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Ca^{2+} or Mg^{2+} nucleotide phosphohydrolases in myometrium: two ecto-enzymes

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A high level of Ca^{2+} or Mg^{2+} nucleotide phosphohydrolase activity is present on the outside surface of intact myometrial cells and is also observed in the isolated plasma membranes. About half of this activity is labile while the remainder is stable. The characteristics of the activities suggest the presence of at least two different ecto-enzymes. The stable component (K_m for Ca^{2+} about 0.1 mM) accepts XTP or XDP as substrate, is not inhibited by *p*-chloromercuriphenylsulfonate or inorganic phosphate, but is inhibited by 20 mM NaN_3. The labile component (K_m for Ca^{2+} nearly 1 mM) cleaves XTP but not XDP, and is inhibited by *p*-chloromercuriphenylsulfonate and inorganic phosphate, but not by NaN_3. The activity of the labile component can be restored by removing the cells from the incubation medium and resuspending them in fresh medium. This suggests that the 'lability' is due to product inhibition, probably by inorganic orthophosphate. While the Ca^{2+} pump of myometrial plasma membranes was inhibited by 0.1 μM oxytocin, these ecto-enzymes were unaffected by oxytocin concentrations up to 10 μM. Because of its high activity and rapid inactivation by product inhibition, the labile enzyme may be involved in the regulation of purinergic receptors.

Introduction

The ATP hydrolyzing activities of plasma membranes from mammalian tissues can be divided into two classes, the ion pumps and the non-pump XTPases. The ion pumps require Mg^{2+} and the translocated ion, have an active site facing the cytoplasm, and use ATP hydrolysis as a power source for the movement of ions across the membrane. The non-pump XTPases can be stimulated by Mg^{2+} or Ca^{2+}, are frequently ecto-enzymes, have broad nucleotide specificity, and are insensitive to inhibitors which inhibit some of the ion transporting ATPases, such as ouabain and vanadate [1–5].

Non-pump XTPases are poorly defined, and there have been reports of a wide variety of properties for these enzymes. Some of them [the enzymes from pig pancreas [6] and rat liver [2,7]] are said to hydrolyze nucleoside diphosphates as well as nucleoside triphosphates. In pancreas [8] the XTPase is inhibited by azide and azide sensitivity was used to distinguish XTPases from XTP diphosphohydrolases in a variety of tissues [9]. The XTPases of other tissues are relatively less sensitive (heart [10]) or not sensitive at all (mammary gland [11]). The sensitivity of these enzymes to mercurial reagents such as *p*-chloromercuriphenylsulfonate (PCMBS) is another variable point, since this sulfhydryl reagent inhibits the Ca^{2+}- or Mg^{2+}-ATPase in mouse brain and in human oat carcinoma cells [9,12] but not in rat liver plasma membrane [2]. In human hepatoma Li 7 cells, PCMBS-sensitive and insensitive components of the ecto ATPases were detected [13].

In rat tissues, including skeletal muscle, heart, spleen, brain and adipocytes, the rate of ATP hydrolysis was not linear as a function of time, due to an ATP-stimulated inactivation of the enzyme [10,14]. Similar time-dependent inactivation was found in bovine aorta, rabbit ear artery and rat myometrium [15,16].

Some studies have shown that non-pump XTPases have their active sites directed toward the outside of the cell [17]. Time-dependent inactivation of some of these ecto-XTPases has also been shown and they can

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be activated by concanavalin A [8,11]. It is possible that all of the non-pump XTPases are, in fact, ecto-XTPases, but it is impossible to say this from the currently available literature. Ecto-XTPases may be involved in the control of the effects of extracellular ATP [17–19]. Extracellular ATP has received increasing attention because it has effects similar to those caused by the activation of non-adrenergic, non-cholinergic nerves in various smooth muscles, including uterus [17,20–22].

Because of the plethora of confusing reports, it is still difficult to tell how many non-pump ATPases are present in mammalian plasma membranes and what their properties are. Careful studies utilizing time courses of activity and measuring the effects of those inhibitors which discriminate between the types of non-pump ATPases are important to advancing this understanding. In this study, we were able to separate the measured nucleotide hydrolysis activity into PCMBS-sensitive XTPase and azide-sensitive XTP diphosphohydrolase activities. The relative contributions of these two activities to the total ATPase activity depends on the assay time. In addition, the effects of oxytocin on the ATPase activities were also monitored since this hormone has an inhibitory effect on the plasma membrane Ca^{2+} pump in both pregnant [23] and non-pregnant myometrium [5].

We will refer to the activities studied here as ATPase, GTPase, or ADPase, depending on the actual substrate for the reaction being discussed. We do not imply the existence of separate enzymes by the use of these names. Indeed, a major conclusion of this study is that only two nucleotide phosphohydrolases are needed to account for the activity observed.

Materials and Methods

Pregnant female rats at 21 or 22 days of gestation were obtained from BioLab (St. Paul, MN, U.S.A.). Percoll was obtained from Pharmacia, and oxytocin from Peninsula Laboratories (Belmont, CA, U.S.A.). [γ-^{32}P]ATP and [γ-^{32}P]GTP (specific activity 30 Ci/mmol) came from New England Nuclear (Boston, MA, U.S.A.). Oligomycin, ouabain, PCMBS, collagenase type I, streptomycin, trypsin inhibitor (soybean), albumin (Fraction V), *p*-nitrophenyl phosphate, diadenosine pentaphosphate and all the lectins and nucleotides were purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.), as were buffers and salts – all reagent grade.

Preparation of plasma membranes

The procedure for preparation of plasma membrane vesicles from pregnant rat myometrium, previously pretreated with oxytocin (up to 10 μM concentration) for 10 min at 37 °C or untreated, was identical to those described previously [5]. A gradient generated from an 18% Percoll solution was used. Storage of the membrane preparation on ice for 16 h, or at −70 °C for weeks, did not alter its nucleotide phosphohydrolase activity. Membrane protein was measured by the method of Lowry et al. [24].

Isolation of myometrial cells

Uterine horns were removed from pregnant rats at the end of gestation (21–22 days). After removal of connective tissue, fat, fetuses and placentas, the endometrium was scraped off and the smooth muscle minced with scissors. The muscle pieces were rinsed by shaking for three successive 5-min periods in a 35 °C water bath, in Hank's solution supplemented with 0.1% BSA [25]. Each 5-min incubation was carried out in fresh solution. The dissociation of the cells was done in a modified Hank's solution. The modifications consisted of omission of the Mg salts and reduction of $CaCl_2$ to 30 μM [25]; also the solution was made 0.1% in BSA, 0.1% in type I collagenase and 0.01% in soybean trypsin inhibitor. All of the solutions were saturated with 95% O_2 and 5% CO_2. The cell dissociation was carried out in four successive 20-min periods, with shaking during each period at 37 °C. The muscle pieces were gently forced through an 18 gauge needle during the change of the dissociation medium after each period. The first two incubation solutions were routinely discarded since these frequently contained debris or damaged cells. Subsequent incubation solutions were collected and filtered through a coarse nylon filter. The cells were harvested by centrifugation at 200 × *g* for 25 min at room temperature, resuspension in modified Hank's solution without any supplement and recentrifugation and resuspension in enough modified Hank's solution to give a suspension containing 1–2 million cells per ml. Over 90% of the cells were found to exclude trypan blue in each cell-dispersion experiment. One uterine horn yielded about 10^6 cells, as measured by counting in a hemocytometer. The cells, kept at 37 °C and gassed with 95% O_2 and 5% CO_2, were used within 2 h. During this time, the appearance of lactate dehydrogenase activity in the medium was very slow. The term 'intact cells' is consequently used to describe this preparation. Total cellular protein was determined by the method of Lowry et al. [24], with the following modifications: Aliquots from the cell suspension were centrifuged in a microcentrifuge at room temperature for 15 s, and the cells, resuspended in fresh Hank's solution containing 1% Na_2CO_3 in 0.1 M NaOH, were incubated for 30 min at 37 °C.

Enzyme assays

Nucleotide hydrolase activity was determined in two different ways:

(a). ATPase and GTPase activities were measured by monitoring the release of inorganic [^{32}P]P_i from [γ-^{32}P]ATP or [γ-^{32}P]GTP as described by Verma and Penniston [26]. Membranes (10–20 μg) or cells ((1–2)·10^5) were suspended in a total volume of 500 μl in a medium containing either 160 mM KCl (in the case of membranes), or 130 mM NaCl and 5 mM KCl (in the case of cells), and 25 mM TES-TEA, 1 mM ouabain, 2 μg/ml oligomycin, 3 mM $CaCl_2$ or 3 mM $MgCl_2$ (if another concentration is not mentioned in the text) at pH 7.2 at 37 °C. After preincubation of the membranes or cells for 10 min at 37 °C, the reaction was started with 3 mM ATP or GTP labeled with γ-^{32}P. The presence of up to 2 mM unlabeled inorganic phosphate in the assay medium did not alter the accuracy of the determination of the radiolabeled phosphate.

(b). XDPase (and sometimes XTPase as well) was measured by determining the liberated inorganic phosphate by a colorimetric method [27]. The assay was carried out in 500 μl under the conditions described in the previous paragraph. The reaction was started with 3 mM unlabeled substrate and stopped with 0.19 ml sulfuric acid/ammonium molybdate (24 mM ammonium molybdate in 4.8 M H_2SO_4). Pellets were sedimented by centrifugation for 10 min at 1500 × *g* at 4 °C. Aliquots of 0.5 ml of clear supernatant were transferred to another tube and mixed with 15 μl of Fiske-SubbaRow reducing agent (obtained from Sigma). After incubation of the reaction mixture for 5 min at 37 °C, the absorbance was determined at 660 nm. For both methods a and b, controls containing no enzyme or containing 1 mM EDTA with no added bivalent cation were used to correct for nonenzymatic hydrolysis.

Lactate dehydrogenase activity was assayed as previously described by Neilands [28] using 10^5 cells or a corresponding amount of cell homogenate.

Sonication of cells

Cells were sonicated on ice three times for 10 s each, using a setting of 40 watts on a Heat Systems W-185 sonicator with a 431A cup horn. Sonication was done in the same solution and at the same cell concentration as was used to store intact cells after isolation. This procedure results in a 50–80-fold enhancement in detectable lactate dehydrogenase activity. Further sonication did not increase this marker enzyme activity.

Results

Non-pump nucleotide phosphohydrolase activity in isolated plasma membranes

Isolated plasma membranes from pregnant rat myometrium have a very high ATPase activity in the presence of either Mg^{2+} or Ca^{2+}. As Fig. 1 shows, the activity is nearly the same regardless of which ion is present. Other divalent cations can also activate the ATPase in these plasma membranes. The order of effectiveness of these ions is $Mg^{2+} \cong Ca^{2+} > Mn^{2+} > Co^{2+} \gg Ba^{2+}$. The effects of Ca^{2+} and Mg^{2+} are not additive (data not shown). The activity declines during the first 10 min, then remains stable. If the activity of the stable component is subtracted, the points represented by the open symbols in Fig. 1 are obtained, showing a labile component with a half-life of about 2 min. A similar biphasic character of a Mg^{2+} ATPase activity was observed by Missiaen et al. in rat myometrium microsomal fraction [15,29].

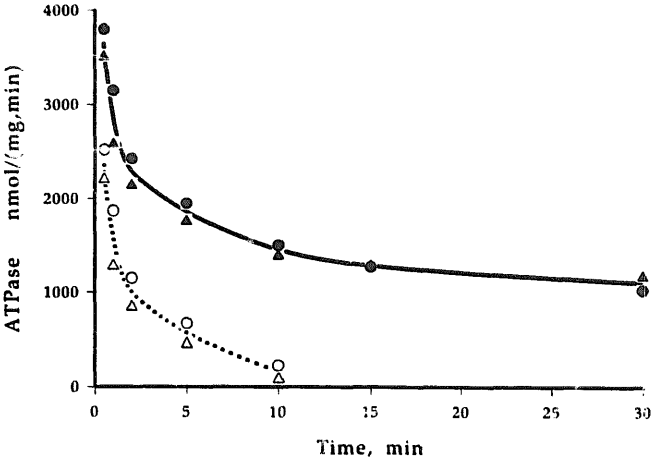

Fig. 1. Ca^{2+}- or Mg^{2+}-activated ATPase in pregnant rat myometrial plasma membranes: 10 μg of plasma membranes were preincubated in the standard assay medium in the presence of either 3 mM Ca^{2+} or 3 mM Mg^{2+} for 10 min at 37 °C. The reaction was then started with 3 mM [γ-^{32}P]ATP. Circles represent the Mg^{2+}-ATPase, while triangles represent Ca^{2+}-ATPase. The filled symbols represent the total activity as measured, while the open symbols represent the labile ATPase after subtraction of the stable component.

As the data of Table I show, the measured ATPase activities were not affected by inhibitors of the different transport ATPases (oligomycin, ouabain, vanadate) nor were they affected by calmodulin or trypsin digestion, which affect the plasma membrane Ca^{2+} pump. The lack of effect of diadenosine pentaphosphate and *p*-nitrophenyl phosphate on the measured activity indicates that neither myokinase nor non-specific phosphatases are involved in this activity. 50 μg/ml of Triton X-100 caused 70–80% inhibition of the activity. This sensitivity to detergent is similar to that reported for nonspecific ATPases in other tissues [14,30].

Since some of the non-specific ATPases can be activated by concanavalin A [8,11], we measured the effect of different lectins on the Ca^{2+}/Mg^{2+}-ATPase activity in rat myometrium as a function of time. Under the same conditions, we also measured the nucleotide triphosphatase activity in the membrane.

Fig. 2 shows the effects of preincubation with ATP and concanavalin A on the ATPase activity. Based on

TABLE I

Effect of various reagents on the labile and stable components of the Mg^{2+}-ATPase

Plasma membranes were preincubated for 10 min at 37 °C with the different reagents in the presence of 3 mM Mg^{2+}. ATP hydrolysis was stopped at 1 min to obtain the total activity or at 20 min to obtain the stable activity. The labile activity was calculated by subtracting the stable activity from the total activity. In the case of trypsin digestion, proteolysis was stopped by adding 250 μg/ml soybean trypsin inhibitor before starting the assay. Data are obtained from three independent experiments. Very similar results (not shown) were obtained when the Ca^{2+}-ATPase was measured. S.D. values are given only if the change is higher than 5%.

Treatment	Mg^{2+}-ATPase (percentage of control)	
	labile	stable
None	100	100
Oligomycin, 2–8 μg/ml	100	100
Ouabain, 0.25–1 mM	100	100
Na_3VO_4, 50 μM	100	100
Calmodulin, 20 μg/ml	100	100
Trypsin, 50 μg/ml	81 ± 8	95
Diadenosine pentaphosphate (AP_5A), 100 μM	93 ± 6	92 ± 5
p-nitrophenyl phosphate, 3 mM	97	98
Triton X-100, 50 μg/ml	28 ± 10	20 ± 7
Triton X-100, 500 μg/ml	4 ± 3	2 ± 4

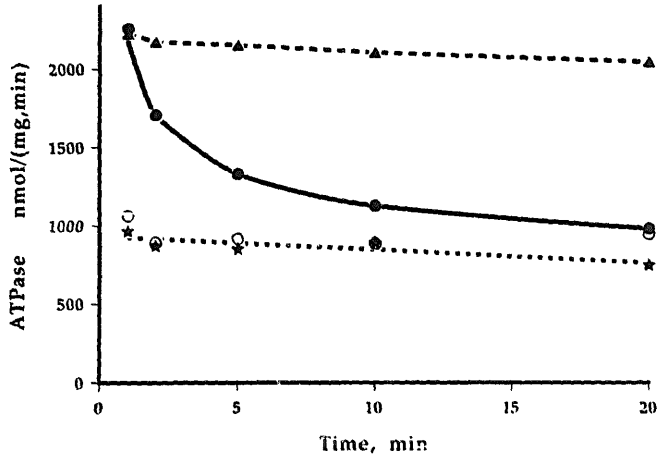

Fig. 2. Effects of concanavalin A and ATP preincubation on Mg^{2+}-ATPase. The filled circles represent the ATPase measured without preincubation, the filled triangles after preincubation with concanavalin A (50 μg/ml), the filled stars after preincubation with 3 mM ATP, and the open circles, preincubation with 3 mM ATP followed by addition of concanavalin A (50 μg/ml) at the start of the assay. The total ATP concentration was 6 mM in these experiments. All tubes were preincubated for 20 min at 37 °C and the assay was begun by adding enough (γ-^{32}P)-labeled nucleotide to bring the final nucleotide concentration to 6 mM. In the case of tubes preincubated with nucleotide, the preincubation concentration was 3 mM of unlabeled ATP and another 3 mM of (γ-^{32}P)-labeled ATP added to initiate the assay. In all other cases, 6 mM (γ-^{32}P)-labeled ATP was added at zero time. In the case of concanavalin A preincubation, the concanavalin A was added in the middle of the 20-min preincubation period.

the results of three independent experiments, preincubation of the membranes with 3 mM ATP eliminated the labile part of the total ATPase. Preincubation with 50 μg/ml of concanavalin A prevented the decrease of ATPase activity with time. When the membranes were preincubated with ATP and concanavalin A was present only during the assay, only the stable component was observed. Another lectin, wheat germ agglutinin, was nearly as potent as concanavalin A. On the other hand, succinyl concanavalin A was ineffective at concentrations up to 150 μg/ml. This suggests that the cross-linking effects of concanavalin A and wheat germ agglutinin are involved in the mechanism by preventing the inactivation of the labile component. Also confirming this picture was the fact that α-methylmannoside reversed the effects of lectins when added to a concentration of 25 mM (data not shown). Similar experiments on GTPase (not shown) gave nearly identical

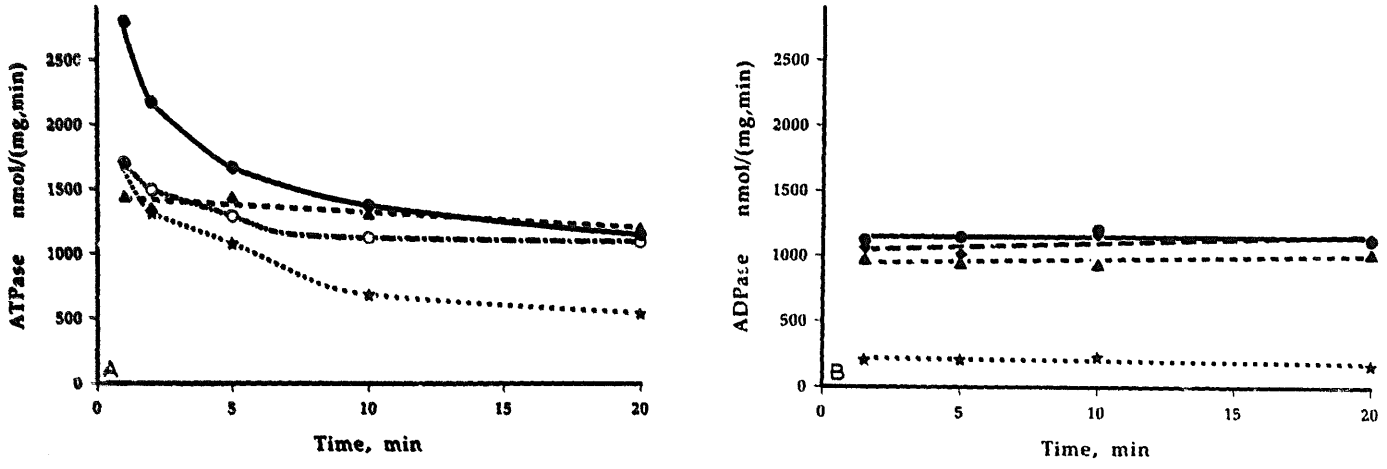

Fig. 3. Comparison of Mg^{2+}-ATPase and Mg^{2+}-ADPase. In panel A, ATPase was measured by release of [^{32}P]P_i from [γ^{32}-P]ATP at 37 °C. The membranes were preincubated for 10 min at 37 °C with 100 μM PCMBS (filled triangles), 500 μM inorganic phosphate (open circles), or 20 mM NaN_3 (stars). The ATPase activity when preincubated without any additive is shown by the filled circles. Panel B shows ADPase activity as measured by colorimetric assay. The symbols have the same meaning as in panel A, except that filled diamonds represent the activity after preincubation with concanavalin A (50 μg/ml).

results. UTP and ITP were less effective substrates than GTP, each giving about 70% of the specific activity given by ATP.

Because of the variety of responses of the non pump ATPases of different tissues (see Introduction) to PCMBS and azide, we measured the ATPase activity in myometrium in the presence of these agents as a function of time. Fig. 3A shows that azide inhibits the stable component of the activity while PCMBS inhibits the labile component. The possibility of product inhibition in the inactivation of PCMBS-sensitive hydrolase activity was also investigated; the effects of all the possible products (ADP, AMP, adenosine and inorganic phosphate) were explored. AMP and adenosine up to 3 mM concentration had no effect on the ATPase activity originating from either the labile or stable phase of the hydrolysis. Inorganic phosphate eliminated most of the labile component ($IC_{50} = 0.3$ mM) while the stable component was not inhibited. Because it is a substrate for the stable component, investigation of product inhibition by ADP was more difficult. Preincubation of the membranes with 3 mM ADP for 20 min significantly reduced the activity of the labile component (up to 70%) while the labile ATPase activity measured in the presence of ADP without preincubation was reduced by only 15–20%. The inhibition observed in these experiments can be attributed to the inorganic phosphate released from the ADP. The stable ATPase activity was reduced by 40–60% in the presence of ADP whether it was preincubated or not. This inhibition was probably due to competition of ADP and ATP for the enzyme. The results concerning ADP do not implicate it as a product inhibiting the labile ATPase, while inorganic phosphate is a strong candidate for that role.

Separate measurements of the ATPase and ADPase activities as a function of time by colorimetric and radiometric assay gave further details as to the relationship between the labile and stable enzyme activity. The ADPase activity shown in Fig. 3B appears to correspond essentially to the stable component of the ATPase activity. It was strongly inhibited by azide, while concanavalin A and PCMBS had little effect. As the colorimetric assay measures the total inorganic phosphate regardless of whether it comes from the α, β, or γ phosphorus, the total ATPase activity measured with this method gave significantly higher values than the same activity measured by radiometric assay (Table II).

When the activities were divided into stable and labile components, the labile component showed essentially the same activity whether assayed by the radiometric or the colorimetric methods, while the stable activity was about twice as great when assayed by the colorimetric assay. These data are consistent with the concept that the labile activity hydrolyzes only the gamma phosphate from ATP, while the stable activity hydrolyzes the gamma phosphate from ATP and the beta phosphate from ADP. Thus, the labile activity might be termed an ATPase or XTPase, while the stable activity could be called a nucleotide diphosphohydrolase. The presence of azide had relatively little effect on the labile activity measured by either method, while it partially inhibited the stable activity regardless of the method of measurement. The azide-sensitive nucleotide diphosphohydrolase observed here may also be similar to that which has been reported by others in different tissues [6,9,31].

5′-Nucleotidase activity (release of inorganic phosphate from AMP) was also monitored along with the measurements in Table II. This activity was lower than the ADPase and ATPase and was not sensitive to azide. When ATPase was measured by the radiometric assay, neither the labile nor the stable components were inhibited by 3 mM AMP. This indicates that 5′-nucleotidase was not a significant component of the ATPase or ADPase activities studied in this report.

Kinetic parameters were calculated for the labile and stable components of the Ca^{2+}-ATPase and Mg^{2+}-ATPase, by fitting each of these activities to the Michaelis-Menten equation. The results further reinforce the concept of two different activities, since the labile component had a much lower apparent affinity for cation than did the stable component. The stable

TABLE II

Comparison of the colorimetric and radiometric assays of the different activities

The activities were measured in parallel on a single membrane preparation, after preincubation of the membranes for 10 min at 37 °C in the assay medium. The assay was started with 3 mM [γ-^{32}P]ATP in the case of radiometric determination, or with 3 mM unlabeled ATP or ADP in the case of the colorimetric method. The labile and stable components were determined as in Table I. The activities are represented as nmol P_i/mg per min.

	Colorimetric assay				Radiometric assay		
	Mg^{2+}-ADPase	Mg^{2+}-ATPase			Mg^{2+}-ATPase		
		total	stable	labile	total	stable	labile
No inhibitor	1029	2898	1812	1078	1899	891	1008
+20 mM NaN_3	246	1600	615	985	1277	417	860

TABLE III

Kinetic parameters of Ca^{2+}- or Mg^{2+}-ATPase in myometrial plasma membranes

The ATPase activity was measured by the radiometric assay, while the ADPase activity was measured by the colorimetric assay. The cation concentrations were varied from 0.001 mM to 3 mM in the presence of 3 mM ATP or ADP. The ATP or ADP concentrations were varied from 0.025 to 3 mM, in the presence of 3 mM $CaCl_2$ or $MgCl_2$. The stable and labile components were calculated as for Table I. Standard deviations were calculated from three independent experiments.

		V_{max} (μmol/ mg per min)	K_a for cation (mM)	K_a for ATP or ADP (mM)
Ca^{2+}-ATPase	stable	1.5 ±0.14	0.128±0.06	0.17±0.03
	labile	2.0 ±0.14	1.01 ±0.02	0.12±0.02
Mg^{2+}-ATPase	stable	1.25±0.057	0.15 ±0.04	0.2 ±0.01
	labile	2.3 ±0.2	1.12 ±0.02	0.25±0.09
Ca^{2+}-ADPase		1.36±0.10	0.08 ±0.03	0.44±0.07
Mg^{2+}-ADPase		1.16±0.12	not measured	0.33±0.06

component had about 0.1 mM K_a for cation regardless of whether the cation was Ca^{2+} or Mg^{2+}, while the labile component had a K_a of about 1 mM. The K_a for substrate (ATP or ADP) was not significantly different between the stable and the labile components. The Ca^{2+} ADPase activity had a K_a of about 0.1 mM for Ca^{2+}, consistent with the idea that it is the same enzyme as the stable component of the ATPase activity (Table III).

Studies were also carried out on the pH and temperature dependence of the two activities. Varying the pH from 6.5 to 8.2 did not significantly alter the V_{max} of the labile and stable components. On the other hand, reducing the temperature from 37 °C to 20 °C strongly inhibited the labile component without any effect on the stable component. The activity of the labile Mg^{2+}-ATPase, measured at 20 °C, was 25% of the activity measured at 37 °C.

Nucleotide phosphohydrolase activity on the surface of whole cells

In order to determine whether the labile and stable activities were directed to the outside of the cell, we measured these activities in enzymatically dispersed cells from pregnant rat myometrium. Fig. 4 shows a comparison of the Mg^{2+}-ATPase activity in intact cells with that in cells which were broken in a manner designed to retain the ATPase activity of the intact cell. Sonication was chosen as the method of cell breakage because the repeated freezing and thawing of cells or the use of detergents significantly inhibited the

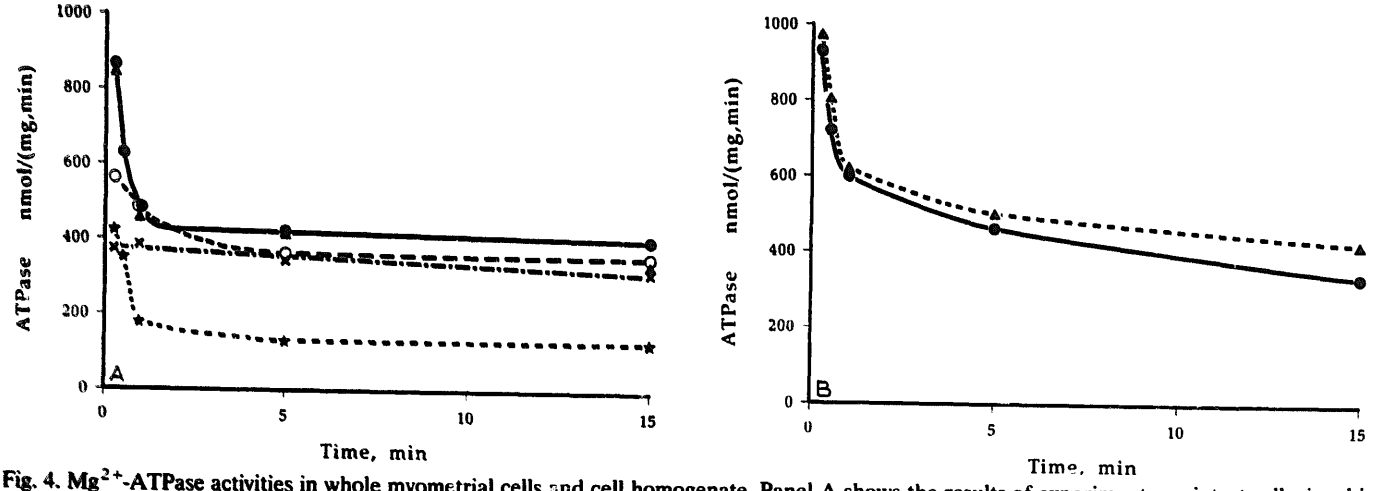

Fig. 4. Mg^{2+}-ATPase activities in whole myometrial cells and cell homogenate. Panel A shows the results of experiments on intact cells, in which 10^5 cells were preincubated for 10 min at 37 °C in the assay medium without any supplement (filled circles), or with 50 μg/ml concanavalin A (filled triangles), 500 μM P_i (open circles), 100 μM PCMBS (crosses) or 20 mM NaN_3 (stars). Panel B shows a similar experiment carried out on cells which had been sonicated. An amount of homogenate equivalent to 10^5 intact cells was used. The symbols have the same meanings as in panel A, except that only the data with and without concanavalin A is shown.

ATPase activity. The sonication procedure gave a 50–80-fold enhancement of lactate dehydrogenase activity without loss of the Mg^{2+}-ATPase activity. The ATPase activity was essentially the same in intact cells as it was in broken cell homogenate. Fig. 4 is representative of three independent experiments showing this retention of activity.

The distinction between the labile and stable components of the ATPase activity is even more clear in the intact cells than it was in isolated membranes. The labile component was fully inactivated after about 2 min and the activity remained almost constant thereafter. The sensitivity of the two activities to inhibitors was also essentially the same in the intact cells as it was in the plasma membranes: PCMBS totally inhibited the labile component, azide inhibited the stable component and inorganic phosphate partially inhibited the labile component. All of these effects were the same as were observed in the plasma membranes, but one notable difference was the effect of concanavalin A. In the intact cells, concanavalin A did not prevent or even retard the inactivation of the labile component. Other experiments using Ca^{2+} as the activating ion and GTP as the substrate (not shown) gave similar results to those shown in Fig. 4A.

The homogenized cells also showed the labile and stable components, but the stable component was less stable than it was in intact cells (Fig. 4B). In the homogenized cells, as in the intact cells, concanavalin A did not prevent the decay of the labile component.

Assays of the incubation medium after the cells were removed from it showed no significant hydrolysis of added ATP, indicating that ATP hydrolysis is not due to an enzyme which is released from the cells. Also, incubation of the cells at 37 °C for 20 min in the presence of 3 mM ATP (conditions which mimic the assay conditions) showed negligible release of lactate dehydrogenase from the intact cells. The lactate dehydrogenase was 0.3 nmol/mg per h in the fresh cells, 0.4 in cells incubated for 20 min and 26.9 in cell homogenate. The fact that the ATPase activity did not increase when the cells were broken by sonication indicates that the ATPase activity measured in the plasma membranes is an ATPase with its active site directed toward the outside of the cell, i.e., an ecto-enzyme. Since concanavalin A did not prevent the decay of the labile component in intact cells or in the cell homogenate, we measured the effect of concanavalin A on isolated plasma membranes incubated in the same medium which was used to prepare the intact cells. Plasma membranes incubated in modified Hank's medium supplemented with 0.1% BSA, 0.1% collagenase and 0.01% soybean trypsin inhibitor for 20 min behaved in the same way as did plasma membranes assayed as shown in Figs. 2 and 3. This made it clear that the difference in response to concanavalin A was

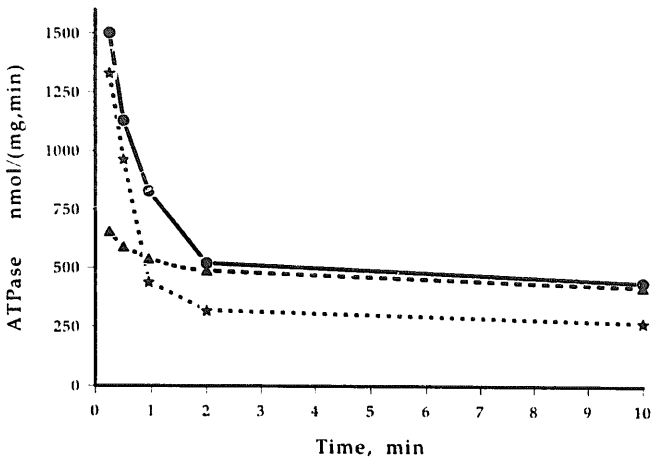

Fig. 5. Reversal of the ATP-induced inhibition of the labile component in intact cells. Preincubation of $2 \cdot 10^5$ cells was done with and without 3 mM ATP for 20 min at 37 °C in the normal assay medium containing 3 mM $MgCl_2$. With half of the cells, the ATPase assay was carried out immediately in the same medium by adding enough [γ-^{32}P]ATP to bring the total ATP concentration to 6 mM. The solid circles represent the data obtained by preincubation in the absence of ATP and the solid triangles that obtained in the presence of ATP. The other half of the cells were washed by centrifugation in the microcentrifuge for 5 s at 10000× *g* and the assay was carried out in fresh reaction medium, starting the reaction with 6 mM [γ-^{32}P]ATP. The stars represent the activity in the washed cells which had previously been preincubated with ATP. Washed cells preincubated without ATP gave similar results.

an intrinsic property of the plasma membranes. In mammary gland, Carraway et al. [11] reported similar differences in the response to concanavalin A between intact cells and plasma membranes. On the other hand, in pancreas, the ecto-ATPase was stimulated by concanavalin A in both intact cells and in plasma membrane fractions [8].

The response of the ATPases to the inhibitors (PCMBS, azide and inorganic phosphate) in the homogenized cells was similar to that measured in intact cells (data not shown).

The fact that preincubation with XTP or inorganic phosphate eliminated or strongly reduced the triphosphatase activity in both membranes and intact cells, raised the question of whether the inactivation of this enzyme is reversible. Fig. 5 is one of three independent experiments, showing that the labile XTPase activity could be nearly fully restored by washing the cells after the preincubation with ATP. In the same experiments, the cells preincubated without ATP were unaffected by the washing step.

Lack of oxytocin effect on ATPase in plasma membranes or intact cells

Pretreatment of the myometrium with oxytocin (0.1 to 10 μM) caused an inhibition in the Ca^{2+} transport in plasma membranes prepared from this myometrium [23]. The same membranes were assayed for the ATPases reported in this paper. Neither the labile nor the

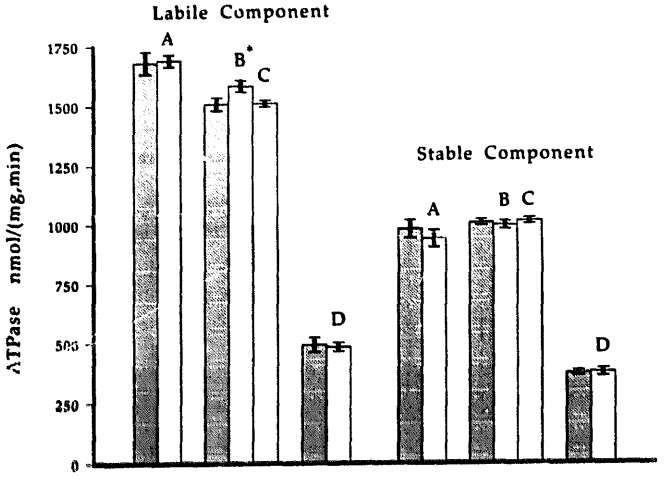

Fig. 6. Lack of effect of oxytocin on Mg^{2+}-ATPase. Activities were measured in plasma membranes and in cells. In all of the experiments, the shaded columns represent the activity of membranes or cells which were not exposed to oxytocin at any point. The columns which were exposed to oxytocin are identified as follows: A, activity in plasma membranes prepared from tissue previously incubated with 10 μM oxytocin, and exposed to 10 μM oxytocin during the assay; B, activity in plasma membranes prepared from untreated tissue, but which were preincubated with 0.1 μM oxytocin before the assay; C, the same as B but the plasma membranes were preincubated with 10 μM oxytocin before the assay; and D, activity in intact cells which were preincubated with 10 μM oxytocin before the assay. Treatment A inhibited Ca^{2+} transport in the same batch of plasma membranes. All of the oxytocin-treated samples showed statistically insignificant differences from their controls ($P > 0.19$) except for the one marked B* ($P \cong 0.01$). In this case, the difference is probably not experimentally significant, since the increase was less than 5% and an increase in the oxytocin concentration did not cause a further increase in activity.

stable part of the ATPase activity was inhibited by oxytocin. Fig. 6 shows both this result and the result of other kinds of treatment with oxytocin. In addition to the treatment of the whole muscle with oxytocin, the isolated plasma membranes and intact cells were also treated with oxytocin. None of these treatments had any effect on the stable or the labile components of the nucleotide phosphohydrolase.

Discussion

A previous report from our laboratory focussing on the Ca^{2+}-pump ATPase in non pregnant myometrium [5] made a clear distinction between that enzyme and the non-pump Mg^{2+}-ATPase activity in the same membrane. In pregnant rat myometrium, we found a very high non-pump ATPase activity [23]. This report shows that this activity is actually due to two separate enzymes, both of which are ecto-ATPases. The non-specificity of the divalent cation requirement, the ability to hydrolyze several nucleotide triphosphates, the sensitivity to Triton X-100 and the insensitivity to ouabain, vanadate and oligomycin all make clear that both of these enzymes are different from the ion pumping ATPases. The relative contributions of these two enzymes to the total activity varies depending on the assay time, because of the lability of one of the activities.

The stable component of the activity comes from a nucleotide diphosphohydrolase which removes inorganic phosphate from either ADP or ATP. This enzyme is insensitive to PCMBS but is inhibited by azide. The existence of such an enzyme has been postulated to explain results obtained in several different types of tissues [6,12,31]. The possibility that such an enzyme might be an ecto-enzyme has also been discussed by other authors [9,32,33].

The labile component of the activity is an XTPase which is inhibited by PCMBS and is insensitive to azide. Such a PCMBS-sensitive enzyme has been reported in a number of tissues [13,14,34–36]. This enzyme may be widespread in plasma membranes of mammalian cells, but use of inappropriate assay conditions would make it difficult to detect. Assays carried out at low temperature, at low divalent cation concentration or for long times will probably not detect this enzyme. Many previous studies have used a fixed reaction time to study ATPases from plasma membranes and, therefore, had difficulty in distinguishing between the activities which might be present. Not all ecto-XTPases are inhibited by PCMBS; Knowles [13] induced a PCMBS-insensitive Ca^{2+}-XTPase by special treatment of Li-7 cells.

The data presented here show that the inactivation of the labile ATPase is probably due to product inhibition caused by the accumulation of inorganic phosphate in the medium. The IC_{50} for phosphate inhibition was about 0.3 mM, an amount of phosphate that could easily be formed during incubation of the membranes with 3 mM ATP under the assay conditions used. The reversibility of the inhibition of the enzyme strongly supports the notion that product inhibition is the mechanism of this lability and rules out any covalent modification of the enzyme. Other GTP- or ATP-dependent modifications of the enzyme are also improbable since preincubation of the membranes with ADP also leads to inactivation of the labile part of the activity. Presumably this occurs because the ADP is being hydrolyzed by the stable ADPase and the inorganic phosphate released from this reaction inhibits the XTPase.

It is not clear why concanavalin A prevents the inactivation of the labile component in plasma membranes, while no such effect was observed in the intact cells. The failure of concanavalin A to have an effect in the isolated cells is not due to the collagenase digestion by which the cells are isolated, since collagenase digestion of the isolated plasma membrane preparation did not alter the concanavalin A effect in these membranes.

Previous studies of the non-pump ATPases of myometrial plasma membranes did not identify the two separate enzyme activities, nor did they identify these activities as ecto-enzymes [5,29]. Our comparison of the activities of intact cells, sonicated whole cell homogenate and isolated plasma membranes shows that essentially all of the activity studied here is due to ecto-enzymes. As was shown in Fig. 4, intact cells, with a cell viability greater than 90%, and very low amounts of released lactate dehydrogenase, showed both kinds of nucleotide phosphohydrolase activity. More than 95% of the activity obtained in the sonicated cell homogenate was present in the intact cells. The fact that the non-penetrating mercurial reagent PCMBS inhibited the labile XTPase in intact cells further demonstrates the external character of this activity.

The extracellular location of the nucleotide phosphohydrolase activities raises the question of whether these enzymes are involved in the action of extracellular ATP in myometrial cells. Extracellular ATP is known to excite pregnant rat myometrium by changing the membrane permeability and the cytoplasmic Ca^{2+} level [21,37,38]. Similar effects of extracellular ATP have also been demonstrated in other cell types [18,39]. Change in cytoplasmic Ca^{2+} level has an extremely important role in the function of late-pregnant myometrial cells. Therefore, we investigated the effect of oxytocin on the ecto-ATPases of these cells. In our assay conditions, neither inhibition nor activation could be detected in either of our activities. Missiaen et al. [29], had reported an oxytocin-induced inhibition of the stable part of the Mg^{2+}-ATPase activity in pregnant rat myometrial microsomes. A microsomal preparation such as that used by Missiaen et al., would contain other ATPase activities, not associated with plasma membranes, and the presence of such enzymes might effect the results obtained.

An ecto-enzyme which binds ATP, such as either of these nucleotide phosphohydrolases, is a potential candidate for being the ATP receptor itself. However, this is probably not the case, since several workers have reported that the effects of extracellular ATP are more effectively mediated in the absence of Mg^{2+} or Ca^{2+}, and are probably due to unchelated ATP^{4-} [18,40–43]. Since ATPases almost invariably require divalent cations, it is unlikely that they are identical with the ATP^{4-} receptor which mediates the actions of extracellular ATP.

Although the functions of these nucleotide phosphohydrolases are not known, their properties suggest possible roles for them in the regulation of nucleotide metabolism at the cell surface. The labile ATPase appears to be inhibited by the products of ATP hydrolysis and it may be involved in regulation of the ATP accessibility to the purinergic receptor. This enzyme could be expected to rapidly hydrolyze small amounts of extracellular ATP, preventing their availability at the purinergic receptor. However, the presence of larger amounts of ATP, such as might actually function as an extracellular signal, would cause a build up of inorganic phosphate, inhibition of the XTPase and activation of the purinergic receptor by ATP. This hypothesis suggests that the ATPase may be physically near the purinergic receptor.

The stable nucleotide diphosphohydrolase may produce AMP which can be further metabolized by 5'-nucleotidase to adenosine. Adenosine can be taken up by intact cells and utilized as a metabolite. Thus, the properties of this enzyme suggest that it is a catabolic enzyme which initiates the breakdown of ATP to products which can be used by later steps in metabolism.

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