CALCIUM UPTAKE AND RELAXING ACTIVITY IN A FRACTIONATED RABBIT MUSCLE HOMOGENATE

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From a rabbit psoas muscle homogenate several fractions have been obtained by differential centrifugation. Strong relaxing activity has been found in the supernatant fraction after high-speed centrifugation. A heavy and a light microsomal fraction also show relaxing activity whereas mainly the heavy microsomes will concentrate calcium. The various fractions of the homogenate have also been tested for cytochrome c oxidase and ATP-ase activities.

Methods. One rabbit is anesthetized with nembutal-curare and then bled to death. The psoas muscles are taken out and rapidly transferred to ice-cold 0.3 M sucrose, weighed and left to cool completely, about 5 min. in the cold room, where the subsequent manipulations are done. The muscles are blotted on filter paper and sliced lengthwise with a razor blade. The slices are washed with sucrose and then chopped in a beaker with scissors. The mince is briefly homogenized (15 - 25 sec.) in a Potter-Elvehjem homogenizer with a glass reinforced teflon pestle with about 4 parts of 0.3 M sucrose. The homogenate is diluted to 9 times the original weight of the muscle and centrifuged in 4 consecutive steps (Lourdes and Spinco refrigerated centrifuges). 1:7 min. at 2000xg. 2: Supernatant from 1 for 15 min. at 20,000xg to remove the mitochondria as efficiently as possible. 3: Supernatant from 2 for 50 min. at 50,000xg. The pellet from this centrifugation is designated "heavy microsomes" (HM). It has

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a thin layer of different material on the top (Top 50,000xg), which easily can be removed with a small amount of sucrose before the rest of the pellet is resuspended. 4: Supernatant from 3 for 1 hr. at 105,000xg. The pellet is designated "light microsomes" (LM) and the supernatant SN

Relaxing activity was measured by following the turbidity change of an actomyosin suspension at 620 mμ. Uptake of calcium was measured as is described in connection with Table II. ATP-ase was measured at 30°C and the orthophosphate was analyzed according to Rockstein and Herron (1951). Cytochrome c oxidase was measured by following the oxidation of reduced cytochrome c at 550 mμ (Smith, 1955).

Results. The heavy microsomes, the light microsomes and the supernatant have been tested for relaxing, ATP-ase and cytochrome c oxidase activities. The heavy and the light microsomes have also been tested for their Ca^{++}-binding capacity. The "mitochondria" and the top layer of the 50,000xg pellet have been tested for cytochrome c oxidase and ATP-ase activities. The distribution of activities is shown in Table I.

Table I.

Activity	"Mitochondria"	HM	Top 50,000xg	LM	SN
Relaxing activity	(+)	+	not tested	+	+
Ca^{++}-pump	not tested	+	not tested	-	not tested
ATP-ase	+	+	+	+	-
Cytochrome c oxidase	+	-	+	-	-

Relaxing activity has been found in both microsome fractions and in the supernatant. Under our conditions the strongest relaxing activity per unit protein is usually found in the LM fraction. Washing of the particles decreases the activity. Preincubation in the presence of ATP increases the activity and the washed particles will regain part of their relaxing activity after 5-10 min. preincubation with ATP. Heating of the supernatant up to 3 min. will not abolish its relaxing activity. Fig. 1.

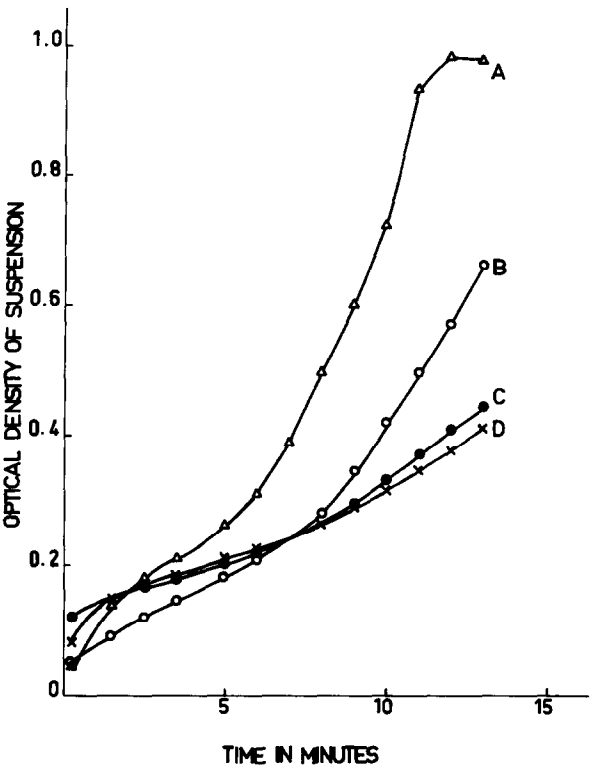

Fig. 1. Contraction of actomyosin suspensions measured as increase in optical density at 620 mμ over the original value of about 0.2. 3 ml of reaction mixture contained 2.3 mg of actomyosin, 0.66 M Tris-acetate buffer pH 7.5, 1.5 mM ATP, 2 mM $MgCl_2$ and where added 0.13 mg of microsomal protein or 0.05 ml (0.33 mg protein) of supernatant. The KCl--concentration was about 0.054 M. Temperature 27°C.

Curve A: without additions, this is the control. Curve B: inhibition of contraction in the presence of the HM fraction, curve C: inhibition of contraction in the presence of LM fraction, curve D: inhibition of contraction in the presence of SN.

Ca^{++}-concentrating activity has been found in the HM fraction, where it is very strong, as compared with the weak activity in the LM fraction (Table II).

Each vessel contained in 2 ml reaction mixture: 0.1 M KCl, 0.025 M Tris-HCl pH 7.5, 4 mM $MgCl_2$, 4 mM K-oxalate, 0.08 mM $^{45}CaCl_2$ and where added, 5 mM ATP. About 1 mg microsomal protein was present in each tube. After incubation the vessels were rapidly cooled and the mixture was centrifuged in the cold. After the centrifugation the supernatant was collected, diluted and counted.

Table II.

ATP-dependent Ca^{++}-uptake in the heavy microsomes

Fraction and Addition	Incubation time (min.)	Residual counts in supernatant (c.p.m.)
HM - ATP	7	11.570
HM + ATP	2	1.290
" "	7	2.420
LM - ATP	7	11.010
LM + ATP	2	9.520
" "	7	10.540

Hasselbach and Makinose (1961) demonstrated that ATP is necessary for the Ca^{++} concentrating activity. As is seen from the table the control values in the absence of ATP are very similar, the small difference between them reflecting the experimental error. In the presence of ATP, the increase of counts with incubation time is assumed to be due to the release of some of the bound Ca^{++}.

ATP-ase activity is obtained in all the particulate fractions from the muscle homogenate in the presence of 5 mM Mg^{++}. Ca^{++} can be substituted for Mg^{++} and gives some quite consistent changes in activity, but those will not be dealt with here. Hasselbach and Makinose (1961) have shown that granules from rabbit muscle can for a short period of time increase their Mg^{++}-stimulated ATP-ase activity upon addition of small amounts of Ca^{++} and that the energy liberated by this extra splitting is about equivalent to the energy necessary to bind the added amount of Ca^{++}. In the HM fraction but not in the LM fraction we have been able to find this increased ATP-ase upon addition of 80 μM Ca^{++}. The difference between the microsomal fractions is here the same as with respect to the Ca^{++}-concentrating activity.

Cytochrome c oxidase activity has been found in the "mitochondria" and also in the top layer of the 50.000xg pellet. Both the heavy and the light microsome fractions showed no or extremely low cytochrome c oxidase activity which is an indication that these fractions contain little or no mitochondria or mitochondrial fragments. Preliminary results from an

electron microscopic investigation of the different fractions indicate that the top layer of the 50,000xg pellet contains an appreciable amount of very small, "intact" mitochondria. This investigation was done in collaboration with Dr. E. Andersson-Cedergren.

Discussion. Since the discovery by Marsh in 1952 that a relaxing factor system is present in extracts from skeletal muscle this activity has been associated with a granular fraction. Ebashi (1960,1961) and Hasselbach and Makinose (1961) have found that the same granular extracts that can cause relaxation have the ability to concentrate Ca^{++} ions from the surrounding medium more than 1,000-fold. This ability has been correlated with the relaxing activity of the granules (Ebashi, 1960).

The SN fraction will even in very low concentration (1-2 volume per cent) cause relaxation in our system, as was shown in Fig. 1. Much higher concentrations of supernatant have recently been reported to be needed by Fuchs and Briggs (1963) for relaxation in a different system. Their report and a recent publication by Mommaerts et al. (1963) give added evidence for the occurrence of a soluble relaxing substance.

In summary, our results would indicate that there is a soluble factor with a relaxing action on actomyosin suspensions present in skeletal muscle and that a relaxing action can exist independently from the Ca^{++}-concentrating activity of the muscle microsomes. Although our results indicate that relaxing activity can exist in a fraction which lacks Ca^{++}-concentrating activity, it may well be that both the Ca^{++}-pump and the activity of the relaxing factor(s) are necessary for obtaining complete relaxation under physiological conditions.

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