THE ARSENATE INDUCED CALCIUM RELEASE FROM SARCOPLASMIC VESICLES

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1. Introduction

The efflux of calcium from calcium loaded sarcoplasmic vesicles into solutions containing low free calcium concentrations gives rise to the formation of a phosphorylated intermediate in the membranes, the phosphoryl group of which can be transferred readily to ADP [1, 3]. Hence, under steady state conditions when the slow calcium efflux is balanced by an ATP driven calcium influx a slow exchange between inorganic phosphate and the γ-P of ATP takes place [2]. When the calcium influx ceases because the energy donators are exhausted or removed, the rate of net calcium efflux i.e. the rate of calcium release remains low as long as there is no ADP present to serve as phosphate acceptor. The transfer of the phosphoryl group to ADP, however, leads to a tremendous acceleration of calcium release which is stoichiometrically related to a net formation of ATP [3]. The calcium efflux dependent ATP formation is neither uncoupled by dinitrophenol nor by azide. On the other hand, all treatments which increase the calcium permeability of the sarcoplasmic membranes by leak formation abolish the osmochemical energy transduction irreversibly. This paper reports the reversible uncoupling of the calcium gradient dependent phosphoryl transfer by arsenate, the classical uncoupler of substrate linked and oxidative phosphorylation.

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Abbreviation:

EGTA: Ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid.

2. Methods

Sarcoplasmic vesicles prepared and purified according to Hasselbach and Makinose [4] were loaded with ^{45}calcium oxalate or ^{45}calcium phosphate with ATP or acetyl phosphate as energy donator as described by Hasselbach and Makinose [3]. The release of calcium was started after the vesicles had used up the energy donators by the addition of a small volume of the solution containing the substance whose releasing activity should be tested. Details are given in the legends. When the calcium release should be studied in media of low phosphate or oxalate content the uptake media were diluted 10–30 fold.

Calcium release was followed by millipore filtration of the vesicle suspension at appropriate time intervals. The ^{45}calcium radioactivity in the millipore filtrate was measured by liquid scintillation counting. The phosphate of ATP formed from inorganic ^{32}phosphate and ADP during calcium release was transferred to glucose (0.1 M). Hexokinase (EC 2.7.1.1) (0.01–0.02 mg/ml) was introduced together with the releasing solutions. The reaction was terminated by adding 2 ml 6% trichloracetic acid. After the removal of excess ^{32}phosphate according to Avron [5] the radioactivity of glucose-6-phosphate and ATP were measured by liquid scintillation counting. Hexokinase (EC 2.7.1.1) and pyruvate kinase (EC 2.7.1.40) was obtained from Boehringer, Mannheim/Germany.

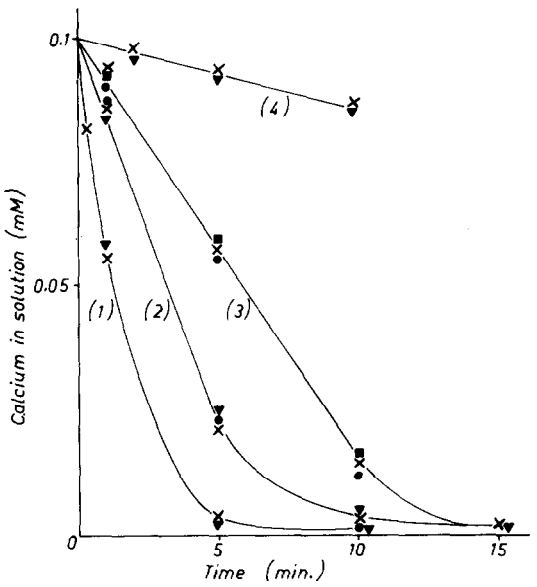

Fig. 1. The depletion of different solutions from calcium by sarcoplasmic vesicles in the presence and in the absence of arsenate. Ordinate: calcium concentration in the solution (mM), abscissa: time (min). Composition of the calcium uptake media: all assays contained: 0.1 mg ves. prot./ml, 20 mM histidine, 40 mM KCl, 5 mM $MgCl_2$, pH 7.0, 0.1 mM $CaCl_2$.
Assay (1): 2 mM K-Li-acetyl phosphate, 3 mM K-oxalate;
Assay (2): 2 mM K-Li-acetyl phosphate, 20 mM Na-phosphate;
Assay (3): 2 mM K-Li-acetyl phosphate, 5 mM Na-phosphate;
Assay (4): 0.01 mM ATP, 3 mM K-phosphoenolpyruvate, 0.01 mg/ml pyruvate kinase, 10 mM Na-phosphate;
Arsenate (mM): (X–X–X): none; (■–■–■): 0.1; (●–●–●): 1.0; (▲–▲–▲): 5.

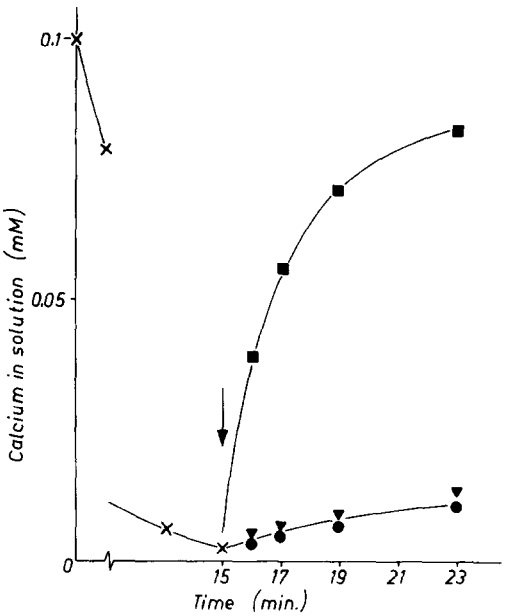

Fig. 2. Arsenate induced calcium release and its abolition by magnesium removal. Ordinate: calcium concentration in the solution (mM), abscissa: time (min). Sarcoplasmic vesicles (0.1 mg prot./ml) were loaded with calcium phosphate for 15 min [3]. The uptake media of 40 ml contained: 2 mM K-Li-acetyl phosphate, 5 mM $MgCl_2$, 20 mM histidine, 40 mM KCl, 20 mM Na-phosphate, pH 7.0, 0.1 mM $CaCl_2$. Calcium uptake was followed by taking 5 samples of 2 ml at appropriate time intervals. The release was started by the addition of 1.5–2.5 ml of the respective test solution to 30 ml uptake medium. The concentrations of the different reagents in the test solution were chosen in such a way that they were present in the following final concentrations in these assays: (●–●–●): 2 mM EGTA; (■–■–■): 2 mM EGTA, 5 mM Na-arsenate; (▼–▼–▼): 2 mM EGTA, 10 mM EDTA, 5 mM Na-arsenate. The pH of the added solutions were adjusted so that pH of the assay remained unchanged.

3. Results

As illustrated in fig. 1 arsenate does not affect the uptake of calcium by the sarcoplasmic vesicles. This ineffectiveness of arsenate depends neither on the kind nor on the concentration of the energy donator, nor on the calcium precipitating anion present in the solution, oxalate or phosphate. Likewise, the total calcium concentration found in the solution after the cessation of net calcium uptake is not affected significantly by arsenate. However, on addition of 0.1–0.3 mM EGTA to the suspension after net calcium uptake has stopped, more calcium is released in the presence of arsenate than in its absence. The enhanced calcium release results in a higher free calcium concentration in the newly attained steady state indicating a reduction of the concentrating ability of the vesicles produced by arsenate. While with phosphate as calcium precipitating anion and ATP as energy donator the free calcium concentration rises only by 30–60% from 0.03 μM to ~ 0.05 μM, it approaches 0.2 μM when the energy is delivered by acetyl phosphate.

In line with these observations, arsenate stimulates

Table 1

The inhibition of the arsenate induced calcium release by ADP.

ADP (mM)	Relative release rate ($V_{rel.}$)
0	100
0.3	100, 100
1.0	35, 51
2.0	27, 31

A procedure was used to estimate the influence of ADP which allowed the removal of calcium precipitating anions from the solution because phosphate as well as oxalate interfere with ADP inhibition. The sarcoplasmic vesicles were loaded with calcium oxalate for 10 min in solutions containing 2 mM ATP, 5 mM $MgCl_2$, 5 mM K-oxalate, 40 mM KCl, 20 mM histidine, pH 7.0, 0.1 mM $^{45}CaCl_2$, 0.2 mg prot./ml. 2 ml of the suspension were sucked through a millipore filter and subsequently the vesicles caught in the filter were rinsed successively with 5 ml of the following solutions which all contained 5 mM $MgCl_2$, 20 mM histidine, pH 7.0: a) 100 mM KCl (2 times); b) 5 mM EGTA, 40 mM KCl; c) 5 mM EGTA, 2 mM Na-arsenate, 40 mM KCl; and d) 5 mM EGTA, 2 mM Na-arsenate, 40 mM KCl plus 2.0 mM, 1.0 mM, 0.3 mM ADP, respectively. The filtration rate was adjusted to approximately 5 ml/min. Solutions a and b liberate $V_a = V_b = 0.05$ μmoles Ca/mg prot./min. The release rate was increased by solution c to $V_c = 0.15$ μmoles Ca/mg prot./min. In solution d the release rate V_d declines depending on its ADP content. Controls were performed showing that the results were not dependent on whether solutions c or d were applied at first. The relative release rates given in the table are defined as follows:

$$V_{rel.} = \frac{V_c - V_b}{V_d - V_b} \times 100.$$

calcium efflux from calcium loaded vesicles in the absence of energy donators as shown in fig. 2. This enhancement of the release is as marked as that produced by the combined action of ADP and phosphate [1]. While ADP is not required for the arsenate effect, magnesium ions must be present. The arsenate induced calcium release is completely reversible. Calcium release stops immediately and calcium uptake starts to re-occur if the system is supplemented with ATP.

The arsenate induced calcium release is inhibited by ADP as well as by phosphate. The inhibition produced by ADP is difficult to measure quantitatively because it shows up clearly only in the absence of phosphate or oxalate. The data given in table 1 were obtained from vesicles precipitated on millipore filters for the complete removal of the phosphate-containing uptake medium. The concentration required for half maximal inhibition is in the order of 1 mM ADP (table 1). On the other hand, the inhibition of phosphate could be analyzed in more detail. As shown by fig. 3 the inhibition of the arsenate induced calcium release by phosphate is noncompetitive. The apparent affinity constant K_m for arsenate has a value of 8 mM at 20° and 2 mM at 30°, indicating an endothermic binding reaction with an enthalpy change of 24,600 cal/mole. The inhibition constant K_i for phosphate, $K_i = 2$ mM, corresponds approximately to the concentration of phosphate giving rise to a half maximal activation of the phosphate-ADP induced calcium efflux [6]. The inhibition constant K_i does not, or only little, change with

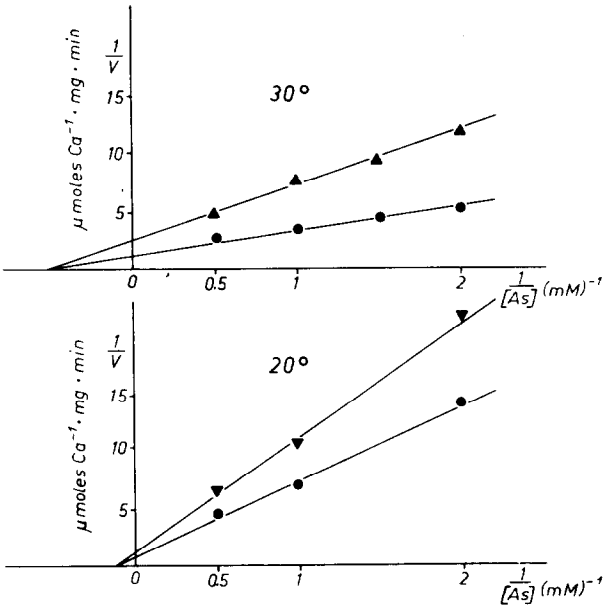

Fig. 3. Double reciprocal plot for the activation of calcium release by arsenate and its inhibition by phosphate at 20° and 30°. Ordinate: reciprocal rate of calcium release ($\frac{1}{V}$) [V] = μmoles calcium/mg prot/min, abscissa: reciprocal arsenate concentration [As] = mM. Composition of the uptake assays as described in fig. 1 except for the concentrations of phosphate which were varied: (●–●–●): 5 mM; (▼–▼–▼): 10 mM; (▲–▲–▲): 20 mM. Calcium release was started by the addition of 1.5–2.5 ml of a concentrated arsenate solution. The release rate was measured as described in Methods.

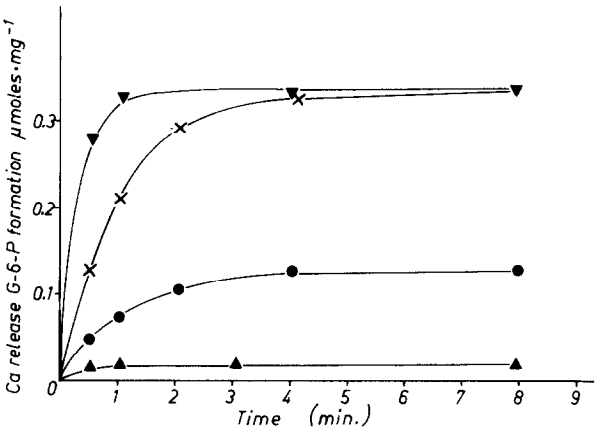

Fig. 4. Inhibition of inorganic phosphate incorporation into ADP during arsenate induced calcium release. The vesicles were loaded for 15 min with calcium oxalate in solutions containing: 0.5 mg ves. prot/ml, 0.5 mM $^{45}CaCl_2$, 1 mM K-oxalate, 1 mM Na-phosphate, 2 mM K-Li-acetyl phosphate, 7 mM $MgCl_2$, 40 mM KCl, 100 mM glucose, volume 30 ml. Under these conditions the calcium load remained low ~ 0.3–0.5 μmoles calcium/mg prot. Calcium uptake was followed by 3 determinations. Calcium release was initiated by adding 6 ml releasing solution to the remaining 24 ml of the uptake medium. The releasing solution contained: 10 mM EGTA, 1 mM Na-phosphate, 2.5 mM ADP, 100 mM glucose, 0.6 mg hexokinase, pH 7.0. The arsenate medium contained in addition 25 mM Na-arsenate. The time course of calcium release was followed by the millipore filtration technique. For the measurement of P-incorporation aliquots were taken and added to equal volumes of 6% TCA to terminate the reaction. P-incorporation was measured by the formation of glucose-6-phosphate which was determined according to Avron [5].Calcium release: (▼–▼–▼): with arsenate, (×–×–×): without arsenate. Glucose-6-phosphate formation: (▲–▲–▲): with arsenate, (●–●–●): without arsenate.

temperature. In spite of the different effects of temperature on arsenate and phosphate binding, the temperature coefficient of the arsenate and the phosphate-ADP induced calcium release prove to be identical: Q_{10} = 2.5–3.0 between 20° and 30°.

The suspected uncoupling of calcium release from phosphate incorporation into ADP by arsenate proves difficult to demonstrate. In the presence of high concentrations of phosphate and/or ADP arsenate does not give rise to an increase of calcium release because ADP and inorganic phosphate compete with arsenate. Therefore, the incorporation of inorganic phosphate

Table 2
Effect of arsenate on orthophosphate incorporation into the sarcoplasmic membranes.

Conditions	Phosphate incorporated into 10^6 g membrane protein (moles)	
	Before the addition of arsenate	After the addition of arsenate
EGTA absent	2.0	1.0
EGTA present	2.1	0.7

Inorganic phosphate was incorporated into the protein of the sarcoplasmic vesicles under the same conditions which give rise to the formation of ATP from P_i and ADP [3]. The assays contained 0.5 mg prot/ml, 2 mM acetyl phosphate, 5 mM $MgCl_2$, 40 mM KCl, 5 mM ^{32}(P) Na-phosphate and 0.2 mM $CaCl_2$, pH 7.0. When net calcium uptake ceased after 15 min at 20°, 2 mM Na-arsenate was added to the mixture. In the second experiment 1 mM EGTA was added 2 min before the addition of arsenate. Immediately before and after the arsenate addition the ^{32}P-incorporation into the membrane protein was measured [10].

into ATP is not affected. The uncoupling effect of arsenate can be detected only when calcium release and phosphate incorporation are submaximally activated by low concentrations of phosphate andADP. Under these conditions calcium release is enhanced by arsenate and the formation of ATP is completely abolished (fig. 4). At intermediate phosphate concentrations arsenate reduces phosphate incorporation partially. As the described experiments suggest, the formation of a phosphorylated intermediate in the membranal protein is suppressed during calcium release stimulated by arsenate (table 2).

4. Discussion

As to be expected from numerous observations showing that the substitution of phosphate by arsenate leads to an uncoupling of the substrate linked as well as the respiratory chain phosphorylation (cf. [7]), arsenate uncouples calcium efflux dependent phosphorylation in the sarcoplasmic membranes. This uncoupling gives rise to a consider-

able acceleration of the calcium efflux. The concentrations of arsenate which produce this effect are somewhat lower than those effecting substrate linked and oxydative phosphorylation [7]. The effect of arsenate is counteracted by phosphate in approximately the same concentration range. However, the inhibition of the arsenate induced calcium efflux by phosphate is not competitive as one should assume if arsenate and phosphate react at the same site of the calcium transport system. The differences between the interaction of phosphate and arsenate are revealed most clearly by the fact that the binding of arsenate is highly endothermic and consequently driven by a large enthropy increase. In contrast, phosphate binding is thermoneutral. The inhibition of the arsenate induced calcium release by ADP is another fact difficult to reconcile with a simple replacement of phosphate by arsenate as proposed for the mechanism of the arsenate stimulated respiration of mitochondria [7].

The different effects which arsenate exerts on the calcium transport system in the steady state, depending on the energy donator by which the transport is supported, suggest a more complex mechanism in which not only phosphate but also the energy donators and their splitting products are involved (cf. [8]). While the ATP driven calcium pump in the steady state is only little effected when arsenate is present and the free calcium concentration is reduced by EGTA, the pump supported by acetyl phosphate releases the stored calcium nearly completely. Obviously, the efflux induced by arsenate cannot be compensated by the acetyl phosphate supported pump when the calcium concentration outside is lowered by EGTA. This is because the acetyl phosphate supported pump has a very low optimal transport rate and it needs a much higher calcium concentration for activation than the ATP supported pump [9]. An explanation for the ineffectiveness of arsenate in the ATP supported system may be found in the observation described in table 2 showing that ADP counteracts the arsenate induced calcium release. Obviously ATP acts not only as a phosphate and energy donator like acetyl phosphate, but ATP as well as its splitting product ADP are at the same time powerful modifiers of the calcium pump.

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