Preliminary Notes

PN 1193

Chemical identification of the soluble muscle-relaxing factor: An enzymic probe

ENGLEHARDT[1] postulated the necessity of an actomyosin–ATP inhibitor in order to have a control mechanism for this reaction. Evidence for such an inhibitor (termed relaxing factor) in homogenates of muscle was provided by MARSH[2]. The relaxing factor was then identified with the particulate fraction of the muscle homogenate[3–5]. LORAND[6] and WEBER[7] pointed out, however, that it was unlikely that a particulate fraction could directly influence the types of actomyosin systems under investigation and that an indirect action had to be considered. Evidence that the indirect action involves the formation of a soluble relaxing factor has been presented by BRIGGS AND FUCHS[8], PARKER AND GERGELEY[9] and NAGAI *et al.*[10]. The structure of the soluble relaxing factor has, however, remained elusive. An enzymic probe of its structure was undertaken in the hope that it would reveal either the identity or the chemical class of the relaxing factor.

Soluble relaxing factor was obtained directly from muscle by the method of FUCHS AND BRIGGS[11]. Its relaxing activity was assayed with single glycerinated rabbit psoas fibers as described by BRIGGS AND FUCHS[8] except that the fibers were treated with 2 mM deoxycholate[12] for 5 min to destroy any contaminating intrinsic relaxing factor. The same solution (30 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 10 mM imidazole, 10 mM potassium phosphate, pH 6.7) was used for the homogenization of the muscle as well as the assay with single fibers except that ATP was added (5 mM). Phospholipase C (EC 3.1.4.3) and D (EC 3.1.4.4) were treated with EDTA and dialyzed before being added to the soluble relaxing-factor preparations.

TABLE I

INFLUENCE OF ENZYMES ON THE RELAXING ACTIVITY OF THE SOLUBLE FRACTION OF A MUSCLE HOMOGENATE

Enzyme	*Concentration*	*Duration*	*Effect on relaxing activity*
Phosphatase* (bacterial)	1500 units*/ml	18 h	none
Phosphodiesterase* (venom)	0.05 mg/ml	18 h	destroyed§
Phosphodiesterase* (spleen)	0.1 μg/ml	2 h	none
Ribonuclease* (pancreas) 5 × crystallized	1 mg/ml	18 h	none
Ribonuclease* + bacterial phosphatase*	1 mg/ml 1500 units*/ml	18 h	none
Phospholipase D** (cabbage)	0.1 mg/ml	10 min	destroyed
Phospholipase C*** (*Clostridium perfringens*)	0.1 mg/ml	10 min	destroyed

* Worthington Biochemical Corporation.
** California Corporation for Biochemical Research.
*** General Biochemicals.
§ Relaxing activity was destroyed in 3 of 6 experiments.

The first enzyme to show any demonstrable activity against relaxing factor was a phosphodiesterase (EC 3.1.4.1) obtained from venom (Table I). The enzyme, however, gave unpredictable results, destroying relaxing factor in only 3 of 6 experiments. Attempts to duplicate the effect of venom phosphodiesterase by a combination of bacterial phosphatase (active on both 3'- and 5'-phosphates) and spleen ribonuclease (active on polynucleotides with a terminal 5'-phosphate) were completely unsuccessful as was spleen phosphodiesterase. This led to an alternative interpretation of the results with venom phosphodiesterase; *i.e.* that the preparation was contaminated by some other venom enzyme. Since phospholipase A (EC 3.1.1.4) was a possible contaminant, samples of phospholipase D and C were tested. At enzyme concentrations of 0.1 mg/ml these phospholipases destroyed the relaxing factor.

Before the enzymic destruction of relaxing factor could be unequivocally accepted it was essential to establish that the observed inactivation was not due to the introduction of calcium with the enzyme. That this was not the case can be derived from the data in Table II. In contrast to the instant reversal of relaxing activity observed when calcium is introduced, phospholipase D at a concentration 0.5 mg/ml required approx. 30 min to initiate any observable reversal of relaxing activity. The non-linearity of this reversal is easily explained by the non-linearity of the effect of relaxing factor on tension generation[11]. In accordance with expectation, when 1 mg/ml phospholipase D (Table II), was used it produced a more prompt reversal. In contrast to the effect of calcium, there was a brief period before reversal of relaxing activity. As noted earlier by EBASHI[13], exposure of the fiber to phospholipase C or D (1 mg/ml) had no effect on its ability to contract or respond to the soluble relaxing factor.

TABLE II

INFLUENCE OF INCUBATION TIME AND ENZYME CONCENTRATION ON RELAXING ACTIVITY

Indicates no reversal of relaxing activity; + to ++++ indicates slight to complete reversal.

Incubation time (min)	*1*	*15*	*30*	*45*	*60*
Control	—	—	—	—	—
Phospholipase D (1 mg/ml)	—	++++	++++	++++	++++
Phospholipase D (0.5 mg/ml)	—	—	+	++	++++
Phospholipase D* (0.5 mg/ml)	+	++++	++++	++++	++++

* The relaxing-factor preparation used in this study was obtained by the technique of gel filtration — see text.

The susceptibility of both the granule[14] and soluble factor to destruction by phospholipase raised the question of whether the soluble relaxing activity is actually due to some particulates, which because of a density close to that of the suspending medium, might not have sedimented. This possibility has been eliminated by showing (Table II) that phospholipase destroys relaxing factor separated from the protein portion of the supernatant by the technique of gel filtration[14]. Both Sephadex G-25 and G-50 have successfully been used for this purpose.

Though the relaxing activity attributed to α-glycerol phosphate[15] and 1,3-diphosphoglyceric acid[16] has not been confirmed[17,18] it may be that these substances produced some relaxation by an indirect action, for example as substrate in the for-

mation of a phospholipid. The observations may support the hypothesis made here that the soluble relaxing factor isolated directly from muscle is a phospholipid.

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1 V. A. ENGLEHARDT, *Advan. Enzymol.*, 6 (1946) 147.
2 B. B. MARSH, *Biochim. Biophys. Acta*, 9 (1952) 247.
3 S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
4 H. PORTZEHL, *Biochim. Biophys. Acta*, 26 (1957) 373.
5 F. N. BRIGGS, G. KALDOR AND J. GERGELEY, *Biochim. Biophys. Acta*, 34 (1959) 211.
6 L. LORAND, J. MOLNAR AND C. MOOS, in *Conference on the Chemistry of Muscular Contraction*, Igaku Shoin, Tokyo, 1957, p. 85.
7 H. WEBER, *Ann. N.Y. Acad. Sci.*, 81 (1959) 409.
8 F. N. BRIGGS AND F. FUCHS, *Biochim. Biophys. Acta*, 42 (1960) 519.
9 C. J. PARKER AND J. GERGELEY, *J. Biol. Chem.*, 235 (1960) 3449.
10 T. NAGAI, K. UCHIDA AND M. YASUDA, *Biochim. Biophys. Acta*, 56 (1962) 205.
11 F. FUCHS AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 51 (1961) 423.
12 F. N. BRIGGS, R. F. KING AND T. L. MCKELLEB, *Federation Proc.*, 21 (1962) 318.
13 S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
14 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1957.
15 B. B. MARSH, *Biochem. Biophys. Res. Commun.*, 3 (1960) 233.
16 H. A. ELLS AND P. FAULKNER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 255.
17 C. J. PARKER, *Biochem. Biophys. Res. Commun.*, 4 (1961) 309.
18 L. LORAND AND J. MOLNAR, in K. RODAHL AND S. M. HORVATH, *Muscle as a Tissue*, McGraw-Hill, London, 1962, p. 97.

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Uridine diphosphate fructose and uridine diphosphate acetylgalactosamine from dahlia tubers

During an investigation of the nucleotides of Dahlia tubers, UDP-glucose and UDP-galactose have been isolated. Evidence is presented here which indicates that these nucleotides are accompanied by an hitherto undescribed compound which appears to be UDP-fructose. Furthermore, UDP-acetylgalactosamine has also been isolated accompanying UDP-acetylglucosamine. Whereas UDP-acetylgalactosamine has been found in bacteria[1] and animal tissues[2,3], it has not been detected in higher plants.

An alcoholic extract of Dahlia tubers was subjected to a preliminary purification by passing it through a column of Dowex-1 X4 in the acetate form, elution being effected by a solution 0.2 *M* NaCl–0.003 *M* HCl. The fraction containing the uridine diphosphate sugars was recovered by adsorption on charcoal, followed by elution with aq. 50% ethanol[4]. Further purification was effected by paper chromatography in ethanol–ammonium acetate, pH 3.8 (ref. 5), followed by rechromatography in ethanol–ammonium acetate, pH 7.5 (ref. 5), or by chromatography on Dowex-1 X4 resin in the chloride form using a gradient elution with NaCl (ref. 6). In this form, two fractions containing mainly UDP-glucose and UDP-acetylglucosamine, respectively, were obtained.