# $Ca^{2+}$-ATPase AND Ca UPTAKE WITHOUT REQUIREMENT FOR $Mg^{2+}$ IN MEMBRANE FRACTIONS OF VASCULAR SMOOTH MUSCLE

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## 1. Introduction

Calcium transport by sarcoplasmic reticulum vesicles of skeletal and cardiac muscles is energized by a $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase (reviewed [1,2]). In membrane fractions from various smooth muscles, an ATP-dependent Ca transport as well as a $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase activity have been described [3–5]. However, due to the presence of a very high 'basal' $Mg^{2+}$-ATPase activity in smooth muscle [3,5,6], the study of a possible ($Ca^{2+}$, $Mg^{2+}$)-ATPase is difficult. Apart from $Mg^{2+}$-ATPase and ($Ca^{2+}$, $Mg^{2+}$)-ATPase activities, $Ca^{2+}$-ATPase activity in the absence of $Mg^{2+}$ has been found in plasma membranes of skeletal muscle [7], cardiac muscle [8] and uterus smooth muscle [9] and in microsomes of vascular smooth muscle [6]. In all these tissues, $Ca^{2+}$-ATPase activity is characterized by a low affinity for $Ca^{2+}$ (mM range).

This is the first report on a high affinity $Ca^{2+}$-ATPase in smooth muscle not requiring $Mg^{2+}$ for activation. This communication also provides the first evidence that membrane fragments from rabbit mesenteric arteries can transport Ca in the virtual absence of $Mg^{2+}$. Finally, data are shown which cast doubt on a coupling between $Mg^{2+}$-ATPase or $Ca^{2+}$-ATPase activities and Ca transport in vascular smooth muscle.

## 2. Materials and methods

Subcellular fractions were obtained by homogenizing pieces of rabbit mesenteric arteries for 15 s in 0.25 M sucrose with a Polytron PT 10 at a rheostat setting of 7. The homogenate was centrifuged at 900 × *g* for 10 min and the supernatant at 200 000 × *g* for 35 min. The precipitate was resuspended in 0.25 M sucrose and layered on a 30 ml 15–45% (w/v) continuous sucrose gradient. After centrifugation of the gradient at 130 000 × *g* for 2 h the fraction included between 16% and 30% sucrose was collected. After a 35 min centrifugation at 200 000 × *g* the fraction was resuspended in $H_2O$.

Unless otherwise stated, ATPase activity and Ca uptake were measured at 37°C in 0.5 ml solution containing KCl 70 mM; histidine–HCl 20 mM (pH 6.8); $NaN_3$ 5 mM and $Na_2ATP$ 5 mM. The rate of ATP hydrolysis was determined by measuring the amount of $P_i$ released from the samples [10]. Ca uptake was estimated by measuring the accumulation of $^{45}CaCl_2$ in the vesicles using the Millipore filter method. Free $Ca^{2+}$ and free $Mg^{2+}$ concentrations were calculated by using the stability constants for CaATP and MgATP given in [11]. When $Ca^{2+}$ at $< 10^{-5}$ M was required, EGTA 0.1 mM was added to the medium and the free $Ca^{2+}$ concentration was calculated as in [12].

*Abbreviations:* EGTA, ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid; pCMB, *p*-chloromercuribenzoate

## 3. Results and discussion

In electron micrographs, the subcellular fraction of rabbit mesenteric arteries used in this study appeared as vesicular structures enclosed by smooth membranes. No intact mitochondria were found. When compared with the first homogenate, the isolated fraction was enriched in ouabain-sensitive *p*-nitrophenylphosphatase activity and in oxalate-activated Ca uptake but

Table 1
Effects of various inhibitors and of various substrates on ATPase activity and Ca uptake in the membrane fraction

| | $Mg^{2+}$-ATPase | $Ca^{2+}$-ATPase | $^{45}Ca$ uptake $MgCl_2$, 5 mM | $^{45}Ca$ uptake $MgCl_2$, 0 |
|---|---|---|---|---|
| Control | 1.99 ± 0.07 | 3.34 ± 0.06 | 60.7 ± 5.3 | 6.23 ± 0.96 |
| $NaN_3$, 0 | 2.30 ± 0.08 | 3.30 ± 0.12 | 66.3 ± 7.2 | 6.66 ± 0.89 |
| pCMB, $10^{-4}$ M | 1.95 ± 0.06 | 3.46 ± 0.12 | 0.91 ± 0.10 | 1.97 ± 0.38 |
| X-537A, 5 μM | 1.88 ± 0.06 | 3.21 ± 0.10 | 1.90 ± 0.35 | 1.84 ± 0.27 |
| CTP, 5 mM | 1.43 ± 0.06 | 1.82 ± 0.02 | 6.5 ± 0.7 | 2.59 ± 0.54 |
| ITP, 5 mM | 1.85 ± 0.10 | 2.65 ± 0.20 | 4.4 ± 0.5 | 2.52 ± 0.31 |
| UTP, 5 mM | 1.75 ± 0.11 | 2.16 ± 0.06 | 10.4 ± 2.6 | 3.26 ± 0.60 |

ATPase activity was measured in the presence of either 5 mM $MgCl_2$ ($Mg^{2+}$-ATPase) or 5 mM $CaCl_2$ ($Ca^{2+}$-ATPase) as in section 2 and is expressed in μmol $P_i$/mg protein · min. Ca uptake was measured after 20 min incubation in the presence of $10^{-6}$ M $Ca^{2+}$ and 10 mM oxalate as in section 2 and is expressed in μmol $^{45}Ca$/mg protein. When CTP, ITP or UTP were used as substrate ATP was absent. All values are the mean ± SEM of 4–5 experiments

contained less cytochrome oxidase activity. The preparation thus probably represents a mixture of membranes derived from the sarcolemma and the endoplasmic reticulum. The low mitochondrial contamination was also evident from the low inhibition caused by $NaN_3$ on $Mg^{2+}$-ATPase activity and Ca uptake (table 1).

While no ATPase activity was detected in the absence of added Mg or Ca, the maximum rate of ATP hydrolysis was higher in the presence of $Ca^{2+}$ than $Mg^{2+}$ (fig.1A). However, ATPase activity showed a greater affinity for $Mg^{2+}$ ($K_m$ $3.5 \times 10^{-6}$ M) than $Ca^{2+}$ ($K_m$ $10^{-5}$ M) (fig.1B). The $K_m$ values for substrate were $10^{-4}$ M for the complex MgATP and $2 \times 10^{-4}$ M for CaATP. The same values were found for free ATP in the presence of $Mg^{2+}$ and $Ca^{2+}$, respectively. $Mg^{2+}$-ATPase and $Ca^{2+}$-ATPase activities exhibited similar properties: absence of significant effects of the Ca ionophore X-537 A and the thiol reagent pCMB and very low specificity for ATP when compared with other substrates (table 1). These similarities together with the observation that addition of $Mg^{2+}$ did not further increase, but rather inhibit, the maximally $Ca^{2+}$-activated hydrolysis (fig.1A) suggest that one enzyme unit might be responsible for both $Ca^{2+}$ and $Mg^{2+}$-stimulated ATPase activities.

While Ca accumulation by the fraction was not influenced by $Mg^{2+}$ in the absence of ATP, the cation strongly stimulated ATP-dependent Ca uptake both in the presence and in the absence of oxalate (fig.2). $Mg^{2+}$ increased the affinity of Ca uptake ($K_{Ca}$ values of $6 \times 10^{-6}$ and $10^{-4}$ M were found in the presence and in the absence of 5 mM $MgCl_2$, respectively) but did not increase the maximum filling capacity of the vesicles (fig.3). In sharp contrast to ATPase activities, Ca uptake (particularly in the presence of $MgCl_2$) showed a high specificity for ATP when compared with other substrates (table 1). Although $Mg^{2+}$-stim-

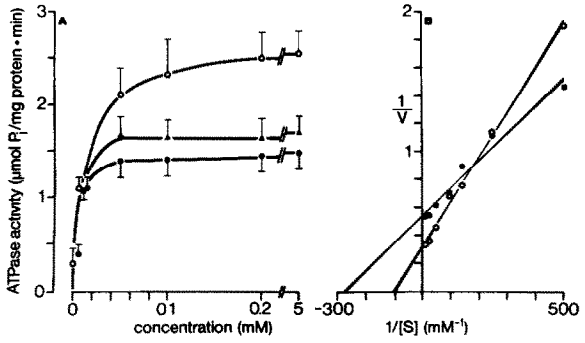


Fig.1. Effects of [$Mg^{2+}$] and [$Ca^{2+}$] on ATPase activity in the membrane fraction. ATPase activity was measured after 15 min incubation as in section 2. (A) Effects of $Mg^{2+}$ alone (•), $Ca^{2+}$ alone (○), and $Mg^{2+}$ plus $Ca^{2+}$ in equimolar concentrations (▲). (B) Lineweaver-Burk plots of ATPase activity at various concentrations of $Mg^{2+}$ (•) and $Ca^{2+}$ (○). $v$ is expressed in μmol $P_i$/mg protein · min. All values are the mean (± SEM in A) of 4 experiments.

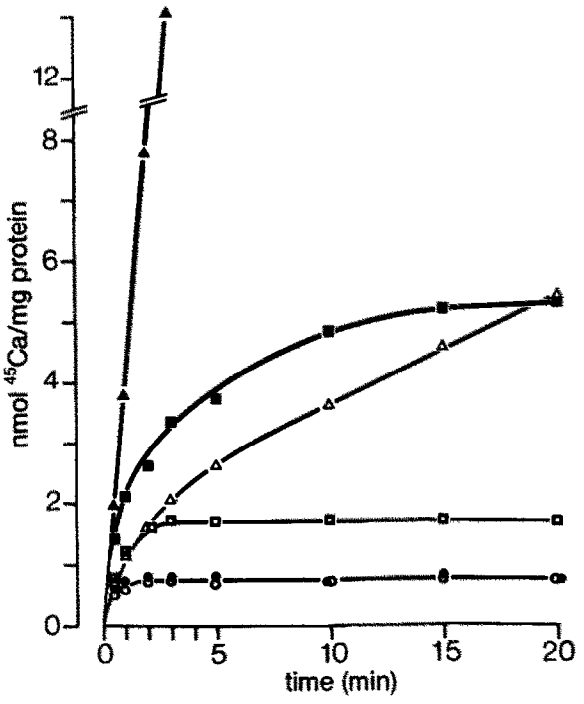


Fig.2. Time curve of Ca accumulation by the membrane fraction in the presence of $10^{-6}$ M $Ca^{2+}$. Ca accumulation was measured as in section 2. Filled symbols: $MgCl_2$ 5 mM; open symbols: no $MgCl_2$. Circles: no ATP, oxalate 10 mM. Squares: ATP 5 mM, no oxalate. Triangles: ATP 5 mM, oxalate 10 mM. All values are the mean of 5–6 experiments.

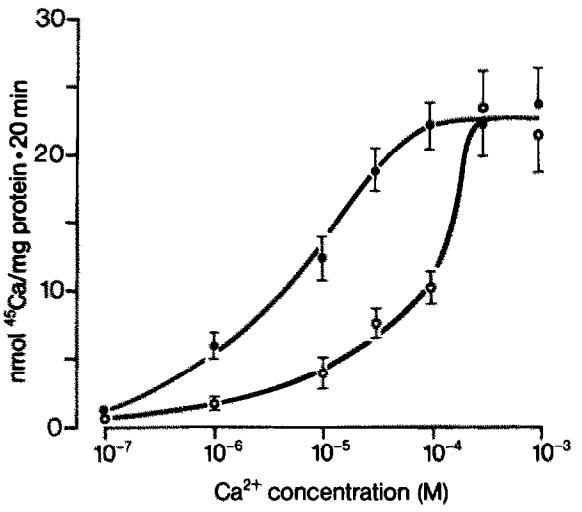


Fig.3. Effects of $[Ca^{2+}]$ on ATP-dependent Ca uptake by the membrane fraction. Ca uptake was measured after 20 min of incubation as in section 2 (no oxalate) and in the presence (•) or in the absence (○) of 5 mM $MgCl_2$. Ca binding (no ATP) as measured under the same conditions was subtracted from every value. All values are the mean ± SEM of 5–8 experiments.

ulation of Ca transport was evident, Ca uptake without added Mg was clearly both ATP-dependent and oxalate-activated (fig.2). Moreover, similar to $Mg^{2+}$-activated Ca uptake, it was depressed by both pCMB and X-537 A. When the whole extraction procedure as well as the measurement of $Mg^{2+}$-independent Ca uptake were carried out in the presence of 0.1 mM EDTA, maximum ATP-dependent filling capacities remained similar in the presence and in the absence of 5 mM MgCl in the incubation medium (not shown). Therefore, trace amounts of $Mg^{2+}$ contained in the vesicles or the solutions do not seem to be responsible for Ca transport as measured in the absence of added Mg.

Beside the absence of sensitivity to thiol reagents shown by ATPase activity (table 1), the fact that the molar ratio between nucleotide splitting and Ca transport varied widely depending on the substrate used is a strong argument against a tight coupling between $Ca^{2+}$-ATPase and Ca transport in the present membrane fraction. At any rate, this coupling ratio would exceed 500; this is a very high value considering that a ratio of 2 is found in sarcoplasmic vesicles of skeletal muscle [1], and its seems unlikely to be entirely accounted for by leakiness or wrong orientation of the vesicles.

In conclusion, while the present results provide evidence that, in vascular smooth muscle, ATPase and Ca transport can be activated by $Ca^{2+}$ in the absence of $Mg^{2+}$, further studies are required to elucidate the function of the $Ca^{2+}$-ATPase and to identify the ATPase enzyme which is coupled with Ca transport.

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