Physiol. Res. 49: 331-338, 2000

Ca-ATPase of Human Myometrium Plasma Membranes

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Received July 13, 1999
Accepted November 5, 1999

Summary

We determined and characterized the Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase (Ca-ATPase) activity in cell plasma membranes from the myometrium of pregnant women, and compared these characteristics to those of the active Ca^{2+}-transport already demonstrated in this tissue. Similarly to the Ca^{2+}-transport system, the Ca^{2+}-ATPase is Mg^{2+}-dependent, stimulated by calmodulin, and inhibited by vanadate. The K_m for Ca^{2+} activation is 0.40 μM, very similar to that found for active calcium transport, i.e. 0.25 μM. Consequently, this Ca^{2+}-ATPase can be responsible for the active calcium transport across the plasma membranes of smooth muscle cells.

Key words

Calcium adenosine triphosphatase • Human myometrium • Smooth muscle.

Introduction

The uterial contractile capacity depends on its smooth muscle layer component. ATP-dependent extrusion of calcium from smooth muscle cells has been associated with their capacity to maintain the state of relaxation (Janis *et al.* 1977). ATP-dependent calcium uptake has been shown in rat myometrium plasma membrane vesicles, suggesting the presence of an active mechanism of calcium transport in these membranes (Grover *et al.* 1982). Regarding this and working with a similar preparation, it has been demonstrated (Enyedi *et al.* 1988) that this tissue possesses a calcium pump with similar characteristics to those of the calcium pump of red cell membranes. However, these authors could not demonstrate Ca^{2+}-ATPase activity in the rat myometrium plasma membranes with characteristics resembling those of the transport system. Specifically, the Ca^{2+}-stimulated ATP hydrolysis that Enyedi *et al.* (1988) could demonstrate in their membrane preparations, was inhibited by the presence of the same amount of Mg^{2+}, which they utilized to measure active calcium transport across the plasma membrane. These results led the authors to suggest that the Ca^{2+}-stimulated ATP hydrolysis measured in their assays was due to the activity of an enzyme different from that one responsible for Ca^{2+}-transport in the rat myometrium. Similar difficulties to demonstrate Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase activity have been found in smooth muscles from other tissues (Akerman and Wikstrom 1979, Soloff and Sweet 1982, Enyedi *et al.* 1989). In the present work, we studied the Ca^{2+}-ATPase activity of human myometrium plasma membranes, in order to compare its characteristics with those of active Ca^{2+}-transport demonstrated in this tissue.

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Methods

Donors

Samples of the human myometrium were obtained by uterine biopsies taken from pregnant patients in the Maternity Hospital "Concepción Palacios" in Caracas, upon delivery by a Caesarean section. The biopsies were taken from healthy patients 23.6±2.8 (S.E.M.) years old and with 39.5±0.3 weeks of gestational age. All the patients had a clinically normal pregnancy, but all had the Caesarean section performed because of an atypical fetus/pelvic ratio, or for a premature membrane rupture. Once obtained, the biopsy samples were placed in a cold solution containing (mM): KCl 100; $MgCl_2$ 5; Tris-HCl 50 (pH 7.4) and immediately transported to the laboratory and processed. The study was approved by the Institutional Review Board of the Maternity "Concepción Palacios", as well as by the Bioethics Committee of IVIC. And all patients gave their informed written consent.

Preparation of plasma membrane enriched fractions

The plasma membrane enriched fractions were prepared at 0 °C, following a modification of the method described by Enyedi *et al.* (1988). Briefly, the myometrial tissue was dissected from the uterine biopsies, minced with scissors, weighted and homogenized in two steps in a solution containing (mM): KCl 100; $MgCl_2$ 5; Tris-HCl 50 (pH 7.4); benzamidine 5; phenylmethyl-sulphonyl fluoride (PMSF) 0.5 and trypsin inhibitor 50 µg/ml. In the first step, the homogenization was carried out with an Ultra Turrax T-25 homogenizer with a dispersing tool S25N for 15 s at 20000 rpm. In the second step, the suspension from the first step was further homogenized with 8 strokes in a Potter-Elvehjem homogenizer, with a teflon pestle at 2500 rpm. Part of the resulting homogenate was dialyzed overnight in the cold, against 1 l of a medium containing (mM): 250 sucrose and 20 Tris-HCl (pH 7.2 at 0 °C). The product of this dialysis constitutes the homogenate (H). The other part of the homogenate was centrifuged for 10 min at 20000 rpm at 4 °C in a RC5B refrigerated Sorvall centrifuge, utilizing a SS34 rotor. The supernatant of this centrifugation was saved and the pellet was resuspended in 5 ml of the same medium, homogenized and centrifuged for a second time. The supernatant of this last centrifugation was added to the first supernatant and the mix was centrifuged for 30 min at 160000 x*g* in a Sorvall Combiplus ultracentrifuge at 4 °C, utilizing a 60Ti rotor. The pellet of this centrifugation was resuspended in 6 ml of a solution containing (mM): 250 sucrose; 5 $MgCl_2$; 10 Tris HCl (pH 7.4 at 0 °C); 5 benzamidine; 0.5 PMSF and 50 µg/ml of trypsin inhibitor. The suspension was homogenized with 4 strokes of a manual homogenizer and divided in 4 parts (1.5 ml each). The 1.5 ml of each fraction was placed on the top of 10 ml of a 20 % percoll solution in the same resuspension medium and centrifuged for 30 min at 33000 x*g*, in a RC5B refrigerated Sorvall centrifuge, at 4 °C. Eleven samples, of 1 ml each, were taken from the top to the bottom of the gradients.

Each fraction was assayed in order to determine the distribution along the gradient of different enzyme markers for different cell membrane organelles. After identification, fractions 1-5 (both included) were pooled constituting fraction F1-5. Fractions 6-11 (both included) were also pooled, constituting fraction F6-11.

Before any assay, the different samples were centrifuged for 60 min at 160000 x*g*, in order to eliminate the percoll present in the preparations. The membranes were resuspended in a solution containing (mM): 250 sucrose and 10 Tris-HCl (pH 7.4 at 0 °C), and then homogenized with 3 strokes of a manual homogenizer. The membrane preparations were divided in 1ml samples, and kept in the freezer at –70 °C until use.

ATPase activities

The Ca-ATPase activity was determined by measuring the quantity of inorganic phosphate liberated from the hydrolysis of ATP, according to the method described elsewhere (Marín *et al.* 1986). Briefly, 180 µl of the incubation medium were preincubated for 2 min at 37 °C, and the reaction was started by addition of 20 µl of membrane suspension. After 10 min incubation, the reaction was stopped by addition of 300 µl of a cold solution contaning: 2.85 % ascorbic acid; 1.76 % HCl; 0.48 % ammonium molybdate; and 2.85 % SDS. The samples were shaken and kept at 0 °C for 10 min. Then, 500 µl of 2 % sodium citrate, 2 % sodium arsenite, and 2 % acetic acid solution were added to each tube, which were then rewarmed, after shaking, for 10 min at 37 °C. The absorbance of each tube was determined in a Milton Roy spectrophotometer at 705 nm. The ATPase activity is expressed in nmoles Pi/(mg prot . min), after subtraction of a blank run in parallel under the same conditions except for the membrane suspension, which was added only after the addition of the ascorbic acid solution. Each sample was run in triplicate or quadruplicate. In all cases, the protein concentration was determined according to the Coomassie blue-dye-

binding-assay method (Bradford 1976). The Ca-ATPase activity was calculated as the difference in the phosphate liberated in a medium containing 200 mM sucrose; 6 mM ATP; 0.1 mM ouabain; 0.125 mM EGTA; 50 mM Tris-HCl (pH 7.4 at 37 °C), and usually 4 mM free magnesium and 30 μM free calcium, minus the one liberated in the same medium, but in the absence of calcium. Purified bovine brain calmodulin was gently supplied by Dr. Gustavo Benaim from Universidad Central de Venezuela.

Other enzyme assays

5'-nucleotidase was determined following the method of Heppel and Hilmoe (1955) and utilized as plasma membrane marker (Daniel 1985, Kidwai 1985). Succinic dehydrogenase activity was utilized as a marker for mitochondrial internal membrane (Kidwai 1985, Malmostöm and Carafoli 1977), and was determined according to the method of King (1967). Acid phosphatase activity was measured following the method described by Hübscher and West (1965) and utilized as a marker for lysosomal membranes (Maunsbach 1966). NADPH-cytochrome c-reductase activity was determined according to the method of Sottocasa et al. (1967) and was utilized as a marker for sarcoplasmic reticulum membranes (Daniel 1985, Kidwai 1985).

Statistical analysis

Statistical analysis was performed by the Student's t-test. All results are expressed as mean ± S.E.M. and (n) represents the number of experiments performed with different membrane preparations. In all cases, the Ca-ATPase activity was calculated from paired data.

Results

Figure 1 presents the 5'-nucleotidase (Fig. 1A) and the NADP-cytochrome *c*-reductase (Fig. 1B) activities of the 11 fractions (from top to bottom) collected from the 20 % percoll gradient. Notice a preferential distribution of the 5'-nucleotidase activity in fractions 3, 4 and 5, indicating the purification of plasma membranes in these fractions. On the other hand, the distribution of the NADP-cytochrome *c*-reductase, a marker utilized to detect the presence of sarcoplasmic reticulum membranes, shows a preferential distribution in fractions 6, 7 and 8 of the gradient.

According to our results and in order to increase the quantity of membranes in each case, we decided to pool fractions 1 to 5 to constitute the fraction 1-5 (F_{1-5}) rich in plasma membranes, and fractions 6 to 11 to constitute fraction 6-11 (F_{6-11}), rich in sarcoplasmic reticulum membranes. The 5'-nucleotidase and the NADPH-cytochrome *c*-reductase activities of fractions F_{1-5} and F_{6-11}, together with their enrichment factors are shown in Table 1. Notice that the higher enrichment factors (fraction activity / homogenate activity) in each case were: 11.1±0.7 for the 5'-nucleotidase activity in fraction F_{1-5} (enriched in plasma membranes), and 3.6±0.3 for the NADPH-cytochrome *c*-reductase activity in fraction F_{6-11} (enriched in sarcoplasmic reticulum membranes). The enrichment factor for the NADPH-cytochrome *c*-reductase activity in fraction F_{1-5}, was only 0.80±0.36.

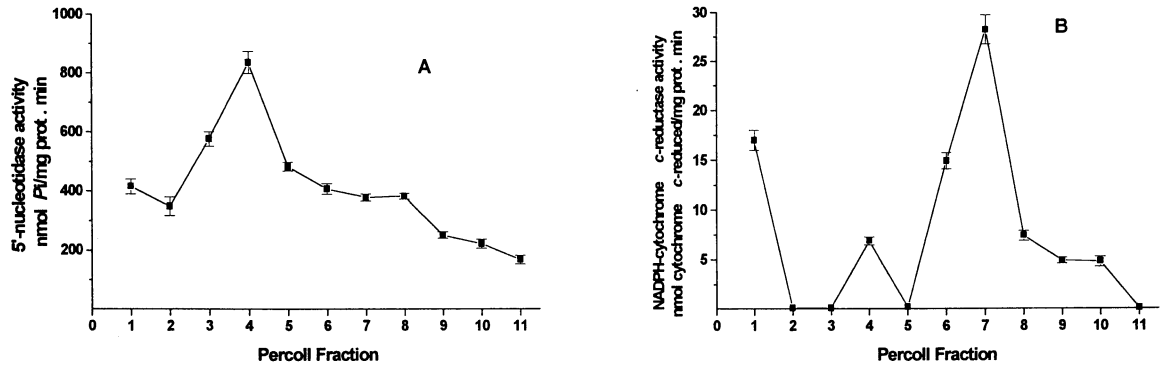

Fig. 1. *(A) Top to bottom distribution of the 5'-nucleotidase activity through the percoll gradient. (B) Top to bottom distribution of the NADPH-cytochrome c-reductase activity through the percoll gradient. Values are means ± S.E.M. of four experiments with different preparations, (n=4).*

Table 1. 5'-nucleotidase and NADPH-cytochrome *c*-reductase activities of homogenates (H) and enriched membrane fractions (F_{1-5} and F_{6-11}) of human myometrium from pregnant patients

Tested enzymes	Enzyme activity			Enrichment factors	
	H	F_{1-5}	F_{6-11}	F_{1-5}	F_{6-11}
5'-nucleotidase[a]	52.0±3.0	578±35	273±11	11.1±0.7	5.3±0.2
NADPH-cytochrome c-reductase[b]	4.4±0.8	3.5±1.6	16±1	0.8±0.4	3.6±0.3

The enrichment factors were calculated as the ratio of enzyme activity in each fraction over the activity in the homogenate. Values are means ± SE of four experiments with different preparations (n = 4). nmoles P_i/(mg prot . min)[a]*; nmol cytochrome c reduced/(mg prot . min)* [b]

The activities of the other tested enzymes in F_{1-5}, as compared to the total homogenate (H), are shown in Table 2. The succinic dehydrogenase activity, marker for inner mitochondrial membranes, was practically zero. On the other hand, the acid phosphatase activity, marker for lysosomal membranes, was enriched by a factor of 13 when compared with the activity of the homogenate. Even when the acid phosphatase assay was carried out in a medium at pH 5.4 and the Ca-ATPase activity was determined in a medium at pH 7.4, it is important to find out if calcium affects the activity of the acid phosphatase.

To answer this question, we studied the acid phosphatase activity of fraction F_{1-5} as a function of the pH value of the incubation medium, either in the presence or in the absence of 30 µM free calcium (Fig. 2). The result of this experiment suggests that 30 µM calcium in the incubation medium has no effect on the activity of the enzyme. Also, at pH 7.4 there is practically no acid phosphatase activity. In conclusion, any Ca^{2+}-stimulated ATP hydrolysis obtained with fractions F_{1-5} could result from the activity of a plasma membrane enzyme.

Table 2. Succinic dehydrogenase and acid phosphatase activities of homogenates (H) and enriched plasma membrane fractions (F_{1-5}) of human myometrium from pregnant patients.

Tested enzymes	H	F_{1-5}
Succinic dehydrogenase[a]	1.5±0.3	N.D.
Acid phosphatase[b]	6.0±0.6	39.0± 2.9

Values are means ± S.E.M. of four experiments with different preparations, expressed as µmol succinate oxidized/(mg prot . min) [a]*; nmoles P_i/(mg prot . min)* [b]

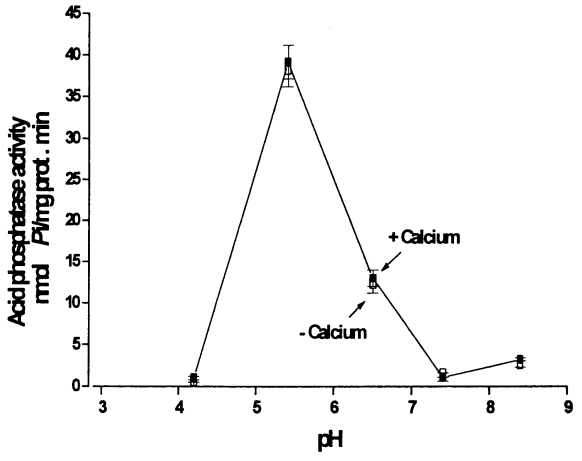

Fig. 2. *Effect of the pH of the incubation medium on the acid phosphatase activity, in the presence or absence of 30 µM free calcium. Values are means ± S.E.M. of four experiments with different preparations.*

Table 3. Hydrolysis of ATP by membrane fractions F_{1-5} and F_{6-11}, determined in the presence of 30 μM free calcium and/or 50 μM free magnesium

Membrane fraction	ATP hydrolysis: nmoles P_i/(mg prot . min) Incubation conditions		
	Mg^{2+}	Ca^{2+}	Mg^{2+}+Ca^{2+}
F_{1-5}	194±10	116±8	81±4
F_{6-11}	165± 4	82±3	104±4

In all the assays, the ATP concentration was 6 mM. Values are means ± S.E.M. of four experiments with different preparations.

It has been shown that the smooth muscle plasma membranes can hydrolyze important quantities of ATP in the presence of Mg^{2+} or Ca^{2+} alone (Enyedi *et al.* 1988, Grover 1985). This represents a technical problem when trying to determine the Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase activity in these tissues. Fraction F_{1-5} was assayed for ATP hydrolysis in the presence of Mg^{2+}, Ca^{2+} or Mg^{2+}+ Ca^{2+} (Table 3.). These experiments were carried out with an ATP concentration of 6 mM. Notice an ATPase activity in the presence of 50 μM free Mg^{2+}, a lower activity in the presence of 30 μM free Ca^{2+}, and even lower activity in the presence of 50 μM Mg^{2+} and 30 μM Ca^{2+}. In the light of these results and considering the absolute requirement of Mg^{2+} for the work of the transporting Ca-ATPase (Carafoli 1991, 1992), we studied the effect of free Mg^{2+} concentration in the incubation medium, on the ATPase activity of fraction F_{1-5}, either in the absence or in the presence of 30 μM free calcium (Fig. 3). The ATPase activity increased with the free Mg^{2+} concentration either in the presence or absence of free calcium. However, the activity was lower in the presence of Ca^{2+} than in its absence for all the tested free Mg^{2+} concentrations below 1 mM. In free Mg^{2+} concentrations higher than 1 mM, on the other hand, the ATPase activity was higher when 30 μM free calcium was added to the incubation medium. Therefore the hydrolysis of ATP stimulated by 30 μM free calcium and dependent of free magnesium concentrations higher than 1 mM, will be referred as Ca-ATPase activity.

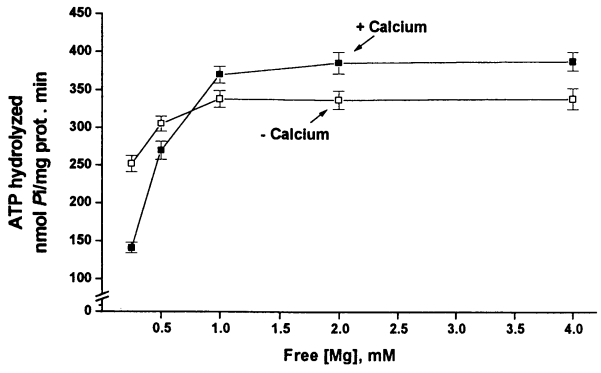

Fig. 3. *Effect of free magnesium concentration in the incubation medium on the hydrolysis of ATP in human myometrium plasma membrane fractions, in the presence or absence of 30 μM free calcium. Values are means ±S.E.M. of four experiments with different preparations.*

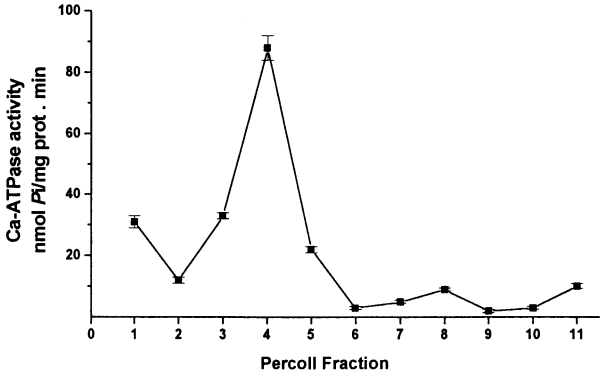

Fig. 4. *Top to bottom distribution of Ca^{2+}-ATPase activity through the percoll gradient. Values are means ± S.E.M. of four experiments with different preparations.*

Once determined the conditions for measuring the Ca-ATPase activity, we studied the distribution of this activity along the different samples of the percoll gradient. The assays were carried out in the presence of 6 mM ATP, 4 mM free Mg^{2+} and 30 μM free Ca^{2+} (Fig. 4). Almost all Ca-ATPase activity is distributed along the fractions 1 to 5, reaching maximal values at fraction 4. This distribution of the Ca-ATPase activity along the percoll gradient shows a clear correlation (r=0.88) with the distribution of the 5'-nucleotidase activity (Fig.1A).

Table 4. Effect of exogenous calmodulin on the Ca^{2+}-ATPase activity of enriched plasma membrane fractions (F_{1-5}) of human myometrium

	ATPase activity: nmoles P_i/(mg prot . min) Incubation conditions		
	Mg^{2+} (a)	Mg^{2+}+ Ca^{2+} (b)	Ca-ATPase (b-a)
– *Calmodulin*	294±15	335±19	41±3*
+ *Calmodulin*	292±16	344±19	52±1*

Ca-ATPase activity was calculated as the difference of the phosphate liberated in the medium containing 200 mM sucrose; 6 mM ATP; 0.1 mM ouabain; 0.125 mM EGTA; 50 mM Tris-HCl (pH 7.4 at 37 °C), 150 nM calmodulin, 4 mM free magnesium and 10 μM free calcium (Mg^{2+}+ Ca^{2+} medium), minus that one liberated in the same medium, but in the absence of calcium (Mg^{2+} medium). Values are means ±S.E.M. of four experiments with different preparations. The S.E.M. for the Ca-ATPase was calculated by paired data. $^{*}P < 0.01$

In general, the Ca-ATPase of plasma membranes is stimulated by calmodulin (Carafoli 1991, Muallem and Karlish 1981). Table 4 shows the ATPase activity of fraction F_{1-5} determined in the presence of Mg^{2+} (Mg-ATPase) or Mg^{2+}+ Ca^{2+}, with and without 150 nM exogenous calmodulin. The difference between the activity measured in the presence of Mg^{2+}+ Ca^{2+} minus that one measured in the presence of Mg^{2+} represents the Ca-ATPase activity. The membranes were treated earlier with EGTA according to Morel *et al.* (1981), in order to release endogenous calmodulin bound to them. While calmodulin does not produce any effect on the Mg-ATPase activity, its presence in the incubation medium produced a significant activation of the Ca-ATPase activity, which increased in about 27 %.

The Ca-ATPase, as well as the other P-type ATPases, are inhibited by vanadate (Carafoli 1992). Hence, we tested the effect of the presence of 20 μM vanadate in the incubation medium on the Ca-ATPase activity of F_{1-5} fraction. It was demonstrated that vanadate inhibits totally the expression of the Ca-ATPase of these membranes (46±3 nmoles P_i/(mg prot . min), in the absence of vanadate vs 5±1 nmoles P_i/(mg prot . min), in the presence of vanadate).

One of the main characteristics of the Ca-ATPase of plasma membranes is its high affinity for calcium. We evaluated the behavior of the Ca-ATPase of the membrane fraction F_{1-5}, as a function of increasing free calcium concentrations in the incubation medium. The results are shown in Figure 5: there is a clear Michaelis-Menten behavior, reaching the enzyme maximal activity values at a concentration of 5 μM free calcium. The K_m of the ATPase for free Ca^{2+} was found to be 0.40±0.02 μM, and the V_{max} of the enzyme was calculated to be 55±3 nmoles P_i/(mg prot . min).

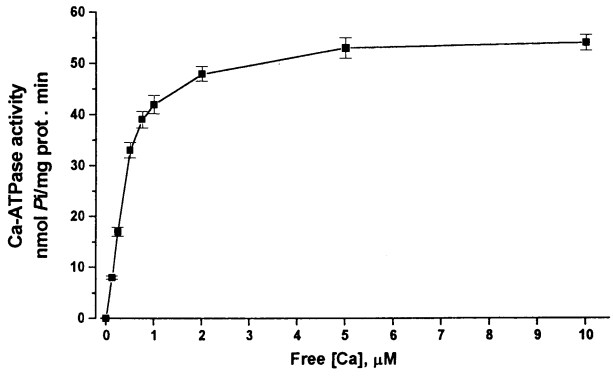

Fig. 5. *Effect of free calcium concentration in the incubation medium on the hydrolysis of ATP by human myometrium plasma membrane fractions. Values are means ± S.E.M. of four experiments with different preparations. K_m for calcium: 0.40±0.02 μM, V_{max} = 55±3 nmoles.*

Discussion

The human myometrium cell membrane fractions F_{1-5} studied in our work are rich in plasma membranes and poor in sarcoplasmic reticulum membranes, as indicated by their high 5'-nucleotidase activity and their low NADPH-cytochrome *c*-reductase activity (Fig. 1). A high 5'-nucleotidase activity has been demonstrated for rat myometrium plasma membranes (Enyedi *et al.* 1989, Grover *et al.* 1980) and is also typical for smooth muscle cells (Grover 1985). Even when the F_{1-5} fraction, on the other hand, shows a high acid phosphatase activity (Table 2) indicating enrichment of lysosomal membranes, this activity is not affected by calcium and, besides, it is practically zero at pH 7.4 (Fig. 2), which is the pH utilized to determine the Ca-ATPase activity.

The F_{1-5} membrane fraction hydrolyzes ATP in the presence of either 30 μM free Ca^{2+} or 50 μM free Mg^{2+}. The presence of both cations in the assay medium gives, however, a lower ATPase activity than the one seen when they are alone (Table 3). Since the presence of Mg^{2+} is essential for the plasma membrane Ca-ATPase to work (Carafoli 1991, 1992), we studied the effect of increasing free Mg^{2+} concentration on the hydrolysis of ATP in the presence of 30 μM free Ca^{2+}. For free Mg^{2+} concentrations lower than 1 mM, the presence of 30 μM free Ca^{2+} in the incubation medium produced a clear inhibition of the ATP hydrolysis (Fig. 3). However, for free Mg^{2+} concentrations of 1 mM or higher, there was a clear and significant stimulation of the ATPase activity by 30 μM free Ca^{2+}.

Ca-ATPase activity in the presence of 4 mM free Mg^{2+} and 30 μM free Ca^{2+} was determined for all the fractions of the percoll gradient. As shown in Figure 4, the activity is practically limited to fractions 1 to 5, being negligible or absent in fractions 6 to 11. This distribution strongly resembles that of the 5'-nucleotidase activity (Fig. 1A, r=0.88), indicating that the Ca-ATPase demonstrated in this work, is present in the plasma membranes of human myometrium. This is corroborated by the stimulatory effect of calmodulin on the ATPase activity (Table 4), by its sensitivity to vanadate, and by its low K_m for Ca^{2+} (0.40±0.02 μM), which are characteristic for the plasma membrane Ca-ATPases (Carafoli 1991, 1992). In conclusion, we have shown that plasma membranes from human myometrium cells possess a Ca-ATPase activity with similar characteristics to those found by Enyedi *et al.* (1988) for calcium transport by plasma membrane vesicles of rat myometrium.

Acknowledgements

This project was partially supported by grant No. H9/181/R427, project 96350, from the World Health Organization.

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Reprint requests

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