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A Mg^{2+}-INDEPENDENT HIGH-AFFINITY Ca^{2+}-STIMULATED ADENOSINE TRIPHOSPHATASE IN THE PLASMA MEMBRANE OF RAT STOMACH SMOOTH MUSCLE

SUBCELLULAR DISTRIBUTION AND INHIBITION BY Mg^{2+}

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Plasma membrane enriched fraction isolated from the fundus smooth muscle of rat stomach displayed Ca^{2+}-stimulated ATPase activity in the absence of Mg^{2+}. The Ca^{2+} dependence of such an ATPase activity can be resolved into two hyperbolic components with a high affinity ($K_m = 0.4$ μM) and a low affinity ($K_m = 0.6$ mM) for Ca^{2+}. Distribution of these high-affinity and low-affinity Ca^{2+}-ATPase activities parallels those of several plasma membrane marker enzyme activities but not those of endoplasmic reticulum and mitochondrial membrane marker enzyme activities. Mg^{2+} also stimulates the ATPase in the absence of Ca^{2+}. Unlike the Mg^{2+}-ATPase and low-affinity Ca^{2+}-ATPase, the plasmalemmal high-affinity Ca^{2+}-ATPase is not sensitive to the inhibitory effect of sodium azide or Triton X-100 treatment. The high-affinity Ca^{2+}-ATPase is noncompetitively inhibited by Mg^{2+} with respect to Ca^{2+} stimulation. Such an inhibitory effect of Mg^{2+} is potentiated by Triton X-100 treatment of the membrane fraction. Calmodulin has little effect on the high-affinity Ca^{2+}-ATPase activity of the plasma membrane enriched fraction with or without EDTA pretreatment. Findings of this novel, Mg^{2+}-independent, high-affinity Ca^{2+}-ATPase activity in the rat stomach smooth muscle plasma membrane are discussed with those of Mg^{2+}-dependent, high-affinity Ca^{2+}-ATPase activities previously reported in other smooth muscle plasma membrane preparations in relation to the plasma membrane Ca^{2+}-pump.

Introduction

In smooth muscles, the plasma membrane has been considered a major site for the regulation of cytoplasmic concentrations of Na^+ and Ca^{2+} via the Na^+-pump (presumably the $(Na^+ + K^+)$-ATPase), Ca^{2+}-pump (presumably the $(Ca^{2+} + Mg^{2+})$-ATPase) and Na^+-Ca^{2+} exchange systems [1,2]. However, the biochemical entities of some of these systems have not been positively identified and characterized in smooth muscle due to their extremely low activities [3–7]. Studies from this laboratory have been focussed upon the characterization of Ca^{2+}-transport by isolated smooth muscle plasma membranes [1]. Using highly purified plasma membrane fractions isolated from a number of smooth muscles, we have demonstrated that the isolated plasma membrane vesicles were capable of transporting Ca^{2+} in the presence of ATP and Mg^{2+} [8–12]. On the other hand, the attempts to measure and identify this Ca^{2+}-pump as a Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase have not been promising due to the presence of a very high level of Mg^{2+}-ATPase in most smooth muscle plasma membranes [6,13]. Recently, several investigators have reported the presence of an

operationally defined (Ca^{2+} + Mg^{2+})-ATPase in microsomal membrane fractions isolated from various smooth muscles [14–16]. In the present study, we have also observed a Ca^{2+} stimulation of Mg^{2+}-activated ATPase activity in the plasma membranes enriched fraction from the rat gastric fundus muscle. However, such a Ca^{2+}-stimulated ATPase activity in the presence of Mg^{2+} can be interpreted in terms of the presence of a previously unidentified high-affinity Mg^{2+}-independent Ca^{2+}-ATPase in the smooth muscle plasma membrane.

Experimental procedures

Materials. Chloride salt of sodium, calcium and magnesium, and sodium azide were from Baker. Vanadate ion-free, di(ethanolamine) or disodium salt of ATP was from Sigma. $^{45}CaCl_2$ was purchased from New England Nuclear. Triton X-100 for liquid scintillation counting was from Amersham. Purified bovine brain calmodulin was a generous gift from Dr. J.S. Law (Georgetown University Hospital, Washington, DC, U.S.A.). All solutions were prepared using deionized and distilled water.

Preparation of plasma membranes. Plasma membrane enriched fraction was isolated from the smooth muscle layer of rat gastric fundus by differential and discontinuous sucrose density gradient centrifugation as described previously in detail [17]. Briefly, in each experiment gastric fundus smooth muscles from 20 to 25 male Wistar rats were homogenized in 250 mM sucrose buffered with 10 mM imidazole at pH 7.0 (sucrose-imidazole) by Polytron and centrifuged at $900 \times g$ for 10 min to remove the cell debris and nuclei. The post-nuclear supernatant (P) was then centrifuged at $10000 \times g$ for 10 min to sediment the mitochondrial membranes (P1) and subsequently at $105000 \times g$ for 30 min to separate the crude microsomal fraction from the soluble protein fraction (S). The crude microsomal fraction was then resuspended and centrifuged at $10000 \times g$ again for 10 min to remove the remaining mitochondrial membrane fragments (P2). The supernatant, which is the refined microsomal membrane fraction (M), was placed on top of a discontinuous sucrose density gradient (15%, 30%, 40% and 60% sucrose layers) and centrifuged at $113000 \times g$ for 2 h. Four submicrosomal fractions were collected at the interphases of the sucrose layers and designated F1 (8%/15%), F2 (15%/30%), F3 (30%/40%) and F4 (40%/60%). All these subcellular fractions have been extensively characterized [17]. The plasma membrane enriched fraction F2 was employed throughout the present work unless otherwise specified.

Enzyme assays. Various marker enzyme activities were determined as described previously [17]. ATPase activities were determined as the release of inorganic phosphate ions from 5 mM ATP in the presence of 5 mM Mg^{2+} for Mg^{2+}-ATPase or 5 mM Ca^{2+} for low affinity Ca^{2+}-ATPase in 50 mM imidazole buffer at 37 °C (pH 6.7). High-affinity Ca^{2+}-ATPase activity was determined under the similar condition with added 100 μM Ca^{2+} and various amounts of EGTA to achieve desired free Ca^{2+} concentrations in the range of 0.2–10.0 μM [18]. Reaction was started by the addition of 50 μl of membrane fraction containing 20–40 μg protein to a final volume of 1.0 ml reaction mixture and terminated by addition of trichloroacetic acid. The ATPase activity was routinely measured against the blank control in which the buffer medium contained ATP, membrane protein and 0.5 mM EGTA. However, in the absence of added divalent metal ions, the blank control values were the same in the presence as well as in the absence of EGTA. The ATPase activity was linear with reaction time up to 30 min and a 15 min reaction time was therefore routinely employed. When the high-affinity Ca^{2+}-ATPase activity was measured in the presence of Mg^{2+}, the basal Mg^{2+}-ATPase activity was subtracted from the total ATPase activity unless otherwise specified. When Triton X-100 treated membrane was employed for ATPase assay, the membrane fraction was preincubated with Triton X-100 (1.0% final concentration) at 5 °C for 30 min prior to the assay for ATPase activity. After preincubation, the membrane suspension was diluted 2-fold with sucrose-imidazole buffer and 50-μl aliquots were used for ATPase assay. Thus, the final Triton X-100 concentration in the reaction mixtures was 0.025%, which did not have significant effect on any of the ATPase activities described above. In studying the effect of calmodulin on high affinity Ca^{2+}-ATPase activity,

20-μl aliquots of 50 mM EDTA or EGTA (pH 7.0) or 20 μl water were added to 1 ml of membrane suspensions and the mixtures were left to stand on ice for 30 min, then diluted 10-fold with sucrose-imidazole buffer and centrifuged at $150000 \times g$ for 45 min. The membrane pellets were resuspended in sucrose-imidazole to desired protein concentrations and used for ATPase assay. The treatment of membranes with these chelating reagents also ensures the removal of endogenous or contaminating Mg^{2+}. To ensure against contaminating Mg^{2+} possibly derived from the buffer solution, the buffer solution with overnight treatment using chelax-100 resin has also been employed. The ATPase activities were not altered by such treatment. Determination of the Mg^{2+} concentrations of the assay medium in the absence of added $MgCl_2$ by atomic absorption spectrophotometry revealed ≤ 3 μM of contaminating Mg^{2+} which was completely eliminated after treatment with chelax-100 resin.

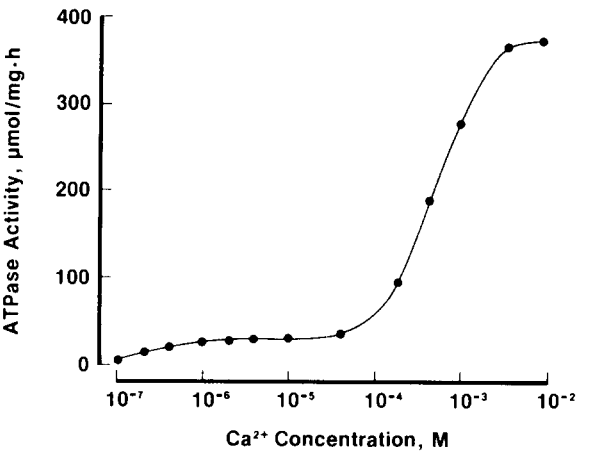

Fig. 1. Ca^{2+} concentrations dependence of ATP hydrolysis by plasma membrane enriched fraction isolated from the smooth muscle of rat gastric fundus. After the initiation of reaction by addition of membrane fraction (40–60 μg/ml), the reaction was incubated for 15 min at 37 °C. Ca-EGTA buffer system [18] was employed for Ca^{2+} concentrations < 10 μM.

Results

Ca^{2+}-concentration dependence of ATPase activity

Fig. 1 shows the hydrolysis of ATP by plasma membrane enriched fraction isolated from gastric fundus smooth muscle in the presence of various Ca^{2+} concentrations in a typical experiment. A biphasic saturation profile was obtained: one saturated at 2–10 μM Ca^{2+} with a low specific activity of 40–60 μmol/mg h and the other saturated at 5–10 mM Ca^{2+} with a substantially higher specific activity of 350–500 μmol/mg h. Analysis of the double reciprocal plots of the saturation profiles in these two regions of Ca concentrations reveals a linear relationship and suggesting that these two classes of Ca^{2+}-ATPase activities follow a Michaelis-Menten kinetic pattern with a high affinity ($K_m = 0.41$ μM) and a low affinity ($K_m = 0.65$ mM) for Ca^{2+}.

Subcellular distribution of Ca^{2+}-ATPase activities

To investigate the subcellular origin of these two classes of Ca^{2+}-ATPase activities in rat gastric fundus muscle, the high-affinity Ca^{2+}-ATPase (Ca^{2+}_H-ATPase) and low-affinity Ca^{2+}-ATPase (Ca^{2+}_L-ATPase) activities were determined in all subcellular fractions isolated from the fundus smooth muscle and compared to various membrane marker enzyme activities including 5′-nucleotidase and phosphodiesterase I (markers for plasma membrane), NADPH-cytochrome *c* reductase (marker for endoplasmic reticulum), Rotenone-insensitive, NADH-cytochrome *c* reductase (marker for outer mitochondrial membrane) and cytochrome *c* oxidase (marker for inner mitochondrial membrane). Both Ca^{2+}_H-ATPase and Ca^{2+}_L-ATPase activities closely paralleled the plasma membrane marker enzyme activities. For brevity, only the distribution of these Ca^{2+}-ATPase activities with that of phosphodiesterase I activity is shown in Fig. 2. Table I clearly indicates that a high positive linear correlation exists between these Ca^{2+}-ATPase activities and the activity of plasma membrane marker enzymes but not that of endoplasmic reticulum or mitochondrial membranes.

Effect of Mg^{2+}

Table II shows that Mg^{2+} is also an effective activator of ATPase. Although 2 μM Ca^{2+} further stimulates the Mg^{2+}-activated ATPase activity, the stimulation is substantially diminished at high Mg^{2+} concentration. Analysis of the double reciprocal plots of Ca^{2+} activation of Ca^{2+}_H-ATPase in the presence and absence of 0.5 mM Mg^{2+}

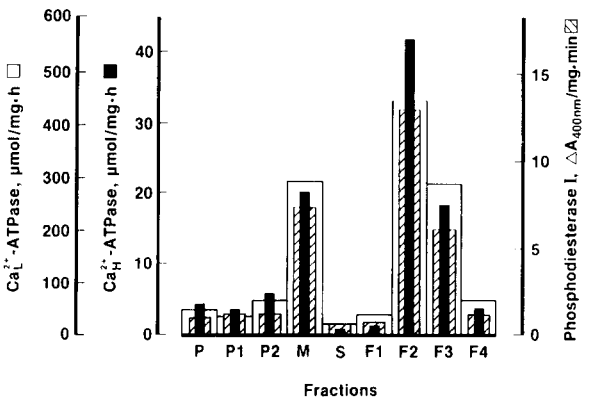

Fig. 2. Subcellular distribution of Ca^{2+}_H-ATPase and Ca^{2+}_L-ATPase activities. See Methods for the abbreviation of various subcellular fractions isolated from the smooth muscle of rat gastric fundus. Measurements of all enzyme activities were made using the fractions from the same preparation.

TABLE II

Ca^{2+} STIMULATION OF ATPase ACTIVITY IN THE PRESENCE OF Mg^{2+}

ATPase activity was measured as release of inorganic phosphate in the presence and absence of 2 μM free Ca^{2+} buffered by the Ca-EGTA system. Ca^{2+}-stimulated ATPase is defined as the difference in activities determined in the presence and absence of Ca^{2+}. Data are expresses as mean $\pm$S.D. from three replicates using the same membrane preparation. Similar results were obtained using different membrane preparations.

Mg^{2+} (mM)	ATPase activity (μmol/mg per h)		
	No Ca^{2+}	2 μM Ca^{2+}	Ca^{2+} stimulation
None	0	53.6 $\pm$ 1.9 *	53.6
0.5	215.7 $\pm$ 3.0	246.4 $\pm$ 6.4 *	30.7
1.0	361.3 $\pm$ 3.5	372.0 $\pm$ 1.5 *	10.7

* Significantly different from activity obtained in the absence of Ca^{2+} (Student's t-test, $P < 0.05$).

indicates that Mg^{2+} inhibited the Ca^{2+}_H-ATPase by approx. 50% at all Ca^{2+} concentrations (0.1–5.0 μM) studied, and the inhibition by Mg^{2+} appeared to be noncompetitive with respect to Ca^{2+} with a K_i of $6 \cdot 10^{-4}$ M.

Effect of azide

Table III shows the effect of 5 mM sodium azide on the ATP-hydrolysis by the plasma membrane enriched fraction from fundus smooth muscle in the presence of 5 mM Mg^{2+}, 5 mM Ca^{2+} and 2 μM Ca^{2+}. Azide potently inhibited the Mg^{2+}-ATPase activity and slightly suppressed the Ca^{2+}_L-ATPase activity. In contrast, azide had no significant effect on the Ca^{2+}_H-ATPase activity in the presence or absence of 0.5 mM Mg^{2+}. Therefore, the Ca^{2+}_H-ATPase activity can be dissociated from the Ca^{2+}_L-ATPase and Mg^{2+}-ATPase activities by the effect of azide.

TABLE I

CORRELATION COEFFICIENTS BETWEEN VARIOUS MARKER ENZYME ACTIVITIES AND Ca^{2+}-ATPase ACTIVITIES

Correlation coefficients, r, were calculated by linear regression using data obtained form the nine fractions shown in Fig. 3. The correlation coefficient between Ca^{2+}_H-ATPase and Ca^{2+}_L-ATPase activities is 0.992 ($P < 0.001$). RI, rotenone-insensitive.

	Ca^{2+}_H-ATPase		Ca^{2+}_L-ATPase	
	r	P	r	P
Phosphodiesterase I	0.986	< 0.001	0.992	< 0.001
5′-Nucleotidase	0.983	< 0.001	0.993	< 0.001
NADPH-cyt. c reductase	0.457	> 0.05	0.428	> 0.05
RI NADH-cyt. c reductase	0.553	> 0.05	0.504	> 0.05
Cytochrome c oxidase	−0.095	> 0.05	−0.096	> 0.05

Effect of Titron X-100 treatment

Table IV shows that Triton X-100 treatment of the plasma membrane enriched fraction resulted in a substantial loss of Mg^{2+}-ATPase and Ca^{2+}_L-ATPase activities. In contrast, Ca^{2+}_H-ATPase retained > 90% of its activities under the same conditions. Again, Ca^{2+}_H-ATPase activity can be dissociated from Ca^{2+}_L-ATPase and Mg^{2+}-ATPase by the effect of Triton X-100 treatment. Fig. 3 further shows that increasing concentrations of Mg^{2+} progressively enhanced its inhibitory effect on the Ca^{2+}_L-ATPase activities in the absence as well as the presence of Triton X-100 treatment. However, the inhibition by Mg^{2+} was more potent using Triton X-100 treated membrane fraction than that using the untreated control membrane fraction (see insets). However, Triton X-100 treatment did not alter the K_m for Ca^{2+} in the absence of Mg^{2+} and the noncompetitive mode of inhibition by Mg^{2+} with respect to Ca^{2+} activation of Ca^{2+}_H-ATPase. Nevertheless, the K_i for Mg^{2+} based

TABLE III

EFFECT ON SODIUM AZIDE ON THE Ca_H^{2+}-ATPase ACTIVITY OF FUNDUS MUSCLE PLASMA MEMBRANE

ATPase activities were determined in the presence and absence of 5 mM sodium azide. Sodium chloride at 5 mM had no effect of any of the ATPase activities. Data are expressed as mean ± S.D. from three replicates. Numbers in the parentheses stand for the relative activities taking the activities in the absence of azide as 100%. Ca_H^{2+}-ATPase activity was measured in the presence of 5 μM free Ca^{2+}. The basal Mg^{2+}-ATPase activity was substracted from the total activity when Ca_H^{2+}-ATPase activity was determined in the presence of 0.5 mM Mg^{2+}.

	ATPase activity (μmol/mg per h)			
	No azide		5 mM azide	
Mg^{2+}-ATPase	477.0 ± 33.4	(100%)	221.9 ± 4.8 *	(44.4%)
Ca_L^{2+}-ATPase	469.3 ± 26.7	(100%)	384.8 ± 9.3 *	(82.0%)
Ca_H^{2+}-ATPase				
No. Mg^{2+}	61.4 ± 0.6	(100%)	61.7 ± 0.4	(100.5%)
0.5 mM Mg^{2+}	32.2 ± 0.8 **	(100%)	33.3 ± 3.8 **	(103.4%)

* Significantly different from values obtained in the absence of azide ($P < 0.05$).
** Significantly different from values obtained in the absence of Mg^{2+} ($P < 0.05$).

upon noncompetitive mode of inhibition was reduced to $1.5 \cdot 10^{-4}$ M under such conditions.

Effect of calmodulin on high-affinity Ca^{2+}-ATPase activity

To investigate whether calmodulin has a stimulatory effect on the Ca_H^{2+}-ATPase activity of the smooth plasma membrane, Ca_H^{2+}-ATPase activities were determined in the presence and absence of 5.5 μg/ml calmodulin using the plasma membrane enriched fractions with and without EDTA pretreatment. Table V shows that calmodulin had no significant effect on the Ca_H^{2+}-ATPase activity at Ca^{2+} concentrations below or near the K_m for Ca^{2+} (0.2 or 0.5 μM, respectively) and at the saturating level (2.0 μM) nor did it affect the Ca_H^{2+}-ATPase activity after the pretreatment of the membrane fraction with EDTA, a procedure effec-

TABLE IV

EFFECT OF TRITON X-100 TREATMENT OF THE ATPase ACTIVITIES

See Method for experimental details of membrane treatment with Triton X-100 (1%). Data are expressed as mean ± S.D. from three replicates. Numbers in the parentheses stand for the percent residual activities remained in Triton X-100 treated membranes.

	ATPase activities (μmol/mg per h)	
	Control membranes	Treated membranes
Mg^{2+}-ATPase	396.6 ± 12.0 (100%)	7.1 ± 3.6 (1.8%)
Ca_L^{2+}-ATPase	385.5 ± 3.1 (100%)	20.2 ± 3.0 (5.2%)
Ca_H^{2+}-ATPase	39.1 ± 1.2 (100%)	35.9 ± 1.7 (92%)

TABLE V

EFFECT OF CALMODULIN ON THE Ca_H^{2+}-ATPase ACTIVITY OF FUNDUS PLASMA MEMBRANE

Ca^{2+}-ATPase activity were measured in the presence and absence of calmodulin using control membrane and EDTA washed membrane obtained from the same preparation. See methods and text for details. Data are expressed as mean ± S.D. from three replicates.

Conditions	Ca_H^{2+}-ATPase activity (μmol/mg per h)	
	No calmodulin	5.5 μg/ml calmodulin
Experiment set 1 (control membranes)		
0.2 μM Ca^{2+}	15.0 ± 1.1	14.6 ± 0.4
0.5 μM Ca^{2+}	25.8 ± 1.3	25.4 ± 1.4
2.0 μM Ca^{2+}	38.1 ± 1.1	39.0 ± 0.6
Experiment set 2 (2.0 μM free Ca^{2+})		
1 mM EDTA pretreated [a]	42.7 ± 0.7	42.5 ± 0.9
Diluted with water and resuspended	40.8 ± 1.3	40.7 ± 1.3

[a] In a separate experiment, similar results were also obtained using membrane fraction pretreated with 1 mM EGTA instead of 1 mM EDTA.

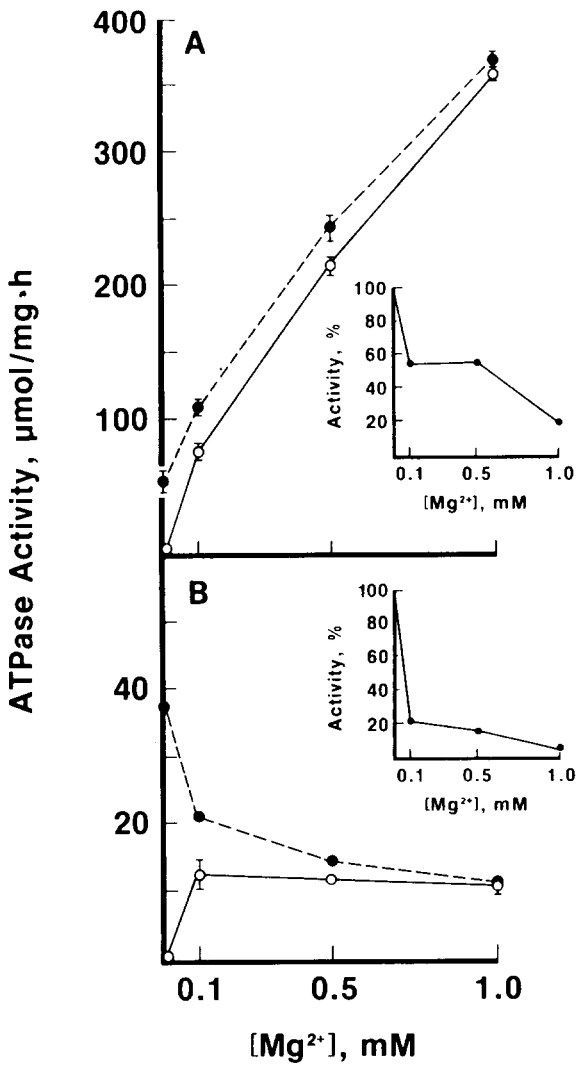

Fig. 3. Effect of Mg^{2+} concentration on the ATP hydrolysis by the fundus muscle plasma membrane; in the presence (●) and absence (○) of 2 μM Ca^{2+}. The ATPase activities were measured using plasma membrane fraction without (A) and with (B) Triton X-100 (1%) pretreatment. The insets show the relative residual Ca^{2+}-stimulated ATPases activities in the presence of increasing concentrations of Mg^{2+}.

tively used to remove the possible endogeneous calmodulin bound to the plasma membranes. Furthermore, the Ca_H^{2+}-ATPase activity was not affected by the calmodulin antagonists, chloropromazine and fluphenazine at 10^{-5} M (data not shown). The lack of effect of EDTA treatment of the membrane fraction on the Ca_H^{2+}-ATPase activity in the absence or presence calmodulin lends additional support that the observed Ca_H^{2+}-ATPase activity has no absolute requirement for Mg^{2+} including endogenously bound Mg^{2+}.

Discussion

The present study, together with our previous findings [6,13] clearly indicates that the plasma membrane isolated from the smooth muscle or rat gastric fundus displays a number of ATPase activities including Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, low-affinity Ca^{2+}-ATPase (Ca_L^{2+}-ATPase) and high-affinity Ca^{2+}-ATPase (Ca_H^{2+}-ATPase) activities. All these ATPase activities were closely associated with the plasma membranes (Fig. 2 and Table I, also see Ref. 13). A high level of Mg^{2+}-ATPase and Ca_L^{2+}-ATPase activities has previously been found in the microsomal fractions from various smooth muscles [19–21] and recently been shown to be of plasma membrane origin [6,12,22]. To our knowledge, the presence of a high-affinity, Mg^{2+}-independent Ca^{2+}-ATPase in the smooth muscle plasma membrane has not been reported previously, although such a Ca_H^{2+}-ATPase has been identified in the cell membrane of other tissues [23–27].

The activation of Ca_H^{2+}-ATPase of smooth muscle plasma membrane does not have an absolute requirement for added Mg^{2+}. Although the addition of Mg^{2+} caused a remarkable stimulation of ATPase activity in the presence of micromolar concentrations of Ca^{2+} (see Fig. 3A), such a stimulation by Mg^{2+} can be primarily accounted for by the high level of Mg^{2+}-ATPase activity in the plasma membrane. When the Ca_H^{2+}-ATPase activity in the presence of Mg^{2+} was corrected for the 'basal' Mg^{2+}-ATPase activity, the Ca_H^{2+}-ATPase was in fact found to be inhibited with increasing Mg^{2+} concentration (see Table II and inset in Fig. 3A). It is unlikely that Mg^{2+} inhibits the Ca_H^{2+}-ATPase by competing for the high-affinity Ca^{2+} binding site because the mode of inhibition by Mg^{2+} was noncompetitive with respect to Ca^{2+} activation. Furthermore, the inhibitory effect of Mg^{2+} on the Ca_H^{2+}-ATPase activity in this work was not affected by azide (Table III) but attenuated by Triton X-100 treatment of the plasma membrane fraction. The K_i for Mg^{2+} decreased

from 0.5–0.6 mM to approx. 0.2 mM when the Triton X-100 treated membrane fraction was employed, whereas the K_m (0.40 μM) for Ca^{2+} was not affected in the absence of added Mg^{2+} using the plasma membrane fraction with or without prior Triton X-100 treatment. This lends additional support that Mg^{2+} interacts with the enzyme at a site different from the high-affinity Ca^{2+} binding site and that it is the Mg^{2+} site not the high-affinity Ca^{2+} binding site that was affected by Triton X-100 pretreatment.

In addition to its high-affinity for Ca^{2+}, Ca_H^{2+}-ATPase activity can be dissociated from the Mg^{2+}-ATPase and Ca_L^{2+}-ATPase by the effect of azide and Triton X-100 treatment (Tables III and IV). However, the relationship between the Ca_H^{2+}-ATPase reported here and the smooth muscle membrane ($Ca^{2+} + Mg^{2+}$)-ATPase reported by other investigators [14–16] deserve special comments due to their possible association with the Ca^{2+}-pump. First of all, in most studies, smooth muscle ($Ca^{2+} + Mg^{2+}$)-ATPase activity was measured in a heterogeneous microsomal membrane fraction. Ford and Hess [16] have attributed this ($Ca^{2+} + Mg^{2+}$)-ATPase activity in bovine aorta to the property of fragmented sarcoplasmic reticulum, though their data can be interpreted equally well in terms of the substantial contamination of their microsomal fraction by the plasma membrane [28–30]. Secondly, the ($Ca^{2+} + Mg^{2+}$)-ATPase activity has been conventionally measured and operationally defined as the difference between the total ATPase activity in the presence of both Ca^{2+} (μM) and Mg^{2+} (mM) and the 'basal' ATPase activity in the presence of Mg^{2+} (mM) only. It is conceivable that the possible presence of the high-affinity Ca^{2+}-ATPase (without added Mg^{2+}) activity has not been considered in such an assay system. This is particularly true when the coupled enzyme system using pyruvate kinase has been employed [14,15] because Mg^{2+} and K^+ are absolutely required for pyruvate kinase activity, whereas Ca^{2+} is nonactivating in the absence of Mg^{2+} and inhibitory in the presence of Mg^{2+} [31]. Therefore, the small ($Ca^{2+} + Mg^{2+}$)-ATPase activity reported in smooth muscle microsomal or plasma membrane fraction in the presence of millimolar concentration of Mg^{2+} is consistent with our present findings and may represent the residual Ca_H^{2+}-ATPase in this presence of Mg^{2+} found in this work.

Calmodulin has been reported to stimulate the smooth muscle plasma membrane Ca^{2+}-pump [32] as well as the ($Ca^{2+} + Mg^{2+}$)-ATPase activity [15,33]. The Ca_H^{2+}-ATPase activity of fundus muscle plasma membrane, however, was not affected by purified bovine brain calmodulin within the effective concentration range, even when the membrane fraction was treated with EDTA or EGTA to remove the endogeneous calmodulin (Table V). However, calmodulin stimulation of Ca^{2+}-ATPase activity may not represent a general characteristic of plasma membrane. It has recently been shown that the high-affinity Mg^{2+}-dependent Ca^{2+}-ATPase from liver plasma membrane [34,35], parathyroid plasma membranes [27], osteosarcoma plasma membranes [36] and corpus luteum plasma membranes [37] was not sensitive to addition of calmodulin even in EGTA washed membranes. On the other hand, calmodulin stimulates not only the sarcolemmal Ca^{2+}-pump [38] but also the sarcoplasmic reticulum Ca^{2+}-pump in cardiac muscle [39]. The high-affinity for Ca^{2+} of the Ca_H^{2+}-ATPase is in excellent agreement with that of the plasma membrane Ca^{2+}-pump from rat gastric fundus smooth muscle [11]. But, the apparent initial rate for the Ca^{2+}-pump was estimated to be 6–10 μmol/g per min whereas the Ca_H^{2+}-ATPase activity was 40–60 μmol/mg per h (equivalent to 600–1000 μmol/g per min). This clearly indicates a stoichiometry of 100 for ATP molecules hydrolyzed per calcium ion transported. Obviously, a large portion of the Ca_H^{2+}-ATPase activity may not be related to the Ca^{2+}-pump considering the fact that the Ca^{2+}-pump from the sarcoplasmic reticulum of skeletal muscle yielded two calcium ions transported per ATP hydrolyzed [40]. On the other hand, if one assumes a stoichiometry of one [14] and calculates the Ca^{2+}-ATPase activity using the initial rate of Ca^{2+}-uptake in rat fundus plasma membrane, a Ca^{2+}-ATPase activity of 0.4–0.6 μmol/mg per h would have been expected. Such an extremely low Ca^{2+}-ATPase activity is beyond the accuracy of the present existing assay methods in light of the high background or basal Mg^{2+}-ATPase activity (400–600 μmol/mg per h) at 5 mM Mg^{2+}. Wuytack et al. [14] reported an activity ≤ 2

μmol/mg per h $(Ca^{2+} + Mg^{2+})$-ATPase in the microsomal fraction from pig coronary artery over a background Mg^{2}-ATPase activitiy of approximately the same magnitude. This is in contrast to our experience with the rat fundus muscle plasma membrane which has a Mg^{2+}-ATPase activity 200–300-times greater than that reported by Wuytack and Casteels on pig coronary artery. Such differences cannot be totally accounted for by the difference in membrane purity and may be related to the different sources of smooth muscle or animal species used. Although it is premature at this stage to attribute this Ca^{2+}_{H}-ATPase to the regulatory function of cytoplasmic level of Ca^{2+}, our results do suggest that future studies of $(Ca^{2+} + Mg^{2+})$-ATPase activity in smooth muscle should consider the possible contribution by the Ca^{2+}_{H}-ATPase activity.

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