Isolation of a plasma-membrane fraction from gastric smooth muscle

Comparison of the calcium uptake with that in endoplasmic reticulum

Luc RAEYMAEKERS, Frank WUYTACK, Jan EGGERMONT, Greet DE SCHUTTER and
Rik CASTEELS

Laboratorium voor Fysiologie, Universiteit Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

(Received 28 June 1982/Accepted 12 October 1982)

1. A plasma-membrane fraction was isolated from the smooth muscle of the pig stomach by using differential and sucrose-density-gradient centrifugations. When the centrifugation was carried out after preloading the crude microsomal fraction with Ca^{2+} in the presence of oxalate, the contamination of the plasma-membrane fraction by endoplasmic reticulum was decreased and a fraction enriched in endoplasmic reticulum vesicles filled with calcium oxalate crystals was obtained. 2. The plasmalemmal and endoplasmic-reticulum membranes could be distinguished by differences in the activity of marker enzymes and in the cholesterol content and by their different permeability to oxalate and phosphate. Oxalate and phosphate stimulated the Ca^{2+} uptake in the endoplasmic reticulum much more than in the plasmalemmal vesicles. In the plasma-membrane vesicles 40 mM-phosphate was more effective for stimulating the Ca^{2+} uptake than was 5 mM-oxalate, but the reverse was seen in the endoplasmic reticulum. 3. The high cholesterol/phospholipid ratio of the crude microsomal fraction suggests that the majority of the vesicles present in the crude microsomal fraction are of plasmalemmal origin. 4. The Ca^{2+} pump of the plasmalemmal and endoplasmic-reticulum vesicles could be differentiated by their different sensitivities to calmodulin. However, the two Ca^{2+} -transport ATPases did not differ by their sensitivity to vanadate nor by the energization of the Ca^{2+} transport by different nucleoside triphosphates.

The contractile state of smooth-muscle cells is regulated by the cytoplasmic Ca^{2+} concentration in the range 0.1–10 μM . During excitation, the Ca^{2+} permeability of the PM increases, allowing a net influx of extracellular Ca^{2+} ions into the cytoplasm. In addition, Ca^{2+} can be released from an intracellular store, probably the ER. In order to explain the return to the original relaxed state, one has to postulate a Ca^{2+} -transport system in the PM which extrudes Ca^{2+} out of the cell and Ca^{2+} -pump sites in the membranes of the intracellular store (Droogmans *et al.*, 1977; van Breemen *et al.*, 1980). In smooth muscle an ATP-dependent Ca^{2+} extrusion across the cell membrane seems to be more important than $\text{Na}^+/\text{Ca}^{2+}$ exchange energized by the inwardly directed Na^+ gradient (Droogmans *et al.*, 1977; Droogmans & Casteels, 1979; van Breemen *et al.*, 1980). The ATP-dependent Ca^{2+} uptake by the ER of smooth muscle has been demonstrated by

using chemically skinned fibres, and this ATP-dependent Ca^{2+} uptake is stimulated by the presence of oxalate (Raeymaekers, 1982). It can be assumed that, as has been shown in sarcoplasmic reticulum of skeletal muscle, oxalate permeates passively through the membranes of this reticulum, resulting in a precipitation of the inwardly transported Ca^{2+} as oxalate crystals (Hasselbach, 1964).

Crude microsomal fractions isolated from smooth muscle also show ATP-dependent and oxalate-stimulated Ca^{2+} uptake, as well as the concomitant Ca^{2+} -stimulated ATPase activity (Wuytack & Casteels, 1980). Such fractions contain vesicles derived from PM and from ER membranes. Subfractions enriched in putative marker enzymes for one of these types of membranes have been prepared by density-gradient centrifugation (Sakai *et al.*, 1981; Carsten & Miller, 1980; Matlib *et al.*, 1979; Wuytack *et al.*, 1978; Stauber & Schottelius, 1975). It has been shown previously that the isolation of ER of gastric smooth muscle is greatly improved by loading the crude microsomal vesicles with Ca^{2+} in the presence

Abbreviations used: PM, plasma membrane; ER, endoplasmic reticulum.

of oxalate, because this loading causes an increase of the density of the ER vesicles, which are oxalate-permeable (Raeymaekers *et al.*, 1980). This observation suggests that the oxalate-loading technique may be useful to improve the purification of PM by removing contaminating ER, as previously observed for heart muscle (Jones *et al.*, 1979). The aim of the present work was to isolate plasma membranes from the stomach smooth muscle by using the oxalate-loading technique and to compare the Ca^{2+} uptake of plasmalemmal vesicles with that of ER vesicles. A preliminary report of the results has been presented (Raeymaekers *et al.*, 1982).

Methods

Preparation of the crude membrane fraction

The smooth muscle of the antrum of the pig stomach was homogenized as described previously (Raeymaekers *et al.*, 1980). A crude vesicular fraction was prepared from the homogenate by centrifugation in a Sorvall GS3 rotor at 6500 rev./min for 15 min. The supernatant (designated post-nuclear supernatant) was further centrifuged in a Sorvall GSA rotor at 13000 rev./min for 15 min, and the supernatant from this step was spun in a Kontron TFT 45 rotor at 35000 rev./min. for 1 h. The microsomal pellets obtained from 35 g of muscle were resuspended in 25 ml of 0.25 M-sucrose. Larger particles were removed from this fraction by centrifugation at 14000 rev./min for 15 min in a Sorvall SS-34 rotor, and this pellet was discarded.

Ca^{2+} loading

The microsomal vesicles were loaded with Ca^{2+} by incubation at 37°C in 60 ml of a medium containing (final concns., mM): KCl 100, Tris/ATP 5, MgCl_2 5, NaN_3 5, imidazole/HCl (pH 6.9) 20, CaEGTA 1, potassium oxalate 5, phosphocreatine 10; and creatine kinase, 100 $\mu\text{g}/\text{ml}$. The Ca^{2+} uptake was terminated after 45 min by cooling in ice. In some experiments this medium was supplemented with either $^{45}\text{CaCl}_2$ or $[^{14}\text{C}]\text{oxalate}$. This allowed the study of the distribution of the intravesicular $^{45}\text{Ca}^{2+}$ or $[^{14}\text{C}]\text{oxalate}$ after fractionation of the crude microsomal fraction by sucrose gradient centrifugation.

Sucrose gradient centrifugation

The microsomal suspension was supplemented with solid sucrose to obtain a final sucrose concentration of 24% (w/w). Volumes (11.5 ml) of this microsomal suspension were then layered between 12 ml of 55% sucrose and 15 ml of 8% sucrose in Beckman SW 27 tubes. Centrifugation was performed at 27000 rev./min for 1 h (procedure A) or for 2 h (procedure B). Five fractions were collected from these gradients: the plasma-membrane fraction, FI,

at the 8%/24%-sucrose interface; FII, the 24% sucrose layer; FIII, the band at the 24%/55%-sucrose interface; FIV, the 55%-sucrose layer; FV, the pellet of calcium oxalate-filled ER vesicles at the bottom of the tube. For further purification of the plasma membranes, fraction FI was diluted in 2 vol. of water and centrifuged in a Beckman 75 Ti rotor at 50000 rev./min for 30 min. The pellets were resuspended in 0.25 M-sucrose/10 mM-imidazole/HCl (pH 6.9), and this further purified PM fraction is designated fraction FIP.

In the experiments on the distribution of $^{45}\text{Ca}^{2+}$ or $[^{14}\text{C}]\text{oxalate}$ in the gradient, samples of the fractions were filtered through Millipore filters for the determination of the intravesicular $^{45}\text{Ca}^{2+}$ or $[^{14}\text{C}]\text{-oxalate}$ content.

For the study of the activity of marker enzymes in the different subfractions of the gradient, the radioactive isotopes were omitted from the Ca^{2+} -uptake solution and the subfractions (except fraction FIP, which was obtained by centrifugation; see above) were dialysed overnight against 0.25 M-sucrose/0.1 M-KCl/10 mM-imidazole HCl (pH 6.9) to remove the substances that had been included in the Ca^{2+} -loading medium.

Enzyme assays

The ATPase activity was measured at 37°C in a solution containing (mM): KCl 100, sucrose 100, imidazole/HCl (pH 6.9) 30, Na_2ATP 5, MgCl_2 5, NaN_3 5, NADH 0.26, phosphoenolpyruvate 1.5; and lactate dehydrogenase, 36 units/ml, pyruvate kinase 40 units/ml, microsomal protein 50–100 $\mu\text{g}/\text{ml}$. The basal Mg^{2+} -ATPase activity was measured in EGTA-containing solution in the presence of 10 μM -ouabain and 10 μM of the Ca^{2+} ionophore A23187. The Ca^{2+} -stimulated ATPase activity was measured by comparing the rate of ATP hydrolysis in the presence of 1 mM-EGTA without added Ca^{2+} and that in the presence of 10 μM - Ca^{2+} buffered by a mixture of 1 mM-EGTA and 0.9 mM- CaCl_2 .

5'-Nucleotidase and NADH-cytochrome *c* reductase (rotenone-insensitive) activities were measured as described by Wuytack *et al.* (1978), except that for the measurement of 5'-nucleotidase the Tris buffer was replaced by glycine buffer, as suggested by Goldman & Slakey (1981).

Ca^{2+} uptake

This was measured in a solution similar to that used for the ATPase activity, except that the coupled enzyme system was omitted and the ATP-regenerating system, consisting of phosphocreatine (5 mM) and creatine kinase (100 $\mu\text{g}/\text{ml}$) was included. This solution was supplemented with 0.5 mM-EGTA and 0.45 mM- $^{45}\text{CaCl}_2$. The vesicles were separated from the solution by Millipore filtration. The filters

were rinsed, dried, and the radioactivity remaining on the filters was counted, with 2,5-diphenyl-oxazole (6 g/l) in toluene as scintillant.

Determination of phospholipid, cholesterol and protein content

Lipid extraction of vesicular suspensions was by the method of Bligh & Dyer (1959). The phosphate content of the extract was determined as described by Jaenicke (1974). The cholesterol content was measured with a cholesterol assay kit (Boehringer, Mannheim, West Germany). Protein was measured by the method of Lowry *et al.* (1951).

Materials

Calmodulin was isolated from bovine brain by the method of Sharma & Wang (1979). Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, phosphocreatine, creatine kinase and nucleoside triphosphates were obtained from Boehringer.

Results and discussion

Density-gradient centrifugation of the crude microsomal fraction and the distribution of intravesicular ⁴⁵Ca²⁺ and [¹⁴C]oxalate in the gradient

The crude microsomal vesicles were allowed to accumulate Ca²⁺ in the presence of oxalate in a medium which also contained trace amounts of either ⁴⁵Ca²⁺ or [¹⁴C]oxalate (see the Methods section). Experiments with these radioisotopes were performed in parallel. After sucrose-density-gradient centrifugation according to procedure A, samples of the subfractions were filtered for the determination of the intravesicular ⁴⁵Ca²⁺ or [¹⁴C]oxalate content. These values are given in Table 1. The higher-density fractions (FIII–FV) contain more than 90% of the total accumulated ⁴⁵Ca²⁺. This can be explained by the fact that most of the intravesicular ⁴⁵Ca²⁺ is

trapped in calcium oxalate crystals in the ER vesicles, which thereby acquire a higher density (Hasselbach, 1964). A similar fraction of ER vesicles obtained by differential centrifugation has been studied previously (Raeymaekers *et al.*, 1980; Raeymaekers & Hasselbach, 1981). However, the present fraction FV shows a higher enrichment in terms of intravesicular Ca²⁺ content.

After centrifugation according to procedure B (centrifugation for 2h) instead of by procedure A (centrifugation for 1h), the intravesicular ⁴⁵Ca²⁺ and [¹⁴C]oxalate contents of fraction FV were about 40% lower. This is probably due to mechanical disruption of the oxalate-filled vesicles at the bottom of the tube by the high centrifugal forces. Loss of intravesicular calcium oxalate was negligible during centrifugation by procedure A.

In the different fractions of the gradient, the ratio of the intravesicular [¹⁴C]oxalate to ⁴⁵Ca²⁺ (mol/mol) is about 1, except in fraction FI, in which the oxalate content is only about 25% of the ⁴⁵Ca²⁺ content. This observation suggests that this fraction contains vesicles which have a very low oxalate permeability. These vesicles are probably derived from the PM, because they are recovered from lower densities in the gradient, as has also been observed in other studies of subcellular fractions of smooth muscle (Wei *et al.*, 1976; Wuytack *et al.*, 1978; Grover *et al.*, 1980; Morel *et al.*, 1981). Moreover, this fraction is enriched in 5'-nucleotidase (see below), a marker enzyme which is typical of the plasma membrane.

Further purification of the plasma-membrane fraction, FI, and its comparison with the endoplasmic-reticulum fraction, FV

The activities of 5'-nucleotidase and of NADH-cytochrome *c* reductase (rotenone-insensitive) are summarized in Table 2 for the fractions FI and FV.

Table 1. *Distribution of intravesicular ⁴⁵Ca²⁺ and [¹⁴C]oxalate in membrane fractions separated on a sucrose density gradient*

Crude microsomal vesicles isolated from the pig stomach smooth muscle were loaded with Ca²⁺ in an oxalate-containing solution in the presence of either ⁴⁵Ca²⁺ or [¹⁴C]oxalate and subfractionated on a sucrose density gradient according to procedure A. The distribution of the intravesicular ⁴⁵Ca²⁺ and [¹⁴C]oxalate in the different subfractions of the gradient is given as the percentage of the total intravesicular Ca²⁺ or oxalate in all subfractions and as the amount (nmol) per mg of protein. The values are means ± S.E.M. for three or four observations.

Fraction	Percentage of total intravesicular ⁴⁵ Ca ²⁺ and [¹⁴ C]oxalate		Amount of intravesicular ⁴⁵ Ca ²⁺ and [¹⁴ C]oxalate (nmol/mg)	
	⁴⁵ Ca ²⁺	[¹⁴ C]Oxalate	⁴⁵ Ca ²⁺	[¹⁴ C]Oxalate
FI	0.93 ± 0.06	0.22 ± 0.02	34 ± 4.3	7.7 ± 0.45
FII	5.0 ± 0.36	3.6 ± 0.4	18 ± 3.2	14 ± 2.4
FIII	10.6 ± 1.1	8.9 ± 0.9	101 ± 22	125 ± 29
FIV	36 ± 2.1	38 ± 2.3	1445 ± 90	1276 ± 101
FV	47 ± 2	50 ± 1.6	8780 ± 1210	10 700 ± 2180

Table 2. *Activities of 5'-nucleotidase and of NADH-cytochrome c reductase (rotenone-insensitive) in different membrane fractions isolated from the pig gastric muscle*

Results are means $\pm$ S.E.M., for the numbers of observations shown in parentheses.

	5'-Nucleotidase		NADH-cytochrome c reductase (rotenone-insensitive)	
	(nmol/min per mg)	Enrichment factor	(nmol/min per mg)	Enrichment factor
Post-nuclear supernatant	2.9 $\pm$ 0.5 (5)	1	60 $\pm$ 3 (6)	1
Crude microsomal fraction	8.0 $\pm$ 1.1 (6)	2.8	177 $\pm$ 16 (6)	3
Fractions isolated by procedure A				
FI	20.4 (2)	7	—	—
FIP	42.5 $\pm$ 6 (4)	14.7	243 $\pm$ 26 (4)	4
FV	3.7 $\pm$ 0.7 (3)	1.3	529 $\pm$ 41 (3)	8.8
Fractions isolated by procedure B				
FIP	99 $\pm$ 18 (3)	34	331 $\pm$ 41 (3)	5.5

Table 3. *Phospholipid and cholesterol contents of the crude microsomal fraction and of the isolated endoplasmic reticulum (FV) and plasma-membrane (FIP) fractions*

Results are means $\pm$ S.E.M. for the numbers of observations shown in parentheses.

	Phospholipid/protein ratio (μ mol/mg)	Cholesterol/protein ratio (μ mol/mg)	Cholesterol/phospholipid ratio (mol/mol)
Crude microsomal fraction	0.101 $\pm$ 0.007 (4)	0.048 $\pm$ 0.0009 (3)	0.44 $\pm$ 0.025 (3)
FIP (plasma membranes)	1.12 $\pm$ 0.18 (4)	0.576 $\pm$ 0.073 (4)	0.524 $\pm$ 0.031 (4)
FV (endoplasmic reticulum)	0.428 $\pm$ 0.045 (7)	0.083 $\pm$ 0.004 (7)	0.207 $\pm$ 0.025 (7)

The 5'-nucleotidase activity in fraction FI was found to be enriched 2.5-fold as compared with the crude microsomal fraction. Differential centrifugation of the PM-enriched fraction FI yields a pellet (designated fraction FIP) which is further enriched about 2-fold in terms of 5'-nucleotidase activity as compared with fraction FI. The PM fractions prepared by procedure B show a higher enrichment in 5'-nucleotidase activity than do the corresponding fractions prepared by procedure A. The specific activity of this enzyme reaches a value 12 times that of the crude microsomal fraction and 34 times that of the post-nuclear supernatant. In contrast, the specific activity of 5'-nucleotidase in the ER fraction FV is low.

It should be noted that the high enrichment of the specific activity in fraction FIP is due not only to the separation of different types of membranes, but also to the lower content of contractile proteins in the subfractions of the gradient. It was previously shown that these contractile proteins constitute an appreciable fraction of the total microsomal protein (Raeymaekers *et al.*, 1980).

NADH-cytochrome c reductase has been used as a marker for internal membranes in liver (Hogeboom, 1949). Therefore it is not surprising to find the highest enrichment of this enzyme in the ER

fraction FV. However, the enrichment is only about 3-fold as compared with the crude microsomal fraction, in contrast with the very high enrichment in terms of Ca²⁺ content in this fraction. This discrepancy can possibly be explained by the fact that rotenone-insensitive NADH-cytochrome c reductase is also present in mitochondrial outer membranes (Sottocasa *et al.*, 1967), which are not concentrated in fraction FV. In addition, it is possible that different subpopulations of ER exist which have different ratios of Ca²⁺ uptake to NADH-cytochrome c reductase activity, and that only vesicles having a relatively high Ca²⁺-transport activity are concentrated in fraction FV.

The values for the phospholipid and cholesterol content of the crude microsomal fraction, the PM and the ER fractions are given in Table 3. The low lipid content of the crude microsomal fractions might be due to its high content of non-membrane protein (probably contractile proteins, as mentioned above). The cholesterol/phospholipid ratio in the PM fraction is 2.5 times that in the ER fraction, but it is not much different from the value found for the crude microsomal fraction. This may indicate that the vesicles present in the crude microsomal fraction are mainly derived from the PM and that the ER membranes are present in smaller amounts. A

similar conclusion was drawn by Wibó *et al.* (1981) from the effect of digitonin on the phospholipid distribution.

Ca²⁺ uptake in the plasma-membrane fraction FIP and the effect of oxalate

The ⁴⁵Ca²⁺ uptake in the PM fraction FIP depends on the presence of ATP and reaches a plateau of about 240 nmol/mg of protein in FIP prepared by procedure B (Fig. 1) and 120 nmol/mg in FIP prepared by procedure A (results not shown). The values for the Ca²⁺-uptake capacity of these subfractions FIP and that of FI (Table 1) correlate very well with the enrichment in 5'-nucleotidase activity (Table 2).

Fig. 1 shows that the Ca²⁺ uptake is only slightly stimulated when 5 mM-oxalate is included in the solution, as can be expected from the low [¹⁴C]-oxalate uptake in this fraction (see above). After 60 min this uptake has increased by a factor of 1.6 as compared with the control. It is possible that this limited effect of oxalate is due to some contamination of the PM fraction by ER vesicles.

In order to study the effect of the use of the calcium oxalate-loading technique on the purity of the PM fraction FIP, another FIP fraction was prepared in the same way as the control, except that oxalate had been omitted from the incubation medium during the Ca²⁺ loading. Fig. 1 shows the effect of oxalate on the Ca²⁺ uptake by this fraction. The stimulation of the Ca²⁺ uptake by oxalate is higher than in the control, suggesting that it contains more ER vesicles. This observation suggests that the oxalate potentiation of the Ca²⁺ uptake in PM fractions of smooth muscle that has been described previously (Grover *et al.*, 1980) is not a property of the plasmalemmal membranes, but is most likely due to a contamination with ER vesicles. However, it should be noted that oxalate loading removes only sealed ER vesicles, because leaky vesicles will not form intravesicular calcium oxalate crystals.

Effect of phosphate on the Ca²⁺ uptake by the plasma-membrane vesicles

The potentiation of the Ca²⁺ uptake by 5 mM-oxalate and by 40 mM-P_i was compared. Phosphate was used at a higher concentration than oxalate because the stimulation of the Ca²⁺ uptake by Ca²⁺-precipitating anions is a function of the solubility product of their calcium salts, as has been shown for sarcoplasmic reticulum of skeletal muscle (Martonosi & Feretos, 1964; Hasselbach & Makinose, 1965).

ER vesicles of smooth muscle are also highly permeable to oxalate and phosphate, as indicated by the fact that the Ca²⁺ uptake in the presence of one of these anions induces the formation of intravesicular calcium oxalate or calcium phosphate

Fig. 1. ⁴⁵Ca²⁺ uptake in plasma-membrane vesicles (FIP) isolated from pig gastric smooth muscle by procedure B

The symbols refer to different conditions during the assay of the Ca²⁺ uptake. The different lines refer to membrane fractions prepared after Ca²⁺ loading under control and under modified conditions. The ⁴⁵Ca²⁺ uptake was measured at 37°C in the absence of Ca²⁺-precipitating anions (O, ●), in the presence of 5 mM-oxalate (▲) or in the presence of 40 mM-phosphate (■). The open squares (□) represent the Ca²⁺ uptake in the absence of ATP. Calmodulin was not added, except for the open circles (O), which represent the Ca²⁺ uptake in the presence of 10 µg of calmodulin/ml. The full lines represent the Ca²⁺ uptake by a control fraction FIP prepared after loading with Ca²⁺ in the presence of 5 mM-oxalate. The dotted and the dashed line show the Ca²⁺ uptake in FIP prepared under modified conditions: dotted line, Ca²⁺ uptake in fraction FIP prepared after loading with Ca²⁺ in the absence of oxalate; dashed line, Ca²⁺ uptake in fraction FIP prepared after loading with Ca²⁺ in the presence of 40 mM-phosphate instead of 5 mM-oxalate. The vertical bars show the S.E.M. for three experiments. Curves without S.E.M. bars are the mean of two experiments.

deposits (Raeymaekers *et al.*, 1980, 1981); 5 mM-oxalate has a larger potentiating effect on the Ca²⁺ uptake than does 40 mM-phosphate. This difference cannot be demonstrated unequivocally on the isolated ER fraction because, in order to isolate this

Fig. 2. Effect of 5 mM-oxalate and 40 mM-phosphate on the $^{45}\text{Ca}^{2+}$ uptake in the crude microsomal fraction. The Ca^{2+} uptake was measured at 37°C , (●) in the absence of Ca^{2+} -precipitating anions, (■) in the presence of 40 mM-phosphate, or (▲) in the presence of 5 mM-oxalate. The open squares (□) show the effect of omission of ATP.

fraction, these vesicles would have to be loaded either with calcium oxalate or with calcium phosphate deposits. Therefore the anion-stimulated Ca^{2+} uptake was measured in the crude microsomal fraction, which is a mixture of ER and PM vesicles. However, the oxalate-stimulated Ca^{2+} uptake largely depends on the ER vesicles, as can be deduced from the very low permeability of the PM vesicles to this anion. As shown in Fig. 2, the Ca^{2+} uptake in the crude microsomal fraction is stimulated to a larger extent by 5 mM-oxalate than by 40 mM-phosphate.

In the PM fraction FIP, the potentiating effect of 40 mM-phosphate on the Ca^{2+} uptake is small (only 4-fold after 60 min of uptake), but it is larger than that of 5 mM-oxalate (Fig. 1). The effect of P_i on the Ca^{2+} uptake by the PM fraction FIP cannot be ascribed to the presence of contaminating ER vesicles. This could be demonstrated by preparing fraction FIP after loading with Ca^{2+} in the presence of 40 mM-phosphate instead of 5 mM-oxalate. By this procedure the Ca^{2+} -transporting vesicles with a high phosphate permeability are eliminated from fraction FI, because intravesicular phosphate deposits have been formed in these vesicles. However, the stimulation by phosphate of the Ca^{2+} uptake in the PM fraction FIP prepared by this modified procedure was about the same as in the control (Fig. 1).

Fig. 3. Effect of calmodulin and of different nucleoside triphosphates on the initial time course of the $^{45}\text{Ca}^{2+}$ uptake in the plasma-membrane fraction FIP prepared by procedure B.

The Ca^{2+} uptake was measured in the presence of ATP (●), ATP + calmodulin (10 $\mu\text{g}/\text{ml}$) (○), UTP (□), deoxyATP (■), ITP (△), GTP or CTP (▲) and in the absence of energy-yielding substrate (dashed line). The concentration of all nucleoside triphosphates was 5 mM. The curves represent the mean of three experiments.

That the Ca^{2+} uptake in this modified fraction FIP is stimulated by phosphate, notwithstanding the fact that vesicles containing calcium phosphate deposits were removed during its preparation, can be explained by assuming that the phosphate-stimulated Ca^{2+} uptake in the PM fraction FIP depends on the majority of the vesicles present in that fraction. The additional amount of Ca^{2+} taken up per vesicle in the presence of phosphate will then be too small to induce an increase in their density that would be sufficient to eliminate them from the lighter fraction during the density gradient centrifugation.

Characteristics of the Ca^{2+} pump of the endoplasmic-reticulum fraction FV and plasmalemmal fraction FIP

The ATPase activity of the PM fraction FIP was stimulated about 2.5-fold by the addition of Ca^{2+} . The Ca^{2+} -stimulated ATPase activity of fraction FIP prepared by procedure B was 116 nmol of P_i/min per mg. The ATPase activity was further stimulated by the addition of calmodulin (10 $\mu\text{g}/\text{ml}$) by a factor of 1.65. Also the rate of Ca^{2+} uptake in the PM fraction was stimulated to the same degree by calmodulin (Fig. 3), but the effect of calmodulin on the plateau value of the Ca^{2+} uptake was negligible (Fig. 1). Calmodulin had no effect on the ATPase activity nor on the rate of Ca^{2+} uptake by

Fig. 4. Vanadate inhibition of the ATP-dependent Ca²⁺ uptake and of the Ca²⁺-stimulated ATPase activity. ○, ●, Ca²⁺ uptake. The Ca²⁺ uptake was stopped 30s after the addition of ATP to the medium. □, Ca²⁺-stimulated ATPase activity. ●, □, Plasma-membrane fraction FIP; ○, endoplasmic-reticulum fraction FV. The experimental points are the means of two experiments.

the ER fraction FV as measured in the presence of 5 mM-oxalate. When the experiments were repeated after washing the fraction overnight at 4°C in the presence of 10 mM-EGTA, which is expected to decrease the amount of bound calmodulin, the effect of added calmodulin on the Ca²⁺-stimulated ATPase activity of fraction FV or FIB was not augmented. The absence of an effect of calmodulin on the oxalate-stimulated Ca²⁺ uptake has also been observed with microsomal fractions from pig coronary artery (Wuytack *et al.*, 1980) and from intestinal muscle (Wibo *et al.*, 1981). However, at present it cannot be excluded that the Ca²⁺-stimulated ATPase of the ER could not be affected indirectly by calmodulin through activation of a protein kinase, as has been shown for sarcoplasmic reticulum of cardiac muscle (Lopaschuk *et al.*, 1980). The lack of a calmodulin effect on the Ca²⁺ uptake in the ER suggests that the Ca²⁺-stimulated ATPase isolated from the crude microsomal fraction by affinity chromatography on a calmodulin column (Wuytack *et al.*, 1981) is the Ca²⁺-stimulated ATPase of the plasmalemma.

Because it has been shown that the isolated sarcoplasmic reticulum and plasma membrane from cardiac muscle have a different sensitivity not only to calmodulin, but also to vanadate (Caroni & Carafoli, 1981) and to various energy-yielding substrates (Trumble *et al.*, 1981), the effect of these

agents on the PM and ER fractions of smooth muscle was tested.

The order of potency of different nucleoside triphosphates to support the Ca²⁺ uptake in the PM fraction FIP is ATP > UTP ≈ deoxyATP > ITP > CTP ≈ GTP (Fig. 3). The relative potency of each nucleoside triphosphate to stimulate the Ca²⁺ uptake is very similar to that observed on the oxalate-stimulated Ca²⁺ uptake by the ER vesicles (Raeymaekers, 1982).

The inhibitory effect of different vanadate concentrations on the rate of Ca²⁺ uptake is similar in the ER fraction FV and in the PM fraction FIP. For both fractions, half-maximal inhibition occurs at about 4 μM-vanadate (Fig. 4). The finding that both Ca²⁺-transport ATPases are inhibited to the same extent by vanadate and that different energy-yielding substrates have about the same order of potency suggests that these Ca²⁺-transport enzymes do not have as different properties as those of cardiac muscle.

This work was supported by grant no. 3.0087.74 of the Fonds voor Wetenschappelijk Geneeskundig Onderzoek, Belgium.

References

- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Caroni, P. & Carafoli, E. (1981) *J. Biol. Chem.* **256**, 3263–3270
- Carsten, M. E. & Miller, J. D. (1980) *Arch. Biochem. Biophys.* **204**, 404–412
- Droogmans, G. & Casteels, R. (1979) *J. Gen. Physiol.* **74**, 57–70
- Droogmans, G., Raeymaekers, L. & Casteels, R. (1977) *J. Gen. Physiol.* **70**, 129–148
- Goldman, S. J. & Slakey, L. L. (1981) *Biochim. Biophys. Acta* **658**, 169–173
- Grover, A. K., Kwan, C. Y., Crankshaw, J., Crankshaw, D. J., Garfield, R. E. & Daniel, E. E. (1980) *Am. J. Physiol.* **239**, C66–C74
- Hasselbach, W. (1964) *Progr. Biophys. Mol. Biol.* **14**, 167–222
- Hasselbach, W. & Makinose, M. (1965) *Biochem. Z.* **343**, 360–382
- Hogeboom, G. H. (1949) *J. Biol. Chem.* **177**, 847–858
- Jaenicke, J. (1974) *Anal. Biochem.* **61**, 623–627
- Jones, L. R., Besch, H. R., Fleming, J. W., McConaughy, M. M. & Watanabe, A. M. (1979) *J. Biol. Chem.* **254**, 530–539
- Lopaschuk, G., Richter, B. & Katz, S. (1980) *Biochemistry* **19**, 5603–5607
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martonosi, A. & Feretos, R. (1964) *J. Biol. Chem.* **239**, 648–658
- Matlib, M. A., Crankshaw, J., Garfield, R. E., Crankshaw, D. J., Kawn, C.-Y., Branda, L. A. & Daniel, E. E. (1979) *J. Biol. Chem.* **254**, 1834–1840
- Morel, N., Wibo, M. & Godfraind, T. (1981) *Biochim. Biophys. Acta* **644**, 82–88

- Raeymaekers, L. (1982) *Z. Naturforsch. Teil C* **37**, 481–488
- Raeymaekers, L. & Hasselbach, W. (1981) *Eur. J. Biochem.* **116**, 373–378
- Raeymaekers, L., Agostini, B. & Hasselbach, W. (1980) *Histochemistry* **65**, 121–129
- Raeymaekers, L., Agostini, B. & Hasselbach, W. (1981) *Histochemistry* **70**, 139–150
- Raeymaekers, L., Wuytack, F., De Schutter, G. & Casteels, R. (1982) *Arch. Int. Physiol. Biochim.* **90**, 16–17
- Sakai, Y., McLean, J., Grover, A. K., Garfield, R. E., Fox, J. E. T. & Daniel, E. E. (1981) *Can. J. Physiol. Pharmacol.* **59**, 1260–1267
- Sharma, R. K. & Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 187–198
- Sottocasa, G. L., Kuylensstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
- Stauber, W. T. & Schottelius, B. A. (1975) *Proc. Soc. Exp. Biol. Med.* **150**, 529–533
- Trumble, W. R., Sutko, J. L. & Reeves, J. P. (1981) *J. Biol. Chem.* **256**, 7101–7104
- van Breemen, C., Aaronson, P., Loutzenhiser, R. & Meisheri, K. (1980) *Chest* **78**, 157–165
- Wei, J. M., Janis, R. A. & Daniel, E. E. (1976) *Circ. Res.* **39**, 133–140
- Wibo, M., Morel, N. & Godfraind, T. (1981) *Biochim. Biophys. Acta* **649**, 651–660
- Wuytack, F. & Casteels, R. (1980) *Biochim. Biophys. Acta* **595**, 257–263
- Wuytack, F., Landon, E., Fleischer, S. & Hardman, J. G. (1978) *Biochim. Biophys. Acta* **540**, 253–269
- Wuytack, F., De Schutter, G. & Casteels, R. (1980) *Biochem. J.* **190**, 827–831
- Wuytack, F., De Schutter, G. & Casteels, R. (1981) *FEBS Lett.* **129**, 297–300