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DEMONSTRATION OF A (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY PROBABLY RELATED TO Ca^{2+} TRANSPORT IN THE MICROSOMAL FRACTION OF PORCINE CORONARY ARTERY SMOOTH MUSCLE

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Summary

A (Ca^{2+} + Mg^{2+})-ATPase activity is demonstrated in the microsomal fraction of porcine coronary artery. The characteristics of the ATPase activity are compared with those of the Ca^{2+} transport, both measured in similar solutions. It is concluded that the (Ca^{2+} + Mg^{2+})-ATPase is related to the Ca^{2+} transport because:

1. Both transport and ATPase have similar low K_m values for Ca^{2+} as well as comparable Hill coefficients. The K_m values are respectively 0.34 ± 0.03 μM [4] and 1.17 ± 0.15 μM [6]. The Hill coefficients are n = 1.69 ± 0.09 [4] and n = 1.23 ± 0.17 [6].
2. Ionophores A23187 and X537A stimulate (Ca^{2+} + Mg^{2+})-ATPase activity while they inhibit net Ca^{2+} accumulation by increasing the Ca^{2+} permeability of the membranes.
3. The V values for Ca^{2+} accumulation and for (Ca^{2+} + Mg^{2+})-ATPase are comparable.

Introduction

An ATP-dependent Ca^{2+}-uptake by microsomal fractions of vascular smooth muscle has been described by several authors [1—5]. However it proved more difficult to demonstrate the existence of a corresponding (Ca^{2+} + Mg^{2+})-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PMSF, phenylmethylsulfonylfluoride.

ATPase. Some authors have described a Mg^{2+}-independent Ca^{2+}-ATPase [6] while others have proposed for vascular smooth muscle a (Ca^{2+} + Mg^{2+})-ATPase without characterizing it further [2,3]. A (Ca^{2+} + Mg^{2+})-ATPase has already been demonstrated and investigated in intestinal smooth muscle [7] and myometrium [8].

In the present work we demonstrate the existence of a (Ca^{2+} + Mg^{2+})-ATPase in the smooth muscle cells of the porcine coronary artery and in addition we present some characteristics of this enzyme. We also bring forward some arguments indicating that this enzyme is related to the Ca^{2+} transport system in the microsomes of this tissue.

Experimental

Microsomal fraction. Microsomes were prepared from the combined media and intima layers of the large extramural branches of porcine right coronary arteries. Hearts were obtained from the slaughterhouse and pieces containing the right coronaries were brought to the laboratory in ice-cold, Ca^{2+}-free, modified Krebs solution buffered with Hepes at pH 7.4. The intima-media layers were then isolated and homogenized as described earlier [5]. The homogenization medium had the following composition: 0.25 M sucrose, 1 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% (v/v) ethanol.

Ca^{2+} uptake. $^{45}Ca^{2+}$ uptake was measured as described earlier [5]. The uptake medium contained 100 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5.7 mM $MgCl_2$, 5 mM disodium ATP, 5 mM NaN_3, 1 mM EGTA, and $K_2(COO)_2$ as indicated. When ATP was omitted the Mg^{2+} concentration was kept at 1.0 mM. The microsomal suspension (20 µl containing 20—80 µg protein) was added to test tubes with the uptake medium at 37° C; 1 min later $[Ca^{2+}]_0$ was adjusted to the desired value and the radioactivity to approx. 1 µCi/ml by adding 20 µl $^{45}CaCl_2$. 200-µl aliquots of this incubation medium were filtered on Millipore filters (0.45 µm) after different incubation times. The filtered microsomes were washed once with 2 ml of 0.25 M sucrose solution containing 1 mM disodium EGTA at pH 6.8.

ATPase activity. The ATPase activity was measured as described by Barnett [9] at 37° C in 1 ml of a medium having the following composition: 100 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5.7 mM $MgCl_2$, 5 mM disodium ATP, 5 mM NaN_3, 1 mM EGTA, 1.5 mM phospho*enol*pyruvate, 0.26 mM disodium NADH, 40 I.U./ml pyruvate kinase, 36 I.U./ml lactate dehydrogenase (both enzymes from rabbit muscle). The decrease in absorbance at 340 nm was recorded with an Aminco DW2 spectrophotometer. A molar extinction coefficient of $\epsilon = 6.2 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ was used in the calculations. After termal equilibration of the cuvette, 10 or 20 µl of microsomes (20—80 µg protein) were added. Ouabain, $CaCl_2$ or ionophores were added as concentrated stock solutions (100 times). A23187 and X537A were dissolved in 100% ethanol and alamethicin in 33% ethanol. Ethanol at the concentration used in our experiments had no effect on ATPase activity. To determine the rate of (Ca^{2+} + Mg^{2+})-ATPase as a function of $[Ca^{2+}]$, Ca^{2+} was added in cumulative way with four subsequent additions per cuvette. Usually four different Ca^{2+} concentrations were studied in three similar series of measurements.

TABLE I

EQUILIBRIA AND STABILITY CONSTANTS USED FOR CALCULATING THE Ca^{2+} CONCENTRATIONS

These values have been compiled from the data of Godt [13] and of Sillen and Martell [14].

Equilibrium	log stability constant (M^{-1})
$H^+ + ATP^{4-} = HATP^{3-}$	7.02
$H^+ + HATP^{3-} = H_2ATP^{2-}$	4.02
$Mg^{2+} + ATP^{4-} = MgATP^{2-}$	4.65
$Mg^{2+} + HATP^{3-} = MgHATP^{1-}$	2.65
$Ca^{2+} + ATP^{4-} = CaATP^{2-}$	4.32
$Ca^{2+} + HATP^{3-} = CaHATP^{1-}$	2.13
$H^+ + ADP^{3-} = HADP^{2-}$	6.88
$Mg^{2+} + ADP^{3-} = MgADP^-$	3.15
$Ca^{2+} + ADP^{3-} = CaADP^-$	2.82
$H^+ + EGTA^{4-} = HEGTA^{3-}$	9.43
$H^+ + HEGTA^{3-} = H_2EGTA^{2-}$	8.85
$Mg^{2+} + EGTA^{4-} = MgEGTA^{2-}$	5.20
$Mg^{2+} + HEGTA^{3-} = MgHEGTA^{1-}$	3.36
$Ca^{2+} + EGTA^{4-} = CaEGTA^{2-}$	10.42
$Ca^{2+} + HEGTA^{3-} = CaHEGTA^{1-}$	5.32

Protein. Protein was measured by a modified Lowry method [10].

Ca^{2+} buffering system. The Ca^{2+} uptake and the (Ca^{2+} + Mg^{2+})-ATPase activity were measured in solutions in which [Ca^{2+}] was buffered by means of 1.0 mM EGTA. In all solutions [Mg^{2+}] was kept at 1.0 mM. The equilibria and the stability constants used in these calculations are given in Table I.

$^{45}Ca^{2+}$ was obtained from New England Nuclear. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer. A23187 was a gift of Dr. R.L. Hamill, Eli Lilly Laboratories. X537A was a gift from Dr. G. Haeusler, Hoffman-LaRoche and Co. Alamethicin was obtained from Upjohn. The other reagents were obtained from Sigma.

Results

The Ca^{2+} transport in the microsomal fraction

Microsomes prepared from the intima and media of the porcine coronary artery present an ATP dependent uptake of Ca^{2+}, which increases in the presence of oxalate. This Ca^{2+} uptake is not affected by 5 mM NaN_3 (see also [5]) and can therefore not be due to mitochondria contaminating the microsomal fraction. Fig. 1 shows the time course of the Ca^{2+} uptake under different experimental conditions. If ATP is present, the Ca^{2+} uptake reaches values which are appreciably larger than those obtained in media without ATP (the ATP-independent binding). After 2—10 min this ATP-dependent Ca^{2+} accumulation reaches a plateau. Adding oxalate (2 or 5 mM) increases the Ca^{2+} accumulation and this uptake now proceeds almost linearly with time during the first 20 min. In Fig. 2 we have plotted the normalized rate of Ca^{2+} uptake expressed by v/V during its stimulation by 5 mM oxalate. These data have been obtained in experiments similar to those depicted in Fig. 1 and in which

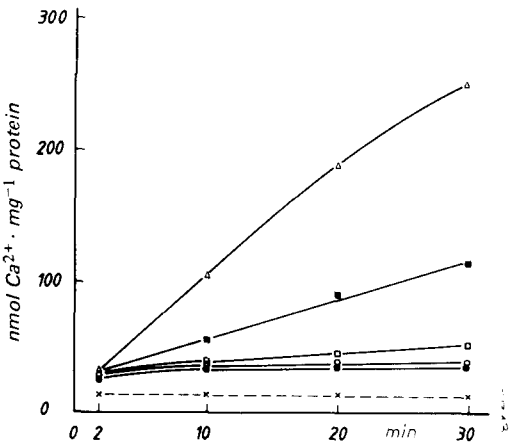

Fig. 1. The $^{45}Ca^{2+}$ uptake by microsomes expressed as nmol · mg^{-1} protein is given for different experimental conditions as a function of time (min). The crosses and the broken line represent the ATP-independent Ca^{2+} binding as obtained in a medium without any ATP. In all other experimental conditions represented by full lines 5 mM ATP had been added, but the oxalate concentration was changed from 0 mM (•), over 0.5 mM (○), 1 mM (◦), 2 mM (■) to 5 mM (△). The composition of the uptake medium is given in the experimental section and $[Ca^{2+}]$ is kept at 10^{-5} M.

the $[Ca^{2+}]$ was varied between 10^{-8} and 10^{-5} M by adjusting the amount of Ca^{2+} added to the solution. The rate of Ca^{2+} uptake in the presence of oxalate was measured from the initial slope of Ca^{2+}-uptake curves. These experimental data were fitted by the Hill equation

$$\frac{v}{V} = \frac{(Ca^{2+})^n}{(K_m)^n + (Ca^{2+})^n} \qquad (1)$$

by means of an iterative non-linear least square method. The following parameters, obtained from four experiments yielding a total of 24 experimental values, provided the best fit for the rate of Ca^{2+} uptake in the presence of 5 mM oxalate: $V = 14.57 \pm 2.38$ (4) nmol · mg^{-1} protein · min^{-1}; $K_m = 0.34 \pm 0.03$ (4) μM; $n = 1.69 \pm 0.09$ (4).

The plateau of Ca^{2+} uptake measured in oxalate-free medium was corrected for the ATP-independent Ca^{2+}-binding. These corrected values have been fitted as a function of $[Ca^{2+}]_0$ by Eqn. 1, but in this case v and V represent the height of the plateau of Ca^{2+} uptake. The following parameters have been calculated from three experiments with a total of 24 experimental values: $V = 32.67 \pm 7.43$ (3) nmol · mg^{-1} protein; $K_m = 0.23 \pm 0.05$ (3) μM; $n = 1.00 \pm 0.10$ (3).

The (Ca^{2+} + Mg^{2+})-ATPase activity of the microsomes

(Ca^{2+} + Mg^{2+})-ATPase was demonstrated and measured in the microsomal fraction using the above described coupled enzyme system. The medium is similar to the one used to study the Ca^{2+} uptake but it contains in addition an ATP regenerating system consisting of NADH, phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase. It was shown in control experiments that these substances did not modify the kinetics of the Ca^{2+} uptake. After addition

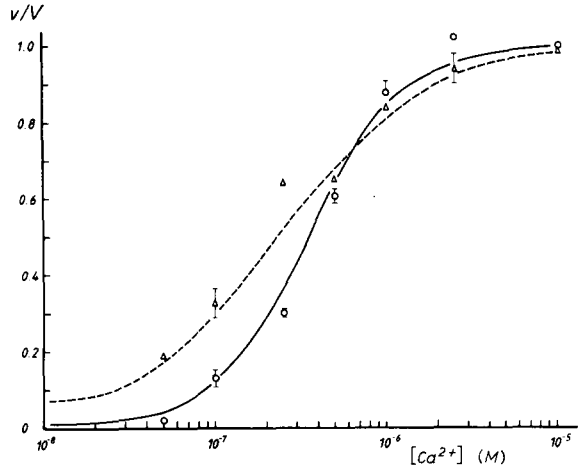

Fig. 2. The rate of $^{45}Ca^{2+}$ uptake was measured in a solution containing 5 mM oxalate and is expressed as v/V on the ordinate. The concentration of Ca^{2+} is given on a logarithmic scale on the abscissa. These results are represented by open circles (○) and the full line is drawn according to Eqn. 1. The plateau of the Ca^{2+} uptake, measured in oxalate-free medium is given on the ordinate by the ratio of the measured value to the maximal value of the uptake. These data are represented by open triangles (△) and the pecked line is drawn again according to Eqn. 1. The parameters of both curves and the number of experiments are given in the text. Vertical bars represent S.E. of mean.

of microsomes to this medium a Mg^{2+}-ATPase activity was recorded. This activity was partly inhibited by 10^{-4} M ouabain. This ouabain-sensitive component was defined as $(Na^+ + K^+)$-ATPase. The remaining 'basal' Mg^{2+}-ATPase activity was increased by increasing $[Ca^{2+}]$ and an additional stimulation was obtained by 10^{-6}—10^{-5} M of the ionophores A23187 or X537A or by 20 μg/ml of the pore-forming ionophore alamethicin (Table II). Because the basal Mg^{2+}-ATPase activity is not affected by the ionophores in Ca^{2+}-free medium, it is likely that these substances act by increasing the Ca^{2+} permeability of the vesicles and hereby reducing the intravesicular $[Ca^{2+}]$. This would diminish the inhibitory action of high intravesicular $[Ca^{2+}]$ on the $(Ca^{2+} + Mg^{2+})$-ATPase, as has been

TABLE II

ATPase ACTIVITY IN THE MICROSOMAL FRACTION

Values are means ± S.E. Number of experiments is given in parentheses. ATPase activities were measured as indicated in experimental section. 'Basal' Mg^{2+}-ATPase is measured in the presence of 10^{-4} M ouabain. $(Na^+ + K^+)$-ATPase is defined as the difference between total Mg^{2+}-ATPase and Mg^{2+}-ATPase after 10^{-4} M ouabain. $(Ca^{2+} + Mg^{2+})$-ATPase is measured in the presence of 10^{-4} M ouabain and at $[Ca^{2+}] = 10^{-5}$ M.

	Activity (nmol ATP · mg^{-1} protein · min^{-1})
'Basal' Mg^{2+}-ATPase	32.99 ± 0.62 (32)
$(Na^+ + K^+)$-ATPase	9.24 ± 0.52 (38)
$(Ca^{2+} + Mg^{2+})$-ATPase	20.04 ± 0.78 (17)
$(Ca^{2+} + Mg^{2+})$-ATPase + X537A 10^{-5} M	27.89 ± 1.64 (9)
$(Ca^{2+} + Mg^{2+})$-ATPase + A23187 10^{-6} or 10^{-5} M	34.79 ± 1.08 (8)
$(Ca^{2+} + Mg^{2+})$-ATPase + alamethicin 20 μg/ml	34.52 ± 0.87 (6)

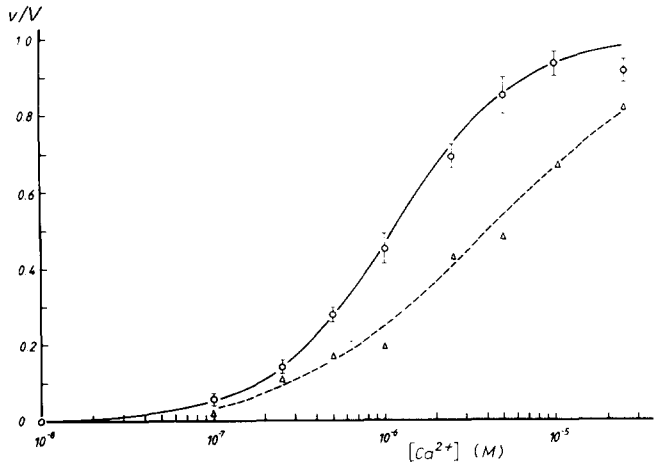

Fig. 3. The $(Ca^{2+} + Mg^{2+})$-ATPase activity measured in the presence (○) or in the absence (△) of ionophore A23187 and expressed as v/V on the ordinate are plotted as a function of the logarithm of $[Ca^{2+}]$. The curves were drawn according to Eqn. 1 with a full line for a medium containing the ionophore and with a pecked line for a medium without. The parameters for both curves and the number of experiments are given in the text. Vertical bars represent S.E. of mean.

reported for fragmented sarcoplasmic reticulum of skeletal muscle [11]. In Fig. 3 we have represented the $(Ca^{2+} + Mg^{2+})$-ATPase activity expressed by v/V as a function of $[Ca^{2+}]$. This ATPase activity observed in the presence of 10^{-5} M of the ionophores A23187 or X537A is represented by the full line. Because the K_m and n values are similar for both ionophores only experiments with A23187 are represented in Fig. 3. These values obtained in six experiments with 70 points are: $K_m = 1.17 \pm 0.15$ μM [6]; $n = 1.23 \pm 0.17$ [6]. The $(Ca^{2+} + Mg^{2+})$-ATPase activity measured in the absence of ionophores (two experiments with 24 points) is represented by a pecked line and has a mean value for K_m of 4.23 μM and for n of 0.78.

Discussion

The main purpose of these experiments was to demonstrate the existence of a $(Ca^{2+} + Mg^{2+})$-ATPase activity in the microsomes of vascular smooth muscle. The data of Table II clearly show that 10^{-5} M Ca^{2+} increases the ATPase activity by a factor of 2 over its basal value. In addition we have tried to relate this enzyme to a Ca^{2+} transport system. This hypothesis is substantiated by the following observations.

1. Both the Ca^{2+} uptake and the $(Ca^{2+} + Mg^{2+})$-ATPase activities have a similar low K_m value for $[Ca^{2+}]$ in the μM range. This K_m of the $(Ca^{2+} + Mg^{2+})$-ATPase has been measured in the presence of a Ca^{2+} ionophore because hereby the inhibitory effect of the intravesicular $[Ca^{2+}]$ is probably prevented. A transport system with a high affinity for Ca^{2+} and a corresponding $(Ca^{2+} + Mg^{2+})$-ATPase could represent two aspects of a mechanism playing an important role in smooth muscle relaxation.

2. Intravesicular Ca^{2+} accumulation might also in smooth muscle micro-

somes, as it has been reported for skeletal muscle microsomes [11], exert an inhibitory effect on the (Ca^{2+} + Mg^{2+})-ATPase. Preventing this accumulation by increasing the Ca^{2+} permeability with Ca^{2+} ionophores not only increases V (Table II) but also diminishes K_m (Fig. 3). If these ionophores (at concentrations exceeding 10^{-6} M) are added to the vesicles before Ca^{2+}, calcium accumulation is prevented.

3. Alamethicin also stimulates the (Ca^{2+} + Mg^{2+})-ATPase. It is believed that this antibiotic at a concentration of 20 μg/ml makes the vesicles permeable not only to Ca^{2+} but also to ATP. It could in this way make also latent ATP-hydrolysing sites accessible in vesicles of reverse sidedness [12].

4. The V of the Ca^{2+} accumulation is rather similar with the V of the (Ca^{2+} + Mg^{2+})-ATPase. The ratio of Ca/ATP = 14.57/20.04 = 0.73. The precision of this ratio can be questioned because the Ca^{2+} uptake has been measured in the presence of oxalate and the (Ca^{2+} + Mg^{2+})-ATPase in the absence of oxalate. We had to follow the latter procedure because oxalate inhibits the pyruvate kinase in our enzyme system. However, we know from control experiments that the rate of Ca^{2+} accumulation measured in a solution containing 5 mM oxalate is a good estimate of the initial rate of Ca^{2+} uptake in oxalate-free medium.

We can conclude from these experiments that (Ca^{2+} + Mg^{2+})-ATPase is present in these microsomes of vascular smooth muscle and that this activity is probably related to an ATP-driven Ca^{2+} transport system.

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