Biochimica et Biophysica Acta, 730 (1983) 276–284
Elsevier Biomedical Press

BBA 71635

EFFECTS OF ADENOSINE DIPHOSPHATE ON Ca^{2+} FLUXES AND Ca^{2+} ACCUMULATION OF SARCOPLASMIC RETICULUM

YAT H. LAU

Departments of Pharmacology and Biochemistry, Upstate Medical Center, State University of New York, 766 Irving Avenue, Syracuse, NY 13210 (U.S.A.)

(Received December 23rd, 1982)

Key words: Ca^{2+} flux; ADP; Ca^{2+} accumulation; (Sarcoplasmic reticulum)

Ca^{2+} transport by sarcoplasmic reticulum vesicles was examined by incubating sarcoplasmic reticulum vesicles (0.15 mg/ml) at 37°C in, either normal medium that contained 0.15 M sucrose, 0.1 M KCl, 60 μM $CaCl_2$, 2.5 mM ATP and 30 mM Tes at pH 6.8, or a modified medium for elimination of ADP formed from ATP hydrolysis by including, in addition, 3.6 mM phosphocreatine and 33 U/ml of creatine phosphokinase. In normal medium, Ca^{2+} uptake of sarcoplasmic reticulum vesicles reached a plateau of about 100 nmol/mg. In modified medium, after this phase of Ca^{2+} uptake, a second phase of Ca^{2+} accumulation was initiated and reached a plateau of about 300 nmol/mg. The second phase of Ca^{2+} accumulation was accompanied by phosphate uptake and could be inhibited by ADP. Since, under these experimental conditions, there was no significant difference of the rates of ATP hydrolysis in normal medium and modified medium, extra Ca^{2+} uptake in modified medium but not in normal medium could not be explained by different phosphate accumulation in the two media. Unidirectional Ca^{2+} influx of sarcoplasmic reticulum near steady state of Ca^{2+} uptake was measured by pulse labeling with $^{45}Ca^{2+}$. The Ca^{2+} efflux rate was then determined by subtracting the net uptake from the influx rate. At the first plateau of Ca^{2+} uptake in normal medium, Ca^{2+} influx was balanced by Ca^{2+} efflux with an exchange rate of 240 nmol/mg per min. This exchange rate was maintained relatively constant at the plateau phase. In modified medium, the Ca^{2+} exchange rate at the first plateau of Ca^{2+} uptake was about half of that in normal medium. When the second phase of Ca^{2+} uptake was initiated, both the influx and efflux rates started to increase and reached a similar exchange rate as observed in normal medium. Also, during the second phase of Ca^{2+} uptake, the difference between the influx and efflux rates continued to increase until the second plateau phase was approached. In conditions where the formation of ADP and inorganic phosphate was minimized by using a low concentration of sarcoplasmic (7.5 μg/ml) and/or using acetyl phosphate instead of ATP, the second phase of Ca^{2+} uptake was also observed. These data suggest that the Ca^{2+} load attained by sarcoplasmic reticulum vesicles during active transport is modulated by ADP accumulated from ATP hydrolysis. ADP probably exerts its effect by facilitating Ca^{2+} efflux, which subsequently stimulates Ca^{2+} exchange.

Introduction

High concentrations of phosphocreatine and creatine phosphokinase are known to be present in muscle [1,2]. This enzyme-substrate system, apart from serving as energy reserve, its role for the rapid removal of intracellular ADP during muscle contraction and relaxation might be of physiological importance. The effects of ADP in stimulating Ca^{2+} exchange between the interior of isolated sarcoplasmic reticulum vesicles and the external

Abbreviation: Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino)ethanesulfonic acid.

medium have been examined previously [3–5]. In this paper, active Ca^{2+} transport of sarcoplasmic reticulum vesicles was studied in the presence and absence of phosphocreatine and creatine phosphokinase. The effect of ADP accumulated from ATP hydrolysis on net Ca^{2+} accumulation as well as Ca^{2+}-flux rates across the sarcoplasmic reticulum membrane were examined. The results are discussed in conjunction with the scheme of Ca^{2+} translocation and substrate hydrolysis of the Ca^{2+}-ATPase.

Methods

Sarcoplasmic reticulum preparation. Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared as previously described [6]. Sarcoplasmic reticulum vesicles suspended in medium of 0.3 M sucrose, 10 mM imidazole at pH 6.8, were stored at −70°C until used.

Ca^{2+}-uptake measurement. Ca^{2+} uptake was determined by Millipore filtration with $^{45}Ca^{2+}$ as tracer. Sarcoplasmic reticulum vesicles at 150 μg/ml were incubated at 37°C in, either a medium that contains 0.15 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, 60 μM $CaCl_2$, 30 mM Tes and 0.1 μCi/ml $^{45}Ca^{2+}$ at pH 6.8, or in a modified medium for removal of ADP by including in addition 3.6 mM phosphocreatine and 33 U/ml of creatine phosphokinase. Ca^{2+} uptake is initiated by adding 2.5 mM ATP. Also, in order to eliminate any contaminating adenylate kinase activity in the sarcoplasmic reticulum preparation, in some assays 80 μM P^1,P^5-di(adenosine-5′) pentaphosphate was included in the media. At various times, 0.5 ml aliquots were passed through Millipore filters and then washed with 5 ml of identical medium except for the omission of ATP and $^{45}CaCl_2$. The filters were dried and dissolved in Insta-gel (Packard) for scintillation counting.

In some experiments, low concentration of sarcoplasmic reticulum (7.5 μg/ml) was used in the assays to minimize the accumulation of ADP and P_i. Also, on some occasions, 1 mM acetyl phosphate or 1 mM ATP plus 1 mM ADP were added to initiate Ca^{2+} uptake.

Ca^{2+}-influx and -efflux measurements. Unidirectional Ca^{2+} influx of sarcoplasmic reticulum in the course of Ca^{2+} uptake was monitored as described by Katz et al. [7]. Sarcoplasmic reticulum Ca^{2+} uptake was set up as described above except nonradioactive medium was used. At various intervals, 1 ml aliquots were removed and added into a test-tube that contained tracer quantity of $^{45}CaCl_2$ in 0.02 ml. The solution was rapidly mixed by vortexing, and the Ca^{2+} influx into sarcoplasmic reticulum vesicles after 10 s was stopped by addition of 2 mM EGTA. 0.5 ml of the sample was filtered through a Millipore filter and washed with 5 ml of nonradioactive medium. The Ca^{2+} retained in the sarcoplasmic reticulum vesicle was determined by scintillation counting. For determining the extent of Ca^{2+} binding not related to Ca^{2+} influx, identical procedures were carried out, except EGTA was added first before the addition of tracer $^{45}Ca^{2+}$ to the sarcoplasmic reticulum vesicles. The Ca^{2+}-efflux rate was obtained by subtracting the net Ca^{2+}-uptake rate, as determined above, from the Ca^{2+}-influx rate.

ATPase measurements. The total ATPase and the Ca^{2+}-insensitive ATPase (in the presence of 2 mM EGTA) were measured under the same conditions as in Ca^{2+}-transport studies. The inorganic phosphate released from ATP hydrolysis was assayed according to the method of Lowry and Lopez [8] with slight modification. At various times, 0.1 ml aliquots were removed and mixed with 0.2 ml saturated ammonium persulfate in sodium acetate buffer (0.025 M sodium acetate and 0.1 M acetic acid) at pH 4.0 in a test-tube. Then 1.8 ml sodium acetate buffer, 0.3 ml 1% ammonium molybdate in 0.05 N H_2SO_4 and 0.3 ml 1% ascorbic acid were added and mixed. The sample was incubated at 26°C for exactly 20 min before its absorbance at 700 nm was determined. As standards, the absorbance of KH_2PO_4 from 5 to 300 nmol in identical medium was also measured.

Phosphate and choline fluxes of sarcoplasmic reticulum vesicles. [^{32}P]Phosphate and [^{3}H]choline fluxes of sarcoplasmic reticulum vesicles were measured according to procedures described by Meissner and McKinley [9]. Sarcoplasmic reticulum at 26 mg/ml was incubated in Ca^{2+}-uptake medium with the presence of 1 mM sodium [^{32}P]phosphate and 1 mM [^{3}H]choline chloride for 20 h or more at 0°C. Then, 56 μl of the sample was diluted into 10 ml of identical but unlabeled medium. The medium was continuously stirred by

a magnetic bar and maintained at 4°C. At 10 s intervals after sarcoplasmic reticulum dilution, 0.5 ml aliquots were filtered through Millipore filters. Each filter was subsequently washed with 5 ml of identical medium. The [^{32}P]phosphate and [^{3}H]choline retained by sarcoplasmic reticulum vesicles were determined by scintillation counting. The data were converted as percentage of the apparent [^{3}H]choline space.

Results

The Ca^{2+} uptake of sarcoplasmic reticulum vesicles, in the presence and absence of creatine phosphate and phosphocreatine kinase (an ADP removal system), is shown in Fig. 1. Ca^{2+} uptake at 37°C reached a maximum plateau of about 120 nmol/mg within 10–30 s, and this level was maintained in medium which lacked an ADP removal system. In contrast, in the presence of an ADP removal system, a second phase of Ca^{2+} uptake initiated following the first plateau. This second phase reached a plateau of 280 nmol/mg at 4 min after the initiation of Ca^{2+} uptake. The amount of Ca^{2+} accumulated in the second phase varied with the sarcoplasmic reticulum preparations, the second plateau was usually reached between 4 and 7 min, and the total Ca^{2+} uptake was 270–450 nmol/mg. The Ca^{2+} accumulated in both the first and second phases could be released upon the addition of $5 \cdot 10^{-6}$ M A23187, a calcium ionophore, to the medium. This implies that the enhancement of Ca^{2+} accumulation in the ADP removal system is not due to increase of Ca^{2+} binding, but rather Ca^{2+} is actively transported into the sarcoplasmic reticulum vesicles. The second phase of Ca^{2+} uptake depends on a functioning ADP removal system: it is not observed in this assay condition if either creatine phosphokinase or phosphocreatine is omitted (Figs. 3 and 4).

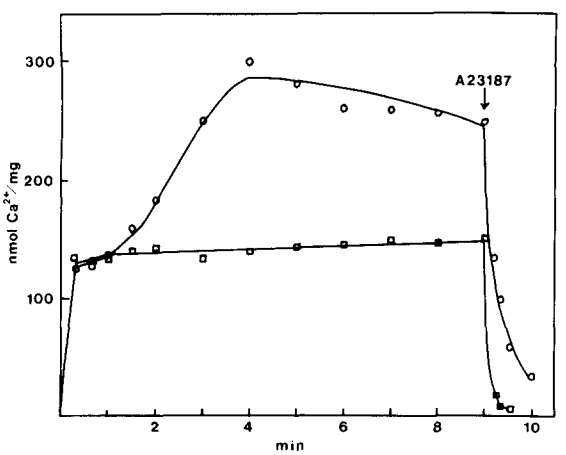

Fig. 1. Ca^{2+} uptake by sarcoplasmic reticulum vesicles. 0.15 mg/ml sarcoplasmic reticulum was incubated at 37°C in a medium containing 0.15 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, 30 mM Tes (pH 6.8) and 60 μM $CaCl_2$ with 0.1 μCi/ml $^{45}CaCl_2$ as tracer. Ca^{2+} uptake was initiated by addition of 2.5 mM ATP (□), or by 2.5 mM ATP, 3.6 mM creatine phosphate and 33 U/ml creatine phosphokinase (○). Ionophore A23187 addition is $2 \cdot 10^{-6}$ M.

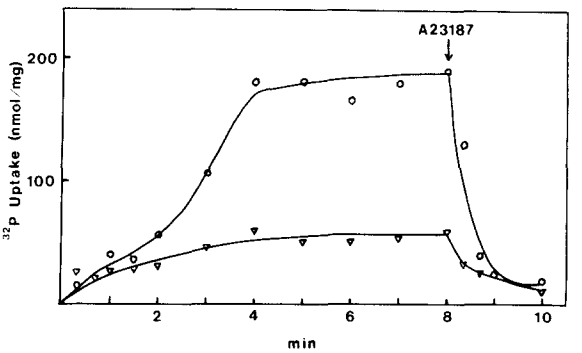

Fig. 2. [^{32}P]Phosphate accumulation by sarcoplasmic reticulum vesicles in Ca^{2+} transport. The protocol was described in Fig. 1, except that ATP added was labeled with [γ-^{32}P]ATP at 0.2 μci/ml. (▽), phosphate accumulation in the presence of 2.5 mM ATP; (○) phosphate accumulation in the presence of 2.5 mM ATP, 3.6 mM phosphocreatine and 33 U/ml of creatine phosphokinase.

The uptake of inorganic phosphate during Ca^{2+} uptake was monitored using [^{32}P]ATP. The experiment shown in Fig. 2 used the same batch of sarcoplasmic reticulum preparation as in Fig. 1 and identical assay media, except ATP was radioactively labeled instead of Ca^{2+}. In the absence of an ADP removal system, there was an accumulation of phosphate to a maximum level of about 58 nmol/mg. The uptake of phosphate was slow compared to the translocation of Ca^{2+}; it did not approach the plateau phase until about 5 min after the initiation of Ca^{2+} uptake. Nonetheless, the accumulation of phosphate appeared to be linked to the accumulation of Ca^{2+}. The Ca^{2+} release induced by ionophore A23187 (Fig. 1) also caused the release or phosphate. In medium with an ADP removal system, two phases of phosphate trans-

port were observed. The first phase, similar to that observed in the absence of an ADP removal system, was much slower that the first phase of Ca^{2+} uptake. However, the onset of the second phase of phosphate uptake as well as the time it reached the plateau were similar to the time course of Ca^{2+} uptake in the second phase. Likewise, the phosphate taken up in the second could be released, accompanying the release of Ca^{2+}, upon the addition of the Ca^{2+} ionophore A23187. Previous studies indicated that the Ca^{2+}-ATPase is about 100 kDa and represents 50–70% of total protein in sarcoplasmic reticulum [10,11]. The Ca^{2+}-ATPase in sarcoplasmic reticulum can, therefore, be estimated to be about 6 nmol/mg. Hence, the binding of [^{32}P]phosphate or [^{32}P]ATP to the Ca^{2+} pump of the sarcoplasmic reticulum vesicles should be insignificant compared to the ^{32}P accumulation observed here. Furthermore, this association could not be attributed to non-specific binding of either [^{32}P]phosphate or [^{32}P]ATP, since it could be released by the presence of $5 \cdot 10^{-6}$ M ionophore A23187. It should be pointed out that the phosphate uptake estimated in the presence of an ADP removal system does not represent the total amount of phosphate taken by the vesicles. This is because the [^{32}P]ATP hydrolyzed will be regenerated by the creatine phosphokinase and phosphocreatine to form nonradioactive ATP. Hence, the phosphate released from the regenerated ATP will not be represented by the [^{32}P]phosphate in the measurement. However, the purpose of this experiment is to demonstrate that there is concomitant phosphate uptake in the second phase of Ca^{2+} accumulation.

Fig. 3 illustrates that the rate of Ca^{2+} uptake in the second phase varies with the concentration of creatine phosphokinase when a fixed concentration of phosphocreatine (3.6 mM) was present. The maximum rate was attained when creatine phosphokinase was increased to 33 U/ml. This implies that the rate of Ca^{2+} transport in the second phase depends on the rate of removal of ADP from ATP hydrolysis.

Further evidence of the role of ADP in the second phase of Ca^{2+} uptake is shown in Fig. 4. In the ADP removal system, ATP is continuously regenerated. However, the second phase of Ca^{2+} uptake appears not to be related to the concentration of ATP. As shown, further addition of 2 mM ATP had no effect on Ca^{2+} uptake. In medium with creatine phosphokinase, but no phosphocreatine, there was no second phase of Ca^{2+} uptake. However, this second phase could be initiated upon the addition of phosphocreatine.

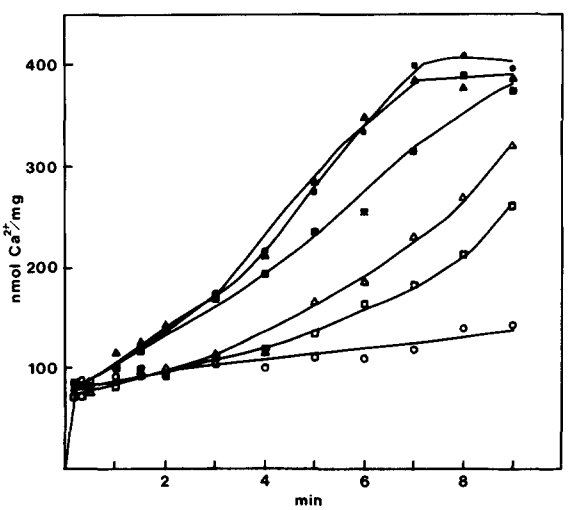

Fig. 3. Dependency of rates of the second phase of Ca^{2+} uptake on the concentration of creatine phosphokinase. Ca^{2+}-uptake condition was as described in Fig. 1 and the phosphocreatine concentration was 3.6 mM in all the assays. The creatine phosphokinase concentrations (U/ml) are: 0 (○); 0.33 (□); 1.1 (△); 11 (■); 33 (▲); 66 (●).

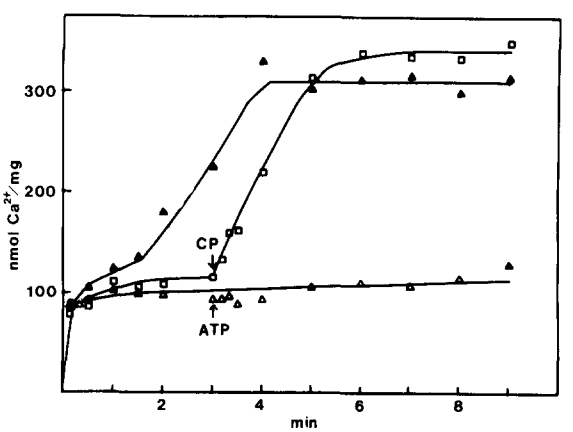

Fig. 4. Ca^{2+} accumulation by sarcoplasmic reticulum vesicles. The protocol was as described in the legend to Fig. 1. (△) ATP alone; (▲) ATP, creatine phosphokinase and creatine phosphate; (□) ATP and creatine phosphokinase. Further additions as indicated are 2 mM ATP and 3.6 mM creatine phosphate (CP).

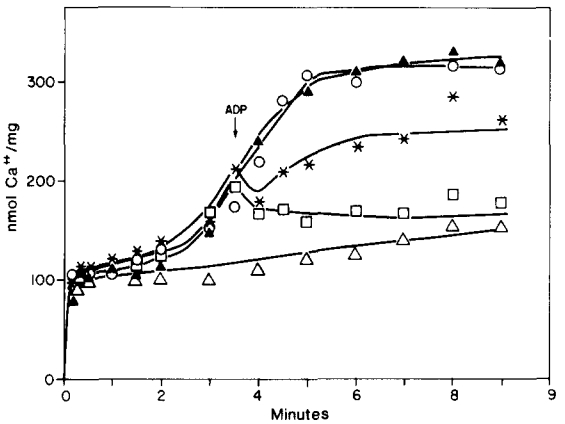

Fig. 5. Ca^{2+} accumulation by sarcoplasmic reticulum vesicles. The protocol was as described in the legend to Fig. 1. (△), ATP alone was added to initiate the Ca^{2+} uptake, and the remaining graphs illustrate Ca^{2+} uptake in the presence of ATP, creatine phosphokinase and creatine phosphate. Further additions of ADP as indicated are 1 mM (○); 3 mM (*) and 4 mM (□).

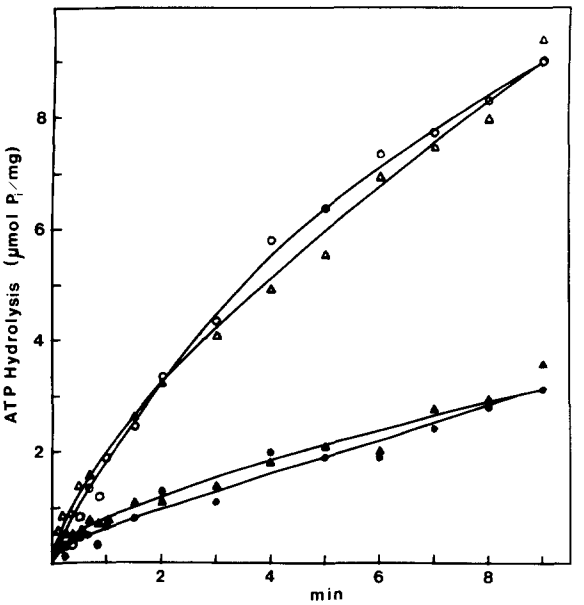

Fig. 6. ATP hydrolysis by sarcoplasmic reticulum vesicles. The experiment was performed under similar condition to the Ca^{2+} uptake described in Fig. 1. ATPase activities in the presence of ADP removal system are indicate by (△,▲) and those without by (○,●). In addition, those with the presence of 2 mM EGTA and no Ca^{2+} in the medium for determining the Mg^{2+}-ATPase activity are (●,▲).

In Fig. 5, the addition of 1 mM ADP had no apparent effect on the second phase of Ca^{2+} uptake. This is expected, since the ADP added would be rapidly converted to ATP by the phosphocreatine and creatine phosphokinase in the medium. However, addition of 3 or 4 mM ADP, which exceeded the capacity of the ADP removal system, inhibited the second phase of Ca^{2+} accumulation.

The second phase of Ca^{2+} transport is coupled to phosphate uptake. It is possible that in medium with ADP removal system, higher rate of ATP hydrolysis results in higher concentration of phosphate in the medium, that subsequently triggers Ca^{2+}-phosphate precipitation in the second phase. The ATPase activity of sarcoplasmic reticulum under identical conditions of Ca^{2+} uptake was determined and the data are shown in Fig. 6. There is no significant difference between the total ATPase activity in medium with ADP removal system and that without. Also, the Mg^{2+}-ATPase activities (in the presence of 2 mM EGTA and no $CaCl_2$) are similar in medium with and without the ADP removal system. This finding suggests that the occurrence of the second phase of Ca^{2+} uptake in medium with the ADP removal system is not solely due to increase of phosphate in the medium.

While we have demonstrated that there is inorganic phosphate accumulation accompanying Ca^{2+} uptake and calcium phosphate precipitation probably occurs at the first plateau (Fig. 2), it is not clear whether the ADP produced eventually prevents further inorganic phosphate influx and, hence, no second phase of Ca^{2+} uptake in the absence of an ADP removal system. In order to examine this possibility, the ion exchange rate of inorganic phosphate of sarcoplasmic reticulum in media with and without ADP were tested. Fig. 7 shows the ion exchange rates for both [^{32}P]phosphate and [^{3}H]choline of sarcoplasmic reticulum vesicles. [^{3}H]Choline permeation rate is slow in sarcoplasmic reticulum. 50% of the trapped [^{3}H]choline was exchanged in about two mins, in agreement with the value determined by Meissner and McKinley [9]. In contrast, about 65% of the inorganic phosphate was exchanged within 10 s, and the remaining 35% had an exchange rate similar to that of choline. The ion exchange rate of inorganic phosphate was not affected by the addition of 2 mM ADP.

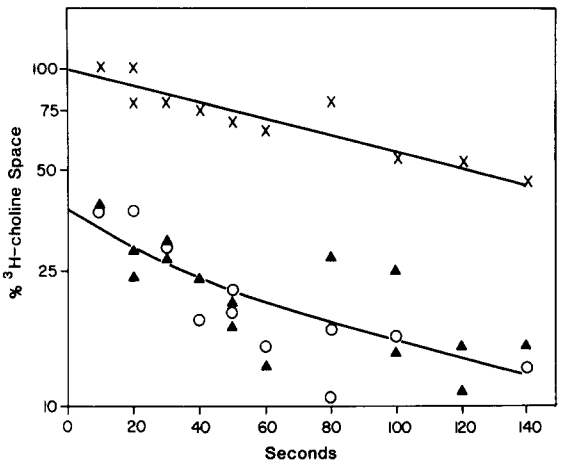

Fig. 7. [^{3}H]Choline and [^{32}P]phosphate exchange rates and isotope spaces of sarcoplasmic reticulum vesicles. The protocol is described in Methods. Data are expressed as percent apparent [^{3}H]choline space extrapolated to zero-time vs. time. (×) denotes [^{3}H]choline exchange rate. (○) and (▲) denote [^{32}P]phosphate exchange rates in the presence and absence of 2 mM ADP, respectively.

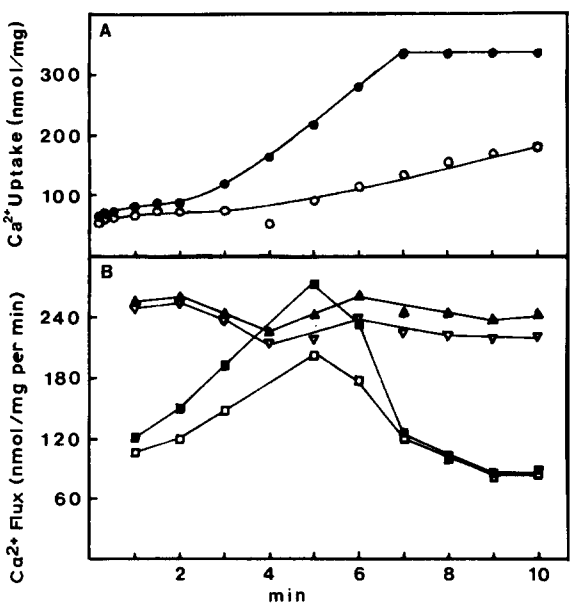

Fig. 8. A. Ca^{2+} uptake of sarcoplasmic reticulum vesicles in the presence (●) and the absence (○) of ADP removal system as described in Fig. 1. B. Ca^{2+} fluxes of sarcoplasmic reticulum vesicles under identical condition to Ca^{2+}-uptake measurement. The flux rates were determined as described in Methods. (▲,■) are Ca^{2+}-influx rates and (□,△) are Ca^{2+}-efflux rates in the presence (□,■) and absence (▲,▽) of ADP removal system.

The unidirectional Ca^{2+} influx and efflux of sarcoplasmic reticulum vesicles in the course of Ca^{2+} uptake had been monitored by pulse labeling with $^{45}CaCl_2$. The data are shown in Fig. 8. In medium that lacked the ADP removal system, the Ca^{2+}-influx rate at the plateau phase was about 240 nmol/mg per min and was balanced by the Ca^{2+} efflux. This Ca^{2+}-exchange rate remained constant in the time course of the assay. In contrast, in medium that included an ADP removal system, the Ca^{2+}-influx rate at the first plateau of Ca^{2+} uptake was about 120 nmol/mg per min; about half of the influx rate observed in medium without the ADP removal system. The Ca^{2+}-efflux rate was about equal to the Ca^{2+}-influx rate. As the second phase of Ca^{2+} uptake started, there was a concomitant increase of Ca^{2+} influx and efflux. The difference between the rates of Ca^{2+} influx and efflux increased gradually until external Ca^{2+} was near depletion. Abruptly, after the second phase of Ca^{2+} uptake reached the plateau, both the influx and efflux rates dropped back to the level at which the first plateau phase of Ca^{2+} was reached. This experiment was repeated again with the presence of 80 μM diadenosine pentaphosphate in the media. Diadenosine pentaphosphate is a potent inhibitor of adenylate kinase but has no effect on the Ca^{2+}-ATPase of sarcoplasmic reticulum [12,13]. The purpose of the experiment is to determine whether the differences in Ca^{2+} accumulation and Ca^{2+} fluxes of sarcoplasmic reticulum, in the presence and absence of ADP, is due to ADP or AMP formed from ADP by contaminating adenylate kinase in the sarcoplasmic reticulum preparation. The results are shown in Fig. 9. In comparing with the data in Fig. 8, inhibition of adenylate kinase activity does not alter the previous observations. This implies that the modulation of Ca^{2+}-uptake and Ca^{2+}-flux rates by ADP is not mediated by adenylate kinase activity.

Net Ca^{2+} uptake and Ca^{2+} exchange of sarcoplasmic reticulum vesicles were also examined under such conditions that inorganic phosphate accumulation was minimized. Ca^{2+}-uptake assays were conducted using 7.5 μg/ml sarcoplasmic reticulum, about one-twentieth of the sarcoplasmic reticulum used in the experiments described previously. As shown in Fig. 10A, the second phase of Ca^{2+} uptake was observed in medium with ATP

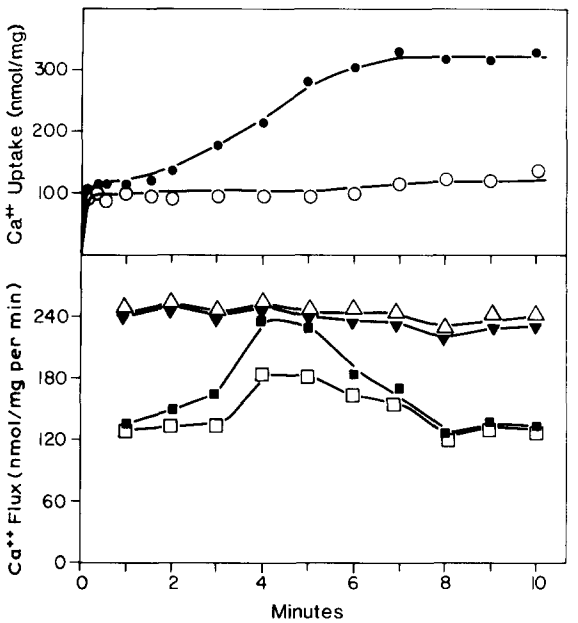

Fig. 9. These experiments were identical to those shown in Fig. 8, except that the medium contain, in addition, 80 μM diadenosine pentaphosphate. A. Ca^{2+} uptake of sarcoplasmic reticulum in the presence (●) and the absence (○) of ADP removal system. B. Ca^{2+} fluxes of sarcoplasmic reticulum vesicles. (△,■) are Ca^{2+}-influx rates and (▼,□) are Ca^{2+}-efflux rates in the presence (□,■) and absence (△,▼) of ADP removal system.

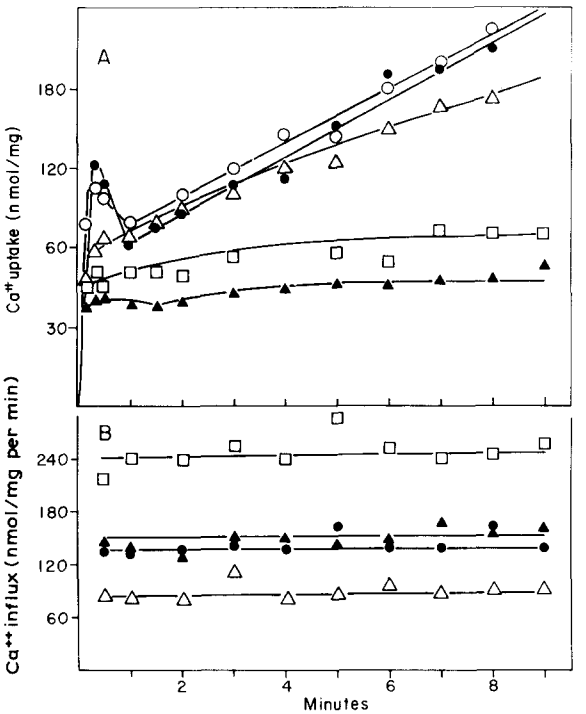

Fig. 10. A. Ca^{2+} uptake by sarcoplasmic reticulum vesicles was determined as described in Fig. 1, except the sarcoplasmic reticulum added is reduced to 7.5 μg/ml. The Ca^{2+} accumulated is activated by the presence of either: 1 mM ATP (○); 1 mM ATP, 1 mM phosphocreatine and 11 U/ml creatine phosphokinase (●); 1 mM acetylphosphate (△); 1 mM ATP plus 1 mM ADP (□); or 1 mM acetyl phosphate plus 1 mM ADP (▲). B. Unidirectional Ca^{2+}-influx rates of sarcoplasmic reticulum vesicles under conditions as in A: (●) medium with 1 mM ATP, 1 mM phosphocreatine and 11 U/ml creatine phosphokinase; (□) medium with 1 mM ATP and 1 mM ADP; (△) medium with 1 mM acetyl phosphate; (▲) medium with 1 mM acetyl phosphate and 1 mM ADP.

alone, the time course and magnitude of Ca^{2+} accumulation were identical to those in medium with ADP removal system. After 2 min, when the second phase of Ca^{2+} uptake had started, the ADP and P_i resulting from ATP hydrolysis were each determined to be not more than 30 μM (data not shown). This implies that the second phase of Ca^{2+} accumulation can occur in low levels of ADP. Furthermore, the occurrence of the second Ca^{2+}-uptake phase with a low concentration of inorganic phosphate suggests that the absence of the second Ca^{2+}-uptake phase in medium with ATP alone and 0.15 mg/ml sarcoplasmic reticulum (Fig. 1) cannot be due to insufficient inorganic phosphate, but rather the accumulation of ADP. When acetyl phosphate was used instead of ATP, the second phase of Ca^{2+} uptake was also observed. However, if 1 mM ADP was added together with either 1 mM ATP or 1 mM acetyl phosphate, there was no second phase of Ca^{2+} uptake. This again suggests that the second phase of Ca^{2+} uptake is inhibited by ADP.

Fig. 10B shows the corresponding unidirectional Ca^{2+}-influx rates under identical conditions to those in Fig. 10A. The influx rate was slower, about 90 nmol/mg per min, in medium with 1 mM acetyl phosphate. The rate was considerably increased to 150 nmol/mg per min if 1 mM ADP and 1 mM acetyl phosphate were in the medium. In medium with 1 mM ATP and the ADP removal system, the average influx rate was about 140 nmol/mg per min. The influx was much increased in medium containing 1 mM ATP and 1 mM ADP, to about 240 nmol/mg per min.

Discussion

This study demonstrates that during active Ca^{2+} transport of sarcoplasmic reticulum vesicles, ADP accumulated from ATP hydrolysis affects both net Ca^{2+} accumulation and Ca^{2+}-exchange rates. In the presence of an ADP removal system, ATP dependent Ca^{2+} uptake of sarcoplasmic reticulum is a biphasic phenomenon. Under assay conditions as described in Methods, sarcoplasmic reticulum Ca^{2+} uptake usually reaches a plateau of 100 nmol/mg within 30 s or less during the first phase. Then, after a delay, a second phase of Ca^{2+} uptake initiated, and reaches another plateau of about 300 nmol/mg. The second phase of Ca^{2+} uptake can be abolished by the presence of ADP.

The second phase of Ca^{2+} uptake is coupled to influx of inorganic phosphate. Assuming that the luminal space of sarcoplasmic reticulum is 3 μl/mg [14], the vesicular Ca^{2+} and inorganic approach 80 and 40 mM, respectively, at the middle region of the second phase of Ca^{2+} uptake (Figs. 1 and 2). Since previous determinations showed that the Ca^{2+} binding sites of sarcoplasmic reticulum are about 100 nmol/mg [10] and the solubility product of calcium phosphate is $7.5 \cdot 10^{-6}$ M^2 [7], calcium phosphate precipitation must occur during the second phase of calcium uptake. The biphasic Ca^{2+} uptake of sarcoplasmic reticulum had been examined by Mermier and Hasselbach [15] and Weber et al. [16]. Both of these teams also attributed the second phase of Ca^{2+} uptake to calcium phosphate precipitation within the vesicles. However, it should be noted that the absence of the second phase of Ca^{2+} uptake in medium that lacks the ADP removal system is not due to insufficient inorganic phosphate accumulation. As shown in Fig. 6, the release of phosphate from ATP hydrolysis in media with and without the ADP removal system are nearly identical. Hence, the initiation of the second phase of Ca^{2+} uptake is not solely due to accumulation of phosphate in the medium. In fact, in assays using low concentrations of sarcoplasmic reticulum, the second phase of Ca^{2+} uptake is observed even when the phosphate accumulated is less that 30 μM. Furthermore, it is unlikely that the absence of the second phase of Ca^{2+} uptake is caused by ADP inhibition of inorganic phosphate influx. This is supported by the results from passive phosphate flux experiments (Fig. 7), indicating that ADP does not affect the inorganic phosphate permeability of sarcoplasmic reticulum membrane.

The effects of ADP examined here appear to be mediated by ADP itself rather than other by-products. Similar effects of ADP are observed in the presence and absence of diadenosine pentaphosphate, an inhibitor of possibly contaminating adenylate kinase in the sarcoplasmic reticulum preparation. Hence the effects of ADP are not mediated by AMP. Further supporting evidence of the direct effects of ADP include: (a) addition of ADP can immediately terminate the second phase of Ca^{2+} uptake and (b) the rate of Ca^{2+} uptake in the second phase is governed by the rate of removal of ADP.

The Ca^{2+}-exchange rate described in these experiments has been studied previously and thought to be mediated by the Ca^{2+} pump [3,5]. Activation of ATP-dependent Ca^{2+} efflux of sarcoplasmic reticulum by ADP was first reported by makinose [3]. According to the generally accepted scheme of Ca^{2+} transport via the Ca^{2+} pump [17,18], both Ca^{2+} influx and efflux are mediated by the phosphorylated enzyme complex: $Ca_2^{2+} \cdot E \sim P$. Ca^{2+} will not be released from the $Ca_2^{2+} \cdot E \sim P$ complex to the external medium unless the enzyme is dephosphorylated first, with ADP as the phosphate acceptor. Hence, depletion of ADP would inhibit the rate of Ca^{2+} efflux. My observation that, at the first plateau of Ca^{2+} uptake, the Ca^{2+}-efflux rate in medium with he ADP removal system is about half of that in medium without (Fig. 8), agrees with previous observations concerning the stimulation of Ca^{2+} efflux by ADP [3,5]. The Ca^{2+} efflux is only partially inhibited in the presence of an ADP removal system. This might be due to inefficient removal of low concentration of ADP from the medium. However, in situations where there is no ADP accumulation, i.e., when Ca^{2+} uptake is supported by acetyl phosphate (Fig. 10B), the Ca^{2+}-efflux rate at 80 nmol/mg per min is still much higher than the passive Ca^{2+} leak from loaded sarcoplasmic reticulum vesicles [16]. This tends to suggest that a portion of the Ca^{2+} efflux may not be mediated by reversal of the same reaction steps for Ca^{2+} influx.

The difference in Ca^{2+}-influx rates in medium

with and without an ADP removal system appears not to be related to intravesicular Ca^{2+} concentration. As shown in Fig. 8, at the first plateau of Ca^{2+} uptake, the Ca^{2+} load of sarcoplasmic reticulum in both media are identical. However, at this state, the Ca^{2+} influx in medium with an ADP removal system is 120 nmol/mg per min vs. a rate of 240 nmol/mg per min in medium without the ADP removal system. It remains to be determined why the inhibition of Ca^{2+} efflux by the removal of ADP also leads to depression of Ca^{2+} influx. Perhaps there exists a coupling mechanism between Ca^{2+} influx and efflux. This study suggests that the initiation of the second phase of Ca^{2+} uptake can be modulated by Ca^{2+} influx and efflux. As Ca^{2+} efflux is partially inhibited by the removal of ADP, a portion of the Ca^{++} influx is linked to phosphate influx and this subsequently leads to Ca^{2+}-phosphate precipitation.

Acknowledgments

I wish to thank Dennis Carlton for his technical assistance. This work was supported by Muscular Dystrophy Association and American Heart Association (Upstate New York Chapter), and was carried out during tenure of NIHLB Young Investigator Award (HL 26036).

References

1 Carlson, F.D. (1963) Progr. Biophys. Mol. Biol. 13, 261–314
2 Baskin, R.J. and Deamer, D.W. (1970) J. Biol. Chem. 245, 1345–1347
3 Makinose, M. (1973) FEBS Lett. 37, 140–143
4 Beiraô, P.S. and De Meis, L. (1976) Biochim. Biophys. Acta, 433, 520–530
5 Waas, W. and Hasselbach, W. (1981) Eur. J. Biochem. 116, 601–608
6 Martonosi, A. (1968) J. Biol. Chem. 243, 71–81
7 Katz, A.M., Dunnett, J., Repke, D.I. and Hasselbach, W. (1976) FEBS Lett. 67, 207–208
8 Lowry, O.H. and Lopez, J.A. (1946) J. Biol. Chem. 162, 421–428
9 Meissner, G. and McKinley, D. (1976) J. Membrane Biol. 30, 79–98
10 MacLennan, D.H and Holland, P.C. (1975) Annu. Rev. Biophys. Bioeng. 4, 377–404
11 Inesi, G. (1972) Annu. Rev. Biophys. Bioeng. 1, 191–210
12 Lienhard, G.E. and Secemski, I.I. (1973) J. Biol. Chem. 248, 1121–1123
13 Feldhaus, P., Fröhlich, T., Goody, R.S., Isakov, M. and Schirmer, R.H. (1975) Eur. J. Biochem. 57, 197–204
14 Kasai, M. (1981) J. Biochem. 89, 943–953
15 Mermier, P. and Hasselbach, W. (1976) Eur. J. Biochem. 64, 613–620
16 Weber, A., Herz, R. and Reiss, I. (1964) Fed. PRoc. 23, 896–900
17 Carralho, M.G.C., Souza, D.G. and De Meis, L. (1976) J. Biol. Chem. 251, 3629–3636
18 De Meis, L. and Boyer, P.D. (1978) J. Biol. Chem. 253, 1556—1559