Characterization of the Mg^{2+} -activated ATPase activity in smooth-muscle membranes

NADH oxidase and adenylate kinase interfere with the NADH-coupled enzyme assay

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The apparent Mg^{2+} -activated ATPase activity measured by the continuous NADH-coupled enzyme assay was studied in a number of microsomal preparations obtained from smooth muscle of the myometrium from pregnant or 17β -oestradiol-pretreated rats, the bovine aorta, the guinea-pig taenia coli, the rabbit ear artery and pig antrum. It was shown that this ATPase assay is prone to the effects of a number of artefacts that are tissue-dependent. The apparent Mg^{2+} -ATPase activity in microsomes (microsomal fractions) from myometrium, aorta and taenia coli declines non-linearly during the assay. Its initial high rate gradually diminishes over 15–60 min, depending on the type of smooth muscle, to a constant value. This decline depends on the presence of ATP and can be partially prevented by concanavalin A. The non-linearity is limited in microsomes from rabbit ear artery. In microsomes from antrum the apparent Mg^{2+} -ATPase activity actually increases with time, albeit gradually. Storage on ice of the microsomes of the aorta, and especially of myometrium of pregnant rats and of taenia coli, is accompanied over a few hours after their preparation by a gradual suppression of the component of the Mg^{2+} -ATPase activity that is inhibited by ATP. The Mg^{2+} -ATPase activity in microsomes from antrum remains constant. NADH oxidase activity accounts for 10 % of the Mg^{2+} -ATPase activity in microsomes from stomach smooth muscle. The apparent initial non-linearity of the Mg^{2+} -ATPase activity in that tissue is due to a time-dependent decrease of a rotenone-sensitive NADH oxidase activity. The adenylate kinase activity, as deduced from the effect of the adenylate kinase inhibitor P^1, P^5 -di(adenosine-5') pentaphosphate, could account for 45.0, 35.0 and 31.0 % respectively of the Mg^{2+} -ATPase activity in microsomes from stomach, myometrium and aorta. No adenylate kinase activity could be detected in microsomes from ear artery and taenia coli. When microsomes from stomach smooth muscle were separated on a sucrose gradient, the contribution of adenylate kinase and NADH oxidase to the Mg^{2+} -ATPase activity was most pronounced in the higher-density fractions. Part of the NADH oxidase activity and of the Mg^{2+} -ATPase activity, and most of the adenylate kinase activity, are not sedimented at $224\,000\,g_{max}$ for 30 min and may therefore be present as soluble enzymes. In conclusion, the different patterns of Mg^{2+} -ATPase activity in microsomes from different types of smooth muscle are due (1) to a variable component of Mg^{2+} -induced ATP hydrolysis that exhibits an inactivation by ATP, (2) to a variable rotenone-sensitive and -insensitive NADH oxidase activity, and (3) in some smooth-muscle microsomes to an adenylate kinase activity.

INTRODUCTION

The ATP-driven Ca^{2+} pumps in the sarco- or endoplasmic reticulum of skeletal, cardiac and smooth muscle and the calmodulin-stimulated ATP-dependent Ca^{2+} pump present in the plasma membranes of many cells depend on a Ca^{2+} -stimulated Mg^{2+} -ATPase activity. In rat myometrial microsomes (microsomal fractions) the study of this Ca^{2+} -stimulated Mg^{2+} -ATPase activity is seriously hampered by the simultaneous occurrence in these membranes of a Ca^{2+} -calmodulin-inhibited Mg^{2+} -ATPase activity [the preceding paper (Missiaen *et al.*, 1988)]. This observation prompted us to investigate further in more detail the Mg^{2+} -ATPase in a number of different smooth-muscle membrane preparations.

The Mg^{2+} -ATPase activity is routinely measured by

following the release of either of the two breakdown products, i.e. P_i or ADP. The ADP production is measured with an assay in which the produced ADP is recycled to ATP. This process is coupled to the oxidation of NADH, and the oxidation of NADH can be easily and continuously monitored at A_{340} . Both the P_i - and ADP-release methods, however, are open to the effects of specific artefacts. An important part of the present work illustrates some of the artefacts encountered in the NADH-coupled enzyme system.

Beeler *et al.* (1985) compared the Mg^{2+} -ATPase activity in microsomal fractions from various rat tissues, and observed that, in all preparations, the Mg^{2+} -ATPase activity declined, as a function of time, as a result of ATP-dependent inactivation of the enzyme. We had observed that the Mg^{2+} -ATPase activity in rat myo-

Abbreviations used: Mg^{2+} -ATPase, Mg^{2+} -activated ATPase; $(Na^+ + K^+)$ -ATPase, $(Na^+ + K^+)$ -activated ATPase; A_pA , P^1, P^5 -di(adenosine-5') pentaphosphate; $(Ca^{2+} + Mg^{2+})$ -ATPase, $(Ca^{2+} + Mg^{2+})$ -activated ATPase.

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metrium varies according to the hormonal priming of the animal [the preceding paper (Missiaen *et al.*, 1988)]. We have now examined different smooth-muscle preparations in order to determine whether the characteristics of the Mg^{2+} -ATPase activity differ between these different preparations.

EXPERIMENTAL

Animals

Myometrium was obtained from Wistar rats, either at day 21 of pregnancy or after ovariectomy and treatment for 2 weeks with 17β -oestradiol as described previously [the preceding paper (Missiaen *et al.*, 1988)]. Vascular smooth muscle was obtained from the ear artery of rabbits and from the intrathoracic part of bovine aorta, between the diaphragm and the ductus arteriosus. Antral smooth muscle was obtained from the stomach of pigs and the taenia coli from guinea pigs. Both porcine antrum and bovine aorta were obtained from the slaughterhouse and transported on ice to the laboratory. After removing connective tissue and non-smooth-muscle structures the muscles were cut in small pieces. Uterine horns were cut longitudinally in strips 1 mm wide under a dissection microscope and the longitudinal smooth-muscle layer was then separated from the circular layer and the endometrium. Rabbit ear artery was cleaned of its periarterial connective tissue and cut into longitudinal strips. Taenia coli was isolated from the caecum of guinea pigs in the usual way (Casteels & Raeymaekers, 1979). During the dissection the tissues were bathed in a physiological solution containing 135.5 mM-NaCl, 5.9 mM-KCl, 1.5 mM- $CaCl_2$, 1.2 mM- $MgCl_2$, 11.5 mM-glucose, 11.6 mM-Hepes (pH 7.3). The tissues were then frozen in liquid N_2 and kept at $-85^\circ C$ until used.

Preparation of the membranes

After thawing, the muscle pieces or strips were minced with a pair of scissors at $0^\circ C$ in a Sorvall SS34 polycarbonate tube in a volume of solution corresponding to 5 times the weight of the tissue. This homogenization medium had the following composition: 100 mM-KCl, 5 mM- $MgCl_2$ and 50 mM-Tris/HCl (pH 7.2). The minced material was homogenized with an Ultra-Turrax instrument (Janke and Kunkel, Staufen, Germany) fitted with an N10 shaft and run at maximal speed for 2×20 s (myometrium, taenia coli and ear artery) or 2×30 s (aorta and stomach). The homogenate was then centrifuged in a Sorvall SS34 rotor for 10 min at $1000 g_{max}$. The supernatant was further centrifuged in a Sorvall SM24 rotor at $10\,500 g_{max}$ for 10 min, and the pellet was discarded. The remaining supernatant was finally centrifuged in a Beckman Ti75 rotor at $224\,000 g_{max}$ for 30 min. The pellet was resuspended in 0.25 M-sucrose. This whole procedure lasted 90 min. The membranes obtained by this experimental procedure contained both plasma membranes and endoplasmic reticulum and are referred to hereafter as 'crude vesicular fraction'. Microsomes from antrum were then put on top of a 15–55% sucrose gradient, which was linear with respect to volume. Centrifugation was performed at $131\,450 g_{max}$ for 15 h at $4^\circ C$ in a SW28 rotor. The fractions were collected and the sucrose concentrations read in a Zeiss hand refractometer. The fractions were diluted 1 in 3 with 8% (w/v) sucrose and pelleted in a

Ti75 rotor at $224\,000 g_{max}$ for 30 min. These pellets were resuspended in 0.25 M-sucrose.

Enzyme assays

The Mg^{2+} -ATPase activity was measured at $37^\circ C$ by using an NADH-coupled enzyme system (Wuytack & Casteels, 1980). Between 10 and $30 \mu g$ of microsomal protein was added to 1 ml of an assay medium of the following composition: 100 mM-KCl, 30 mM-imidazole/HCl, pH 6.8, 5.7 mM- $MgCl_2$, 5 mM-ATP, 1 mM-EGTA, 1.5 mM-phosphoenolpyruvate, 0.26 mM-NADH, 5 mM- NaN_3 , 0.1 mM-ouabain, 40 units of pyruvate kinase and of lactate dehydrogenase and 0.2 mg of saponin. The decline in A_{340} was determined with an Aminco (DW-2a) spectrophotometer. The NADH oxidase activity was measured in the same medium, except that ATP, phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were omitted. 5'-Nucleotidase was measured spectrophotometrically by using a kit from Sigma. Cytochrome *c* oxidase and rotenone-insensitive NADH:cytochrome *c* reductase were measured as described previously (Wuytack *et al.*, 1978). The $(Na^+ + K^+)$ -ATPase activity was calculated from the inhibition of ATP hydrolysis by $100 \mu M$ -ouabain. The maximum adenylate kinase activity was determined as the sum of the Ap_5A -suppressible Mg^{2+} -ATPase activity plus the increment of the Mg^{2+} -ATPase activity induced by $100 \mu M$ -AMP in the absence of the inhibitor. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Materials

NADH, 17β -oestradiol, concanavalin A, DL-dithiothreitol and cyclic AMP were obtained from Sigma; saponin and ouabain were from Merck; AMP, cyclic GMP, Ap_5A and creatine were from Boehringer.

RESULTS

Mg^{2+} -ATPase activity in crude microsomal fractions of different smooth-muscle preparations

Mg^{2+} -ATPase activity as a function of time. Fig. 1 presents the apparent Mg^{2+} -ATPase activity as a function of time after adding to the assay medium the microsomes from rat myometrium, bovine aorta, guinea-pig taenia coli, rabbit ear artery or from porcine antral smooth muscle. The pattern of Mg^{2+} -ATPase activity suggests that it consists of two components: a time-dependent component that gradually declines during the assay and a constant component. Both components are not always present at the same ratio in the different smooth-muscle preparations. The time-dependent component is very pronounced in microsomes from pregnant-rat myometrium (Fig. 1a, ii), from bovine aorta (Fig. 1b), and from taenia coli (Fig. 1c); it is small in myometrial microsomes from rats treated with 17β -oestradiol (Fig. 1a, i), and it is hardly detectable in microsomes from rabbit ear artery (Fig. 1d) and antrum (Fig. 1e). Fig. 1(e), furthermore, illustrates that, in antral microsomes, the Mg^{2+} -ATPase activity slightly increases with time. This Mg^{2+} -ATPase activity at 20 min is increased by $15.6 \pm 1.9\%$ ($n = 5$) as compared with the value at 5 min.

The initial non-linearity of the Mg^{2+} -ATPase activity is caused by an ATP-dependent inactivation of the enzyme. Indeed, preincubating the microsomes with 5 mM- Mg -ATP for 30 min at $37^\circ C$ results in a complete

Fig. 1. Apparent Mg²⁺-ATPase activity as a function of time in microsomes from several smooth-muscle preparations

The experiments were done within the first hour after preparing the membranes. At zero time, between 10 and 30 μg of microsomal protein were added to a cuvette containing 1 ml of medium, and the NADH oxidation was monitored as a function of time. A typical recording is given for myometrial microsomes obtained from 17 β -oestradiol-treated animals (a,i), and from pregnant animals (a,ii), for microsomes from bovine aorta (b), taenia coli microsomes (c), rabbit ear artery microsomes (d) and stomach smooth-muscle microsomes (e). The broken lines in the records (a)–(d) represent the extrapolation of the slow component of the Mg²⁺-ATPase activity to zero time. The broken line in (e) represents the extrapolation of the initial component of the Mg²⁺-ATPase activity. The curves illustrated are typical for five experiments.

Table 1. Initial and steady-state Mg²⁺-ATPase activity in microsomes from different smooth-muscle preparations

The initial rate was determined immediately after adding the membranes to the medium; the steady-state rate was determined after the Mg²⁺-ATPase activity had remained linear for at least 15 min. The rates were measured during the first 1 h after preparing the membranes. The specific activities given are means $\pm$ S.E.M. for the number of observations given in parentheses.

Smooth muscle	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	
	Initial rate	Steady-state rate
Rat myometrium		
17 β -Oestradiol-treated	0.42 ± 0.05 (11)	0.27 ± 0.03 (11)
Pregnant	3.55 ± 0.20 (9)	0.34 ± 0.02 (9)
Bovine aorta	1.21 ± 0.17 (3)	0.09 ± 0.02 (3)
Guinea-pig taenia coli	0.87 ± 0.04 (3)	0.17 ± 0.02 (3)
Rabbit ear artery	1.28 ± 0.20 (3)	0.92 ± 0.08 (3)
Porcine stomach smooth muscle	0.07 ± 0.01 (4)	0.08 ± 0.01 (4)

disappearance of the time-dependent decline of the ATPase [the preceding paper (Missiaen *et al.*, 1988)].

The initial rate of Mg²⁺-ATPase activity (measured immediately after adding the microsomes to the assay

medium) and the steady-state Mg²⁺-ATPase activity are summarized in Table 1.

Concanavalin A has been shown to prevent partially the time-dependent decline in the Mg²⁺-ATPase activity

Fig. 2. Effect of concanavalin A on the apparent Mg^{2+} -ATPase activity in microsomes from different smooth-muscle preparations

The time course of the apparent Mg^{2+} -ATPase activity measured in the absence of concanavalin A is represented by unbroken lines and that in the presence of concanavalin A (20 $\mu g/ml$) by dotted lines. Microsomes were obtained from the myometrium of 17 β -oestradiol-treated (a) and from pregnant rats (b), from bovine aorta (c) and from taenia coli (d). (e) and (f) illustrate the absence of a significant effect of the lectin on the apparent Mg^{2+} -ATPase activity in microsomes from rabbit ear artery and from pig stomach smooth muscle respectively.

(Beeler *et al.*, 1985). Fig. 2 shows the effect of concanavalin A on the Mg^{2+} -ATPase activity in the different smooth-muscle preparations. Because it prevents the decline of the time-dependent component, concanavalin A augments the Mg^{2+} -ATPase activity increasingly in the following series of tissues: pig antrum (Fig. 2f) < rabbit ear artery (Fig. 2e) < myometrial microsomes of oestradiol-treated rats (Fig. 2a) < taenia coli (Fig. 2d) < myometrial membranes of pregnant rats (Fig. 2b) < bovine aorta (Fig. 2c).

The above-described pattern of Mg^{2+} -ATPase activity and its inactivation by ATP was observed immediately after resuspending the microsomal pellet. Fig. 3 illustrates the effect of the duration of storage on ice on the Mg^{2+} -ATPase activity of the various smooth-muscle preparations. This activity remains remarkably constant in microsomes from stomach smooth muscle, but the non-linearity gradually disappears over a few hours in microsomes from myometrium, aorta and taenia coli. This effect is most obvious in microsomes from myometrium of pregnant rats and from taenia coli. The time-dependent decline in the Mg^{2+} -ATPase activity during storage on ice becomes greater on addition of 5 mM-dithiothreitol.

NADH oxidase activity. The Mg^{2+} -ATPase activity is indirectly measured in our assay by coupling it to the oxidation of NADH. It has therefore to be assessed to what extent the NADH oxidase activity contributes to the apparent Mg^{2+} -ATPase activity and whether the

initial non-linearity of the apparent Mg^{2+} -ATPase activity is or is not due to a time-dependent decrease of an NADH oxidase activity. In the absence of ATP and the coupled enzyme system, some NADH oxidase activity is measured in a NADH-containing medium, as shown in Fig. 4(a). The initial phase of the NADH oxidase activity is high in microsomes from stomach smooth muscle and declines over a few minutes to a constant value. It is completely blocked by 10^{-4} M-rotenone, whereas the constant component of NADH oxidase activity is not affected. Fig. 4(b) indicates that the small initial non-linearity observed in pig antral smooth-muscle microsomes is due to a time-dependent decrease of the NADH oxidase activity. Fig. 4(b), furthermore, indicates that 10 % of the apparent Mg^{2+} -ATPase activity in microsomes of antral smooth muscle is due to NADH oxidase activity.

Adenylate kinase, creatine kinase and alkaline phosphatase activity. Because the rephosphorylation of ADP is coupled in our ATPase assay to the oxidation of NADH, an additional production of ADP independent of the true ATPase activities should be recognized as such and corrected for.

Adenylate kinase or myokinase catalyses the reaction:

and could thereby interfere with our ATPase assay. The addition of 100 μM -AMP enhances the apparent Mg^{2+} -ATPase activity by a factor of 6 in stomach smooth-

Fig. 3. Change in the apparent Mg²⁺-ATPase activity of microsomes from the different smooth-muscle preparations during storage on ice

The apparent Mg²⁺-ATPase activity was determined at different intervals after preparing the membranes. These intervals (in h) are indicated at the right side of the tracings. Time '0' indicates the moment of resuspending the membranes in 0.25 M-sucrose and without ATP. Microsomes were obtained from myometrium of 17 β -oestradiol-treated (a) or pregnant rats (b), of bovine aorta (c), guinea-pig taenia coli (d), rabbit ear artery (e) and pig antrum smooth muscle (f). Tracing (g) represents the apparent Mg²⁺-ATPase activity in myometrial microsomes from pregnant animals that were stored on ice in the presence of 5 mM-dithiothreitol.

muscle microsomes and to a lesser extent in the other smooth-muscle preparations. This observation suggests the presence of some adenylate kinase in the microsomes obtained from different smooth muscle. However, it does not yet prove that adenylate kinase contributes to the measured apparent Mg²⁺-ATPase activity under our assay conditions. We have therefore determined the

effect of Ap₅A, a strong and selective inhibitor of adenylate kinase (Feldhaus *et al.*, 1975) on the Mg²⁺-ATPase activity. It was observed that Ap₅A inhibits the apparent Mg²⁺-ATPase activity in a concentration-dependent manner and that a maximum inhibition is observed at 100 μ M-Ap₅A. The inhibition of the Mg²⁺-ATPase activity in a crude vesicular fraction of the

Fig. 4. NADH oxidase activity and apparent Mg^{2+} -ATPase activity in microsomes from pig antrum smooth muscle

(a) Represents the NADH oxidase activity in the presence (i) or the absence (ii) of 10^{-4} M-rotenone. The NADH oxidase activity was measured by the decline in A_{340} in a medium identical with that used to measure the Mg^{2+} -ATPase activity, except that ATP and the coupled enzyme system (phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase) were omitted. (b) Represents the NADH oxidase activity (i) and the apparent Mg^{2+} -ATPase activity (ii) on the same time scale. The broken line represents the extrapolation of the apparent Mg^{2+} -ATPase activity and of the NADH oxidase activity to zero time.

different types of smooth muscle by $100 \mu M$ - Ap_5A is given in Table 2. These values indicate that no ADP is produced by adenylate kinase activity in microsomes from taenia coli and rabbit ear artery. However, the contribution of adenylate kinase activity to the Mg^{2+} -ATPase activity is significant in membranes from myometrium, aorta and stomach smooth muscle.

Also, creatine kinase, which catalyses the reaction:

might, via the production of ADP, augment NADH oxidation and thereby the apparent Mg^{2+} -ATPase activity. Because 10 mM-creatine triples, in stomach smooth-muscle microsomes, the production of ADP, creatine kinase must also occur in these microsomes. However, no specific inhibitor of this enzyme is available, and it is therefore not possible to quantify its contribution to ADP production. It is unlikely that this contribution is significant, because the contamination of the membranes by creatine should be limited. Creatine can only be formed by a recycling of creatine phosphate to creatine via creatine kinase.

Table 2. Ap_5A -induced inhibition of Mg^{2+} -ATPase activity in the different types of smooth muscle expressed as a percentage of the control value

Ap_5A ($100 \mu M$) was added to the assay medium after the disappearance of the labile component of the Mg^{2+} -ATPase activity, and the percentage decrease of the rate of Mg^{2+} -ATPase activity was established. Because the Mg^{2+} -ATPase activity in microsomes from stomach smooth muscle increases with time, Ap_5A was here added to the assay medium at zero time, and the percentage inhibition was calculated from the difference in Mg^{2+} -ATPase activity from samples with and without Ap_5A , measured 15 min after adding the microsomes to the assay medium. The results (percentage inhibition by Ap_5A) are means $\pm$ s.e.m. for the numbers of observations given in parentheses.

Smooth muscle	Inhibition (%)
Pregnant-rat myometrium	35.0 ± 2.6 (3)
Bovine aorta	31.0 ± 3.8 (3)
Guinea-pig taenia coli	0.0 ± 1.1 (3)
Rabbit ear artery	0.9 ± 0.9 (2)
Porcine stomach smooth muscle	45.0 ± 5.7 (3)

An effect of alkaline phosphatase activity on ADP production from ATP is also unlikely, because 5 mM- β -glycerophosphate, which is the preferred substrate for this phosphatase activity, does not reduce the Mg^{2+} -ATPase activity.

Mg^{2+} -induced ATP hydrolysis in microsomes from stomach smooth muscle. The microsomes of the stomach, in contrast with those of other smooth muscles, do not present a labile component of the Mg^{2+} -ATPase activity that is inactivated by ATP. Fig. 5(a) illustrates the apparent Mg^{2+} -ATPase activity and the Mg^{2+} -ATPase activity of a crude vesicular fraction from stomach smooth muscle in the presence of $100 \mu M$ - Ap_5A , but without correction for the NADH oxidase activity. The apparent Mg^{2+} -ATPase activity increases with time, whereas it declines in the presence of Ap_5A and reaches a constant value after 15 min. This observation suggests that stomach muscle contains a component of Mg^{2+} -ATPase declining as a function of time, as has also been observed in other smooth muscles. However, in the stomach microsomes this component is probably masked by a time-dependent increase in apparent Mg^{2+} -ATPase activity. This transient inactivation revealed by the presence of Ap_5A can, as in other smooth-muscle tissues, be prevented by concanavalin A ($20 \mu g/ml$) (Fig. 5b).

Subcellular localization of the different components of the Mg^{2+} -ATPase activity

We have compared the distribution of the activity of NADH oxidase, adenylate kinase, the true Mg^{2+} -ATPase and of plasma-membrane and mitochondrial markers after separating microsomes from stomach smooth muscle on a sucrose density gradient (Fig. 6). It is assumed that the particulate adenylate kinase is located in the mitochondria and that Mg^{2+} -ATPase is a plasma-membrane enzyme. In order to separate these two types of membranes, we did not apply the digitonin treatment described by Raeymaekers *et al.* (1985) because the plasma membranes would then be shifted by digitonin

Fig. 5. Uncorrected Mg²⁺-ATPase activity and Mg²⁺-induced ATP hydrolysis in microsomes from stomach smooth muscle: effect of concanavalin A on the Mg²⁺-induced ATP-hydrolysis

(a) Mg²⁺-induced ATP hydrolysis (measured in the presence of Ap₅A) (i) and uncorrected Mg²⁺-ATPase activity (measured in the absence of Ap₅A) (ii) in microsomes from stomach smooth muscle. The broken line represents the extrapolation of the initial Mg²⁺-ATPase activity and the constant Mg²⁺-induced ATP hydrolysis respectively. (b) The uncorrected Mg²⁺-induced ATP hydrolysis in the absence of concanavalin A is represented by the continuous line, and the Mg²⁺-induced ATP hydrolysis in the presence of concanavalin A (20 µg/ml) by the dotted line. The tracings have not been corrected for the microsomal NADH oxidase activity.

to the higher-density fractions in which the mitochondria occur. The high specific activities of the marker enzymes 5'-nucleotidase and (Na⁺ + K⁺)-ATPase in the lower-density fractions [19.5–26.5% (w/v) sucrose] indicate that the plasma membranes equilibrate at this sucrose concentration, whereas the mitochondria equilibrate in the higher-density fractions [32.5–48.0% (w/v) sucrose]. In the latter fractions both the NADH oxidase activity and the adenylate kinase activity contribute appreciably to the total amount of apparent Mg²⁺-ATPase activity. However, the largest amount and the highest specific activity of adenylate kinase activity is recovered in the low-density fraction. In this fraction a large amount of NADH oxidase activity is also present, and at a high specific activity. It can also be inferred from Fig. 7 that saponin at 20 µg/ml unmasks latent adenylate kinase activity in the mitochondrial fractions. Saponin does not significantly affect the Mg²⁺-ATPase activity measured in the presence of Ap₅A, but it enhances the Mg²⁺-ATPase activity when measured without Ap₅A. This effect is even more apparent in the presence of 100 µM-AMP, i.e. under optimal conditions for measuring the adenylate kinase activity. The absence of a stimulation by saponin in the low-density fraction again suggests that the adenylate kinase in this fraction occurs as a soluble enzyme. Watanabe *et al.* (1979) also described a soluble and a mitochondrial component of adenylate kinase in porcine heart. Fig. 6, furthermore, illustrates the distribution of the Mg²⁺-ATPase activity. Its distribution is similar to that of the plasma-membrane markers, except in the lowest-density fraction, where an appre-

ciable amount of Mg²⁺-ATPase activity can be recovered. Part of the Mg²⁺-induced ATP hydrolysis may depend on a soluble enzyme.

When highly purified plasma membranes were prepared after digitonin treatment (Raeymaekers *et al.*, 1985), the contribution of adenylate kinase to Mg²⁺-ATPase activity in the plasma-membrane-enriched fraction amounted to 57.3 ± 3.2% (*n* = 4).

DISCUSSION

It is often very difficult to determine (Na⁺ + K⁺)-ATPase activity (Turi & Török, 1985) or (Ca²⁺ + Mg²⁺)-ATPase activity (Daniel, 1985) in microsomes isolated from various smooth-muscle preparations. A major complicating factor is the very high apparent Mg²⁺-ATPase activity. Furthermore, the study of the (Ca²⁺ + Mg²⁺)-ATPase activity associated with Ca²⁺ transport can be hampered by the inhibition of this apparent Mg²⁺-ATPase activity by Ca²⁺, as has been described in microsomes of the myometrium [the preceding paper (Missiaen *et al.*, 1988)] and the liver (Fleschner & Kraus-Friedmann, 1986).

A major conclusion from the present study is that the Mg²⁺-ATPase activity, as measured with the classic NADH-coupled enzyme system, is subject to artefacts. Both NADH oxidation that is not coupled to ATPase activity and the adenylate kinase activity result in an appreciable overestimate of the activity of the Mg²⁺-ATPase. The spontaneous oxidation of NADH, reported here and described for rat liver microsomes (Boutin,

Fig. 6. Density distribution of protein, 5'-nucleotidase, cytochrome *c* oxidase, (Na⁺ + K⁺)-ATPase, rotenone-insensitive NADH:cytochrome *c* reductase, NADH oxidase, Mg²⁺-induced ATP hydrolysis and adenylate kinase activity in microsomes from stomach smooth muscle subfractionated on a sucrose density gradient

●, Percentage of total activity (left ordinate). ×, Specific activities in nmol/min per mg of protein (or munits/mg of protein for cytochrome *c* oxidase) (right ordinate). In the very bottom panel the percentage contribution of the adenylate kinase, the NADH-oxidase activity and the Mg²⁺-induced ATP-hydrolysis to the total apparent Mg²⁺-ATPase activity is represented. These data are experimental results typical for six experiments.

1986), has to be taken into account when calculating the true Mg²⁺-ATPase activity. This NADH oxidase activity is hardly detectable in the ATPase assay of most tissues because of the presence of the much larger ATPase-linked NADH oxidation. However, in microsomes from pig stomach smooth muscle, in which the Mg²⁺-ATPase

activity is unusually low, this NADH oxidase activity amounts to 10% of the total apparent Mg²⁺-ATPase activity and is responsible for the initial non-linearity of the apparent Mg²⁺-ATPase activity.

A second and quantitatively more important artefact recognized in the present study is that not all ADP

Fig. 7. Effect of saponin on the Mg²⁺-ATPase activity in microsomes from stomach smooth muscle subfractionated on a sucrose density gradient

The open column represents the specific activity of the Mg²⁺-ATPase activity in the absence of saponin; the filled column represents the increment of the Mg²⁺-ATPase activity obtained by adding saponin (20 µg/ml). This unmasking effect of saponin was determined either in the presence of 100 µM-Ap₅A (a), in control conditions (b) or in the presence of 100 µM-AMP (c). The sucrose density of the fractions is given underneath the columns. The specific activities are expressed in nmol/min per mg of protein. The results are those for a typical experiment.

molecules in the assay reaction are produced by Mg²⁺-induced ATP hydrolysis; adenylate kinase also contributes to this ADP production. A continuous production of AMP by the microsomes must therefore be assumed. During the initial period after adding the membranes to the assay medium the Mg²⁺-ATPase activity can be very high, and the ADP production resulting from adenylate kinase activity is then only of minor importance. The contribution of the ADP generated by adenylate kinase becomes increasingly more important as the labile component of the Mg²⁺-induced ATP hydrolysis becomes inactivated. Because microsomes from stomach smooth muscle present a low rate of ATP hydrolysis from the outset, adenylate kinase activity already contributes significantly to the ADP production during the initial period. The error induced by the adenylate kinase activity is quantitatively the most

important and may be corrected for by addition of Ap₅A.

The function of the basal Mg²⁺-ATPase activity is not known. Hamlyn & Senior (1983) have proposed that Mg²⁺-ATPase is an ectoenzyme capable of hydrolysing extracellular ATP. However, it is most likely that the Mg²⁺-induced ATP hydrolysis observed in our microsomes is due to more than one enzyme [see the preceding paper (Missiaen *et al.*, 1988)]. This is supported by the finding that the Mg²⁺-ATPase activity of stomach smooth-muscle membranes does not distribute in the density-gradient as do other membrane markers, but instead equilibrates largely at the top of the gradient.

Barron & DiSalvo (1979) first noticed the non-linear behaviour of the Mg²⁺-ATPase activity in microsomes from bovine aorta. Beeler *et al.* (1983) confirmed this in their kinetic analysis of the Mg²⁺-ATPase activity in microsomes from rat skeletal muscle, and they extended their observations to various rat tissues (Beeler *et al.*, 1985). We recently observed that, in myometrial microsomes, this non-linear behaviour of the Mg²⁺-ATPase activity was hormone-dependent [the preceding paper (Missiaen *et al.*, 1987)]. The absence of a non-linearity in microsomes from stomach smooth muscle seemed at first to be an exception, but this discrepancy can be explained by the important contribution of adenylate kinase activity to the ADP production in that tissue.

A crude vesicular fraction can be prepared from smooth muscle in about 90 min, but the preparation of subfractions that are highly enriched in plasma membranes requires more than 24 h (Raeymaekers *et al.*, 1985). Our results indicate that the properties of the apparent Mg²⁺-ATPase activity can be affected by storage of the microsomes on ice for 24 h.

Adenylate kinase and, to a lesser extent also NADH oxidase activity, lead to a considerable overestimation of the Mg²⁺-ATPase activity in the fractions enriched in mitochondria, equilibrating between 32.5 and 41.0% sucrose. Addition of saponin (20 µg/ml) to the assay medium in order to unmask any latent enzyme activity strongly stimulates the adenylate kinase activity in all but the low-density fraction. This suggests the presence of two types of adenylate kinase: a soluble fraction and a membrane-bound fraction, the latter distributing along with the mitochondria.

Because of these artefacts that occur in the coupled enzyme assay, measurements of Mg²⁺-ATPase activity by the P_i release method might seem a valuable alternative. Unfortunately also the P_i-release method suffers from its own artefacts (L. Missiaen, F. Wuytack, H. Van Belle, J. Minten & R. Casteels, unpublished work).

In conclusion, each of the different smooth-muscle membrane preparations which we have investigated presents a Mg²⁺-ATPase activity that consists of at least three different components: (1) a true Mg²⁺-ATPase activity that inactivates on contact with millimolar concentrations of ATP; the function of this enzyme activity is not known, but it most likely consists of several distinct enzymes; (2) an 'artefactual' Mg²⁺-ATPase activity due to ADP production by adenylate kinase activity; this component can be suppressed by Ap₅A; (3) an 'artefactual' ATPase activity due to NADH oxidation and not related to ATPase activity. The relative contribution of the three components varies among the different tissues.

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Received 8 May 1987/7 September 1987; accepted 4 November 1987