochem. biophys. Res. Comm.* **7**, 132–136.

HASSELBACH, W. & MAKINOSE, M. (1963). Über den mechanismus des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339**, 94–111.

JÄRNFELT, J. W. W. (1962). Properties and possible mechanism of the Na^+ and K^+-stimulated microsomal adenosine-triphosphatase. *Biochim. biophys. Acta*, **59**, 643–654.

LEE, K. S. & YU, D. H. (1963). A study of the sodium and potassium activated adenosine-triphosphatase activity of heart microsomal fraction. *Biochem. Pharmacol.* **12**, 1253–1264.

MARSH, B. B. (1951). A factor modifying muscle-fibre syneresis. *Nature, Lond.*, **167**, 1065–1067.

MARTEL, A. E. & SCHWARZENBACH, G. (1956). Adenosinephosphate und Triphosphat als Komplexbilder für Calcium und Magnesium. *Helv. chim. acta*, **39**, 653–661.

MARTONOSI, A. & FERETOS, S. (1964). Sarcoplasmic reticulum. *J. biol. Chem.* **239**, 648–658.

MARUYAMA, K. & WATANABE, S. (1962). The role of magnesium in the superprecipitation of myosin B. *J. biol. Chem.* **237**, 3437–3442.

MELCHIOR, N. C. (1954). Adenosine triphosphate, equilibrium studies. *J. biol. Chem.* **208**, 615–627.

MUSCATELLO, U., ANDERSON-CEDERGREN, E. & AZZONE, G. F. (1962). The mechanism of muscle fiber relaxation, adenosine triphosphatase and relaxing activity of the sarcotubular system. *Biochim. biophys. Acta*, **63**, 55–74.

NAGAI, T., MAKINOSE, M. & HASSELBACH, W. (1960). Der physiologische Erschlaffungsfactor und die Muskelgrana. *Biochim. biophys. Acta*, **43**, 223–238.

NANNINGA, L. B. (1961). The association constant of the complexes of adenosinephosphate with magnesium, calcium, strontium and barium ions. *Biochim. biophys. Acta*, **54**, 330–338.

PORTER, K. R. (1961). The sarcoplasmic reticulum. *J. biophys. biochem. Cytol.* **10**, no. 4 suppl. 219–226.

PORTER, K. R. & PALADE, G. E. (1957). Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *J. biophys. biochem. Cytol.* **3**, 269–299.

POST, R. L., MERRITT, C. R., KINSOLVING, C. R. & ALBRIGHT, C. D. (1960). Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. biol. Chem.* **235**, 1796–1802.

SKOU, J. C. (1957). The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. biophys. Acta*, **23**, 394–401.

SKOU, J. C. (1962). Preparation from brain and kidney of the enzyme involved in active transport of Na^+ and K^+. *Biochim. biophys. Acta*, **58**, 314–325.

SMITH, R. M. & ALBERTY, R. A. (1956). The apparent stability constants of ionic complexes of various adenosine phosphates with monovalent cations. *J. phys. Chem.* **60**, 180–184.

WALAAS, E. (1958). Stability constants of metal complexes mononucleotides. *Acta chem. scand.* **12**, 528–538.

WEBER, A. (1959). On the role of calcium in the activity of adenosine-5′-triphosphate hydrolysis by actomyosin. *J. biol. Chem.* **234**, 2764–2769.

WEBER, A. & WINICUR, S. (1961). The role of calcium in the superprecipitation of actomyosin. *J. biol. Chem.* **236**, 3198–3202.

WEBER, A., HERZ, R. & REISS, I. (1963). On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. *J. gen. Physiol.* **46**, 679–702.

WEBER, A. & HERZ, R. (1963). The binding of calcium to actomyosin systems in relation to their biological activity. *J. biol. Chem.* **238**, 599–605.