INHIBITION OF MYOFIBRILLAR ADENOSINETRIPHOSPHATASE AND SYNERESIS BY 1,3-DIPHOSPHOGLYCERIC ACID[1]

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The identity of the relaxing substance (RS)[3] of muscle remains obscure in spite of intensive investigation. Although many compounds known to be metabolic intermediates have been screened (Gergely, 1959; Marsh, 1960), none except pyridoxal phosphate appears to have RS activity as judged by the criteria of inhibition of myofibrillar ATPase and inhibition of the contractile response of myofibrils to ATP. However, it has not been shown that pyridoxal phosphate could be formed by systems known to form RS. Bendall (1960) failed to confirm Marsh's (1960) report that α-glycerophosphate was the RS. By incubating the granular component of the rabbit muscle relaxing factor system with ATP and Mg^{++} and removing the granules by centrifugation, Parker and Gergely (1960) prepared a protein-free solution containing RS. A dialyzable cofactor was sometimes required for RS formation. The identity of RS was not established although some of the properties were described.

Experiments performed in this laboratory suggest that 1,3-diPGA may be the RS. This paper constitutes a preliminary report of our study and a tentative interpretation of the findings in the light of other recent work.

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[3] Abbreviations: RS, relaxing substance; ATP, adenosinetriphosphate; 1,3-diPGA, 1,3-diphosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; PGKase, phosphoglyceric kinase; P, inorganic orthophosphate.

EXPERIMENTAL

Rat and rabbit myofibrils were prepared by the method of Perry and Grey (1956). The reaction system for myofibrillar ATPase assay contained 0.02 M Tris(hydroxymethyl)-aminomethane, 0.152 M K^+, 0.035 M Na^+, 0.003 M Mg^{++}, 0.005 M ATP and 8.5 mg myofibrillar protein. The pH was 7.0 and the final volume 2 ml. The K, Na and Mg were added as the chlorides. The medium was designed to approximate the intracellular cationic composition insofar as this is known. Following a 15 minute incubation period at 37° the reaction was stopped by adding 0.2 ml of 10 N H_2SO_4. Unreacted ATP and other nucleotides were removed by adding 5 ml of a 40 mg/ml suspension of acid-washed norite and the total volume made up to 10 ml. After 10 minutes contact with the norite with occasional shaking, the samples were filtered and inorganic phosphate was determined in aliquots of the filtrates by the method of Fiske and SubbaRow (1925). Appropriate controls and standards were run with each assay. The 1,3-diPGA used in these studies was prepared by a modification of the method of Krimsky (1959) or was generated *in situ* in the ATPase reaction mixture by adding 3-PGA to a final concentration of 0.011 M and 40 µg of crystalline yeast PGKase (Sigma Chemical Co., St. Louis, Mo.). The hydroxamic acid method of Lipmann and Tuttle (1945) was employed to estimate 1,3-diPGA using Krimsky's (1959) value for the extinction coefficient. Syneresis was measured by the time required for visible clumping to occur following the addition of 0.0025 M ATP (final concentration) to a myofibrillar suspension (21.5 mg myofibrillar protein in a final volume of 5 ml) containing 0.05 M K succinate, pH 7.0, and 0.01 M $MgCl_2$. Protein was determined by the method of Lowry et al (1951).

RESULTS AND DISCUSSION

Adenosinetriphosphatase activity of rabbit myofibrils was markedly inhibited by 1,3-diPGA formed *in situ* enzymatically or added in low concentrations, as shown in Table I. In the experiment in which 1,3-diPGA was generated enzymatically the inhibition by 3-PGA alone was found to be due to the presence of PGKase in the myofibrils even

TABLE I

INHIBITION OF MYOFIBRILLAR ADENOSINETRIPHOSPHATASE

BY 1,3-DIPHOSPHOGLYCERIC ACID

Experiment	Added to ATPase Reaction system*	ATPase activity (μmoles P formed)
1	Nothing	4.75
	PGKase	4.70
	3-PGA	3.59
	PGKase + 3-PGA	3.04
2	Nothing	3.26
	0.11 μmole 1,3-diPGA	1.37
3	Nothing	3.37
	0.08 μmole 1,3-diPGA	3.10
	0.2 μmole 1,3-diPGA	2.22

*The composition of the reaction system, additions, and other conditions of the assay are described in the experimental section.

though the myofibrils had been washed 5 times. Following incubation of 3-PGA and ATP with a myofibrillar suspension, 1,3-diPGA was detected in the reaction mixture.

In an experiment with rat myofibrils, syneresis occurred in 5-6 minutes in the control tube while in the presence of 0.04 μmoles/ml 1,3-diPGA syneresis required 12-13 minutes. This effect of 1,3-diPGA resembles the RS inhibition of syneresis of rabbit myofibrils (Parker and Gergely, 1960). Difficulty in obtaining and storing 1,3-diPGA has limited our studies of syneresis thus far to rat myofibrils.

The stability of the RS of Parker and Gergely (1960) to heat and acid would seem to rule out identity with 1,3-diPGA, which is an extremely labile compound (Negelein, 1955). We cannot at present reconcile this with our hypothesis that 1,3-diPGA is RS. It should be pointed out, however, that other substances present in Parker and Gergely's impure preparation might influence the stability of RS. It is noteworthy that RS was extremely heat-labile in the presence of the granular component of the relaxing factor system.

Marsh (1960) claimed identity of RS with α-glycerophosphate but Bendall (1960) and Parker (1961) could not confirm this report. Their studies bear examination in the light of the findings reported in this paper. Marsh used washed muscle fibers while Bendall tested the ability of α-glycerophosphate to act as RS on myofibrils. Marsh's system may have possessed the potential for the accumulation of 1,3-diPGA from α-glycerophosphate under the proper conditions. Inorganic phosphate would be required, which is consistent with the requirement for inorganic phosphate for the demonstration of the RS activity of α-glycerophosphate. Assuming that Marsh was dealing with 1,3-diPGA formed by a multienzyme system, the failure of Bendall and Parker, using myofibrils, to confirm Marsh's finding is not surprising.

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