PHOSPHORYLATION OF SKELETAL MUSCLE MICROSOMES BY ACETYLPHOSPHATE

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The phosphoryl group of acetylphosphate is transferred to the membranal protein of the sarcoplasmic vesicles during active calcium transport. Although the phosphoprotein formed cannot be distinguished from that obtained in the presence of ATP, the conditions for ATP and acetylphosphate hydrolysis are different from each other.

1. Introduction

The phosphorylation of the membranal protein of the sarcoplasmic vesicles by ATP or other NTP* has been found to be closely connected with the activity of the sarcoplasmic calcium transport system [1–3]. The phosphoryl group transferred to the outer surface of the membrane is split off when the calcium is liberated inside the vesicles after the translocation across the membrane.

When the membranal protein is fixed in the phosphorylated state, the transferred phosphate exists, presumably, as an acylphosphate in the protein. The true nature of the phosphorylated compound which participates directly in the active calcium translocation, however, remains to be ascertained.

The recent studies that acetylphosphate (AcP) is hydrolyzed by kidney and brain microsomes [4, 5] as well as by sarcoplasmic vesicles [6] may be taken as an argument for the assumption that an acylphosphate in fact is involved in ion translocation. The sarcoplasmic membranes are the only structure, with which one could demonstrate not only the hydrolysis of AcP but also active ion transport coupled with the AcP consumption. This communication describes experiments which were designed to get some information concerning the mode of usage of the two different families of energy donator during calcium translocation by the sarcoplasmic membrane.

* *Abbreviations:*
NTP : nucleoside triphosphate
EGTA: ethyleneglycol-bis-(β-aminoethyl)-*N,N'*-tetraacetate
NEM : *N*-ethyl-maleinimide

2. Methods

The vesicles of the sarcoplasmic membrane were prepared according to Hasselbach et al. [1] with slight modifications. (^{32}P)-AcP was synthesized enzymatically as described by Israel and Titus [5]. Phosphoprotein formation and AcPase activity were determined under the same conditions in media containing 20 mM histidine buffer (pH 7.0) 2 mM acetylphosphate, 5 mM magnesium and 0.4 mg vesicular protein per ml assay. 0.2 to 0.5 mM EGTA were used in order to adjust the concentrations of calcium ion in assay. All experiments were carried out at 37°. AcPase activity was determined by more than three successive measurements of unhydrolysed AcP by the method of Lipmann and Tuttle [7]. The phosphoprotein formation was measured according to Makinose [3].

3. Results and discussion

As observed for ATP, the phosphoryl group of AcP can be transferred only if both magnesium and calcium ions are present in the medium (table, fig. 1). The magnesium ions can be replaced by manganese ions. Furthermore, phosphoryl transfer

Table
Acetylphosphatase activity and phosphoprotein formation.

$MgCl_2$ (M)	Ca^{2+} (M)	Modifications	E–P (μmoles/10^6 g prot)	AcPase activity (μmoles/mg prot/min)
0	0	–	∿ 1.0	0.03
$5X10^{-3}$	0	–	∿ 1.0	0.15
0	$3X10^{-6}$	–	∿ 1.0	0.03
		–	8.0 ∿ 12.0	0.35
		ADP $2X10^{-3}$ M	0.7	0.03
		Prenylamine $2X10^{-4}$ M	4.7	0.2
$5X10^{-3}$	$3X10^{-6}$	NEM*	0.5	0.0
		Phospholipase A**	0.5 ∿ 0.7	0.52
		Ditto, washed with albumin	–	0.03

* The vesicles were incubated with NEM (0.3 μmoles/mg prot.) for 15 min at pH 8.5 before use.
** The vesicles were treated with one hundredth amount of phospholipase A for three hr at pH 7.2 before use.

from both substrates is affected if the membranal free SH groups are blocked by NEM or if the membranal lipids are removed after digestion with phospholipase A. Under optimal conditions the steady state level of phosphoprotein is reached with both substrates – ATP or AcP – in less than three sec (fig. 1). The phosphoprotein complex isolated after acid denaturation displayes the properties of an acylphosphate – i.e. stability at low pH and fast decomposition with hydroxylamine at pH 5.5 – irrespectively of the phosphate donator.

Fig. 2 illustrates that the amount of the phosphoprotein formed with AcP as phosphate donator depends in nearly the same manner on the concentration of ionized calcium in the medium as observed in the ATP system. It should be noticed that the maximal amount of the phosphoprotein formed in the AcP system is 2 or 3 times higher than in the ATP system. The most likely explanation for the apparently low phosphorylation efficiency of ATP on the one hand and for the high efficiency of AcP on the other hand arises from observations of the effect of ADP. ADP, which accumulated inevitably more or less in the ATP system as a splitting product, is a potent phosphate

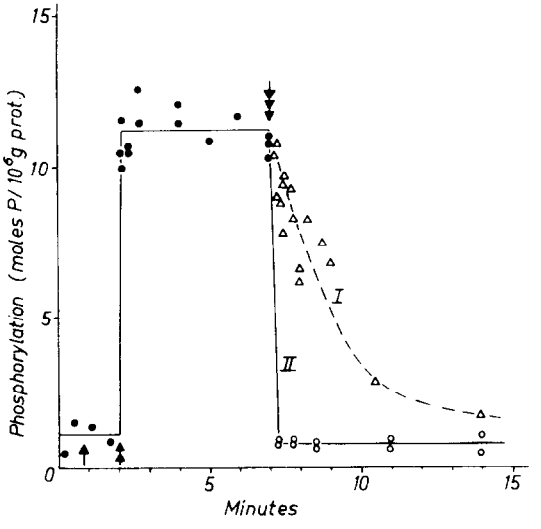

Fig. 1. Formation and decomposition of the phosphoprotein in the AcP assay. *Initial assay*: 20 mM histdine (pH 7.0), 0.2 mM EGTA, 0.2 mM EDTA, 60 mM KCl, 2 mM AcP and 0.4 mM membranal protein per ml assay. Additions: single arrow 5 mM $MgCl_2$, double arrow 0.4 mM $CaCl_2$ and triple arrow 4 mM EGTA (*curve* I) or 2 mM ADP (*curve* II). Each addition was followed by a phosphoprotein determination within 3 sec.

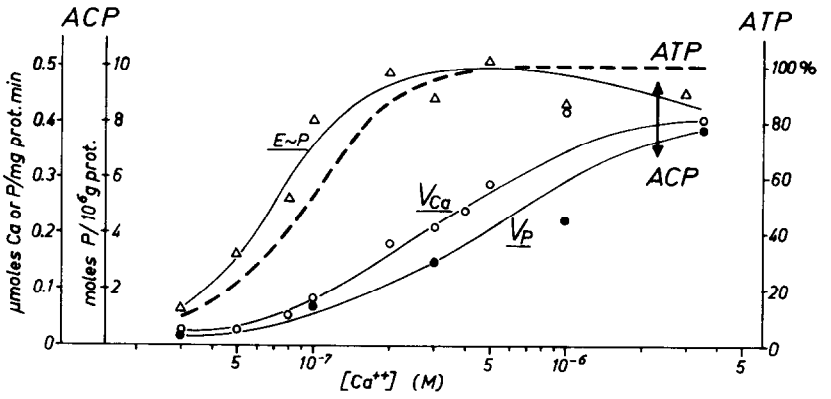

Fig. 2. Activation of the AcP driven calcium pump by calcium ions. V_p and V_{Ca}: the rate of extra-AcP consumption and calcium uptake respectively, extreme left ordinate. E ~P: phosphoprotein formation, left ordinate. The composition of the assays is as described in Methods. The assay for V_{Ca} measurement contains 4 mM oxalate additionally. - - - -, average relative activity of extra-ATPase, ATP driven calcium transport and phosphoprotein in the ATP assay [1–3], right ordinate.

acceptor, while acetate is quite ineffective. Even when concentrations of ADP are as low as they exist in the presence of ATP regenerating system, the phosphorylation level could be suppressed certainly. In agreement with this explanation, ITP is a phosphate donator which is more efficient than ATP [8], because IDP is also a much weaker phosphate acceptor than ADP.

Likewise, ADP suppresses the phosphoprotein formation in AcP system very severely (table). Fig. 1 shows that the addition of ADP causes a sudden and complete disappearance of the phosphoprotein formed in the AcP containing medium, while 30 to 40% phosphate still remain attached to protein in the ATP system [3]. This suppression of phospoprotein formation in the AcP system by ADP, however, is not due to the action of ADP as the potent phosphate acceptor. The table presented shows that ADP inhibits simultaneously the rate of AcP decomposition. A phosphate acceptor could lower the level of the phosphoprotein formation and, consequently, inhibit the rate of inorganic phosphate liberation but not the rate of the decomposition of AcP in the assay. After addition of ADP, AcP should be decomposed further on with an unchanged (or even enhanced) rate, if ADP would accept the energy rich phosphate transferred from AcP to the protein.

The activity of the extra-AcPase, i.e. increment of the AcP splitting rate induced by the addition of calcium to the magnesium containing system, does not exceed a value of 0.2 μmoles/mg prot/min – less than one fifth of the maximal extra-ATPase activity (table). Fig. 2 illustrates that much higher calcium ion concentrations are required to activate maximally the extra-AcPase and calcium transport in the AcP system than that needed to activate extra-ATPase and ATP driven calcium transport. When the calcium ion concentration is reduced suddenly by the addition of EGTA, the phosphoprotein formed from AcP decays with a half life time of about 120 sec (fig. 1), while in the ATP system the decay is over in a few seconds. As compared with ATP hydrolysis, the rate determining reaction steps for the AcP hydrolysis required higher calcium concentrations and is characterized by a lower maximal turnover rate.

In summary: The observations described suggest that the vesicles hydrolyse ATP and AcP by different enzymatic processes.

The following observation supports this assumption. In contrast to the extra-ATPase activity, which is severely inhibited by prenylamine [9, 10], the AcPase activity is only 40% inhibited by the drug. On the other hand, the phosphoprotein formation, using ATP as substrate, is influenced only very little by prenylamine. Using AcP as substrate, prenylamine lowers the formation of the phosphorylated intermediate to 40%.

Because prenylamine exerts its effect via the unsaturated fatty acids such as oleic acid or arachidonic acid in the membranal lipids [11], this result indicates that the calcium dependent ATPase activity is charac-

terized by another type of protein lipid interaction than calcium dependent AcPase activity. This conclusion is further supported by the change of the activity pattern produced by lipid hydrolysis. For the ATP system, it has been found that, after treatment with phospholipase A, the calcium storing capacity of the vesicles is destroyed, while neither the calcium magnesium activated ATPase activity nor the phosphoryl transfer reaction is affected. The AcP driven calcium accumulation is likewise destroyed by lipase digestion. However, the AcPase activity is enhanced by this treatment while, at the same time, the level of phosphoprotein formed is strongly reduced (table). A similar activity pattern has been observed for the interaction of ATP with sarcoplasmic membranes after lipid depletion and reconstitution by lysolecithine [9].

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