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Subcellular fractionation of pig stomach smooth muscle. A study of the distribution of the (Ca^{2+} + Mg^{2+})-ATPase activity in plasmalemma and endoplasmic reticulum

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Isolated membrane vesicles from pig stomach smooth muscle (antral part) were subfractionated by a density gradient procedure modified in order to obtain an efficient extraction of extrinsic proteins. By using this method in combination with digitonin-treatment, an endoplasmic reticulum fraction contaminated with maximally 10 to 20% of plasma membranes was isolated, together with a plasma membrane fraction containing at most 30% endoplasmic reticulum. The endoplasmic reticulum and plasma membrane fractions differed in protein composition, reaction to digitonin, binding of wheat germ agglutinin, activities of marker enzymes and in the characteristics of the Ca^{2+} uptake. The Ca^{2+} uptake by the endoplasmic reticulum was much more stimulated by oxalate than the uptake by plasma membranes. Both fractions showed a (Ca^{2+} + Mg^{2+})-ATPase activity, but the largest amount of this enzyme was present in the plasma membranes. The study of the phosporylated intermediates of the (Ca^{2+} + Mg^{2+})-ATPases by polyacrylamide gel electrophoresis revealed two phosphoproteins one of 130 kDa and one of 100 kDa (Wuytack, F., Raeymaekers, L., De Schutter, G. and Casteels, R. (1982) Biochim. Biophys. Acta 693, 45–52). The 130 kDa enzyme was predominant in the fraction enriched in plasma membrane whereas the distribution of the 100 kDa polypeptide correlated with the endoplasmic reticulum markers. The 130 kDa ATPase was the main ^{125}I-calmodulin binding protein detected on nitrocellulose blots of proteins separated by gel electrophoresis. The (Ca^{2+} + Mg^{2+})-ATPase activity of the plasma membranes was higher than the (Na^+ + K^+)-ATPase activity, suggesting that the Ca^{2+} extrusion from these cells depends much more on the activity of the (Ca^{2+} + Mg^{2+})-ATPase than on Na^+-Ca^{2+} exchange.

Introduction

Ca^{2+}-transport systems which regulate the Ca^{2+} metabolism during contraction and relaxation in smooth muscle are present both in the endoplasmic reticulum and in the plasmalemma. Important questions which are at present unsolved not only bear on the mechanism of these Ca^{2+} transport systems but also on their relative importance under different conditions [1–5]. A possible approach to solve these questions is to isolate the relevant subcellular membranes involved in Ca^{2+} transport. Many studies have been carried out on the Ca^{2+} uptake in crude membrane preparations and in plasma membrane-enriched fractions. However, it has proven to be more difficult to purify to

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetatic acid.

a sufficient degree the endoplasmic reticulum membranes from intestinal and vascular smooth muscle. Isolation of the endoplasmic reticulum from stomach smooth muscle by means of loading it with calcium oxalate [6–8] introduces a serious drawback for the study of various aspects of Ca^{2+} transport, e.g. the presence of intravesicular calcium oxalate deposits interferes both with Ca^{2+} uptake and release in these membranes. We have therefore tried to obtain endoplasmic reticulum vesicles by a method which does not imply calcium loading. The present method also allows a more efficient extraction of extrinsic proteins, mainly actin and myosin, from the membrane vesicles. In addition we have investigated the distribution of the $(Ca^{2+} + Mg^{2+})$-ATPase activity which is the enzymatic basis for the ATP-dependent Ca^{2+}-transport. This activity has only recently been described in crude membrane preparations [9]. A study of its distribution over the different subfractions could provide more precise information on Ca^{2+} transport than the measurement of the Ca^{2+} uptake alone, because the latter does not only depend on the activity of the Ca^{2+} pump, but also on the permeability, the size and the sidedness of the isolated vesicles.

Methods

Preparation of membrane fractions

Muscles of the antral part of pig stomachs were homogenized by passing them through a press as described by Wuytack et al. [10]. The mince was dispersed in 3 volumes 0.25 M sucrose containing 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride and centrifuged in a Sorvall GSA rotor at $13\,000 \times g_{max}$ for 30 min. The pellet and a floating layer were discarded and the supernatant (designated as 'post mitochondrial supernatant') was directly applied below a density gradient. In some experiments, 120 mg digitonin, dissolved in 10 ml ethanol, was added to 1200 ml of the supernatant. This amount corresponds to a digitonin/cholesterol molar ratio of 0.7–1. Solid sucrose and KCl were added to the supernatant up to a final concentration of 50% (weight/weight) and 0.6 M, respectively. This material was loaded into a Beckman Ti15 zonal rotor below 250 ml of a 15 to 45% sucrose gradient which was linear with respect to volume and which contained 0.6 M KCl and 1 mM dithiothreitol. The central part of the rotor was filled with 150 ml 0.6 M KCl. Centrifugation was performed at $105\,000 \times g_{max}$ for 20 h at 4°C. Fixed volume fractions were collected and each fraction was assigned a '% sucrose' value as read in a Zeiss hand refractometer. It should be noted that the density corresponding to these values is slightly higher (about 0.01 g/cm^3) than that of solutions of pure sucrose giving the same reading in the refractometer. A small volume of these fractions was saved for the determination of the Ca^{2+} uptake, since it has been observed that pelleting reduces the Ca^{2+} uptake [11]. The rest was diluted three times with 0.6 M KCl containing 1 mM dithiothreitol and pelleted in a Kontron TFT45.94 rotor at $140\,000 \times g_{max}$ for 1 h. The pellets and the tubes were rinsed to remove KCl, and the pellets were resuspended in 0.25 M sucrose containing 1 mM dithiothreitol.

Calcium oxalate-loaded vesicles were prepared from a crude membrane mixture recovered between 20 and 25% sucrose. The suspension was diluted with 3 volumes of a solution of such composition that the following final concentrations were obtained (mM): KCl 150, NaN_3 5, imidazole-HCl (pH 6.9) 20, ATP 5, $MgCl_2$ 5, potassium oxalate 5, CaEGTA 2 (equal concentrations of $CaCl_2$ and EGTA), phosphocreatine 10, creatine kinase 50 μg/ml. The suspension was incubated at 37°C for 30 min and then cooled in ice. While cooling, solid sucrose was dissolved to a final concentration of 30% in order to avoid sedimentation of non-loaded vesicles. Subsequent centrifugation in a Kontron TFT45.94 rotor at $45\,000 \times g_{max}$ for 20 min yielded a small white pellet of calcium-oxalate loaded vesicles.

Inside-out plasmalemmal vesicles from pig erythrocytes and sarcoplasmic reticulum from pig skeletal muscle were prepared as described by Wuytack et al. [12].

Enzyme assays

All enzyme activities were assayed by continuously monitoring the reaction rate at 37°C in a Varian Cary 219 or in an Aminco DW2 spectrophotometer. 5'-Nucleotidase was measured using a kit obtained from Sigma. NADH-cytochrome *c* reductase (rotenone-insensitive) was measured as

described [10]. NADPH-cytochrome *c* reductase was determined similarly, except for the replacement of NADH by NADPH. Mg^{2+}-ATPase activity was measured by following the decrease in absorbance at 340 nm of a solution containing (mM): NaCl 95, KCl 10, NaN_3 5, imidazole-HCl(pH 7.5) 30, Tris-ATP 5, $MgCl_2$ 6, EGTA 0.5, NADH 0.26, digitoxigenin 0.05, phospho*enol*-pyruvate 1.5, lactate dehydrogenase 36 units/ml, pyruvate kinase 40 units/ml. Digitoxigenin was included to inhibit the $(Na^+ + K^+)$-ATPase activity, and NaN_3 to inhibit the mitochondrial ATPase. The Mg^{2+} ATPase activity was not linear with time, but slowly decreased. The rate values given in the results were calculated from the decrease of the absorbance observed 2.5 min after the start of the reaction.

The $(Na^+ + K^+)$-ATPase activity was calculated from the inhibition of the rate of ATP splitting by ouabain (10 μM) or digitoxigenin (50 μM). $(Ca^{2+} + Mg^{2+})$-ATPase activity is defined as the difference between the rate of ATP-splitting in the presence of 0.5 mM EGTA without added Ca^{2+} and that after addition of Ca^{2+} resulting in about 10^{-5} M of free Ca^{2+}. The composition of the medium was the same as that used for the basal Mg^{2+} ATPase. In order to circumvent the difficulty created by the non-linear reaction rate of the basal Mg^{2+}-ATPase, the activities were measured by recording the difference in the absorbance between a sample and a reference cell of identical composition, except for the presence or absence of digitoxigenin or ouabain (for $(Na^+ + K^+)$-ATPase) or Ca^{2+} (for $(Ca^{2+} + Mg^{2+})$-ATPase). For the determination of the $(Ca^{2+} + Mg^{2+})$-ATPase activity, $CaCl_2$ from a 50 mM stock solution was added to the reference cell in small aliquots until the stimulation of the reaction rate was maximal (at about 0.45 mM). Each time, the same amount of 0.1 M HCl was added to the other cell to obtain a similar pH shift (about 0.1 unit) as that induced by the liberation of H^+ ions from EGTA by Ca^{2+}. It should be mentioned that our subcellular fractions contain two types of $(Ca^{2+} + Mg^{2+})$-ATPase and that the conditions in which the activity is maximal may be different for these two enzymes. In order to obtain the maximum activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasmalemma, 0.6 μM calmodulin was routinely added. Changing the pH from 7.4 to 6.9, or substituting the Na^+ by K^+ had little effect on the $(Ca^{2+} + Mg^{2+})$-ATPase activity. Saponin (100 μg/ml) was included in the medium to open vesicles and to expose latent ATPase sites. This substance exerted a greater stimulatory effect than the detergents Triton X-100, deoxycholate, or the pore-forming antibiotic alamethicin [13].

Electrophoresis and electroblot techniques

Phosphorylation and separation of the phosphoproteins in acid gels were performed as described by Wuytack et al. [12]. For Laemmli-type sodium dodecyl sulphate polyacrylamide gel electrophoresis [14] 10% slab gels of 0.75 mm thickness were used. The gels were either stained in Coomassie Brilliant Blue R-250 or used for the transfer of the separated proteins to nitrocellulose sheets. The binding of ^{125}I-calmodulin to the blots was carried out as described earlier [15]. The radioactive spot was cut out for determination of its radioactivity.

For the binding of wheat germ agglutinin, blots were pretreated with Tris-buffered saline (0.9% NaCl/10 mM Tris-HCl (pH 7.6)) containing 0.1% Tween 20 for 1.5 h with three changes of the solution, and then incubated in TBS-Tween (0.05% Tween 20 in Tris-buffered saline) containing 20 μg/ml wheat germ agglutinin. In a control experiment 0.1 M *N*-acetylglucosamine was added. The lectin was washed away with five applications of TBS-Tween (40 min in total). The blots were then incubated with TBS-Tween containing 15 μg/ml anti wheat germ agglutinin antibodies for 1 h, and washed for 40 min with TBS-Tween. The bound antibodies were visualised as described [16].

Other assays

Ca^{2+} uptake was measured at 37°C in a medium containing (mM) KCl 150, $NaN_3$5 (to block eventual Ca^{2+} uptake in contaminating mitochondria), imidazole-HCl (pH 6.9) 30, Tris-ATP 5, $MgCl_2$ 6, ^{45}CaEGTA 1, with or without 5 mM potassium oxalate. The protein concentration was 25 μg/ml. The vesicles were separated from the solution by Millipore filtration. The filters were rinsed and the ^{45}Ca remaining on the filters was counted.

Cholesterol was measured as described previously [8]. Protein was measured by the method of

Lowry et al. [17] using bovine serum albumin as a standard.

Materials

Calmodulin and ^{125}I-calmodulin were prepared as described [15]. Wheat germs agglutinin and rabbit antibodies to this lectin were obtained from E-Y Laboratories. *N*-Acetylglucosamine was from Sigma, digitoxigenin from Fluka and saponin from Merck. Digitonin from Merck was further purified as described [18]. Rabbit skeletal muscle lactate dehydrogenase and pyruvate kinase were obtained from Boehringer. Alamethicin was obtained from Upjohn. Molecular weight standards for the gel electrophoresis were obtained from Boehringer.

Results

The effect of extraction at high ionic strength on activities of the membrane enzymes

The inclusion of 0.6 M KCl in the density gradient significantly increased the specific activity of the enzymes and the uptake of Ca^{2+} by the isolated membrane fractions (Table I). This effect can be explained by a more efficient removal of extrinsic proteins from the membranes. Gel electrophoresis of isolated fractions showed that mainly bands of 43 and 200 kDa (presumably actin and myosin, respectively) and an as yet unidentified band of 70 kDa were extracted (Fig. 1). A second effect of the use of 0.6 M KCl was a shift of the Ca^{2+} uptake and the marker enzyme activities to lower buoyant densities (data not shown). The inclusion of 0.6 M KCl also induced a narrowing of the density range in which the marker enzymes and the Ca^{2+} uptake were recovered, resulting in a better separation between endoplasmic reticulum and plasma membranes.

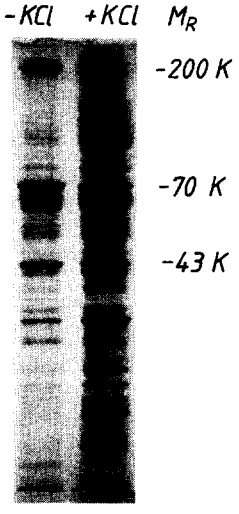


Fig. 1. Sodium dodecylsulphate polyacrylamide gel electrophoresis (10% gel) of membrane fractions recovered between 23 to 26% sucrose from a density gradient with or without 0.6 M KCl. In the absence of 0.6 M KCl, larger amounts of 43, 70 and 200 kDa proteins are present.

The distribution of marker enzymes in density gradients containing 0.6 M KCl and the effect of digitonin

The largest amount of membrane protein was recovered between 25 and 35% sucrose (Fig. 2A).

TABLE I

MAXIMUM SPECIFIC ACTIVITIES OF Ca^{2+} UPTAKE, 5'-NUCLEOTIDASE AND (Ca^{2+} + Mg^{2+})-ATPase OBSERVED IN SUCROSE GRADIENTS WITH AND WITHOUT 0.6 M KCl

Post-mitochondrial supernatant was applied on sucrose gradients with and without 0.6 M KCl. 12 fractions between 15 and 45% sucrose were collected and analysed for protein content, Ca^{2+} uptake, and enzyme activities. The highest specific activities are tabulated. Data in the last line represent mean ± S.E. for three to five determinations.

Sucrose gradient	Maximum specific activities in gradient			
	Rate of Ca^{2+} uptake + oxalate ($nmol \cdot mg^{-1} \cdot min^{-1}$)	Plateau of Ca^{2+} uptake without oxalate ($nmol \cdot mg^{-1}$)	5'-Nucleotidase ($nmol \cdot mg^{-1} \cdot min^{-1}$)	(Ca^{2+} + Mg^{2+})-ATPase ($nmol \cdot mg^{-1} \cdot min^{-1}$)
−KCl Expt. 1	4.5	22	165	183
Expt. 2	15.5	28	208	210
+0.6 M KCl	87 ± 7.0	153 ± 45	410 ± 120	779 ± 170

The protein distribution correlated better with the plasma membrane markers (Fig. 2B) than with the putative endoplasmic reticulum markers (Fig. 2C).

In the absence of digitonin, the largest fraction of the putative endoplasmic reticulum markers was recovered at lower densities than the plasma membrane markers, but the separation was not complete. The $(Na^+ + K^+)$-ATPase and 5′-nucleotidase activities did not present exactly the same distribution. The reason for this discrepancy is not

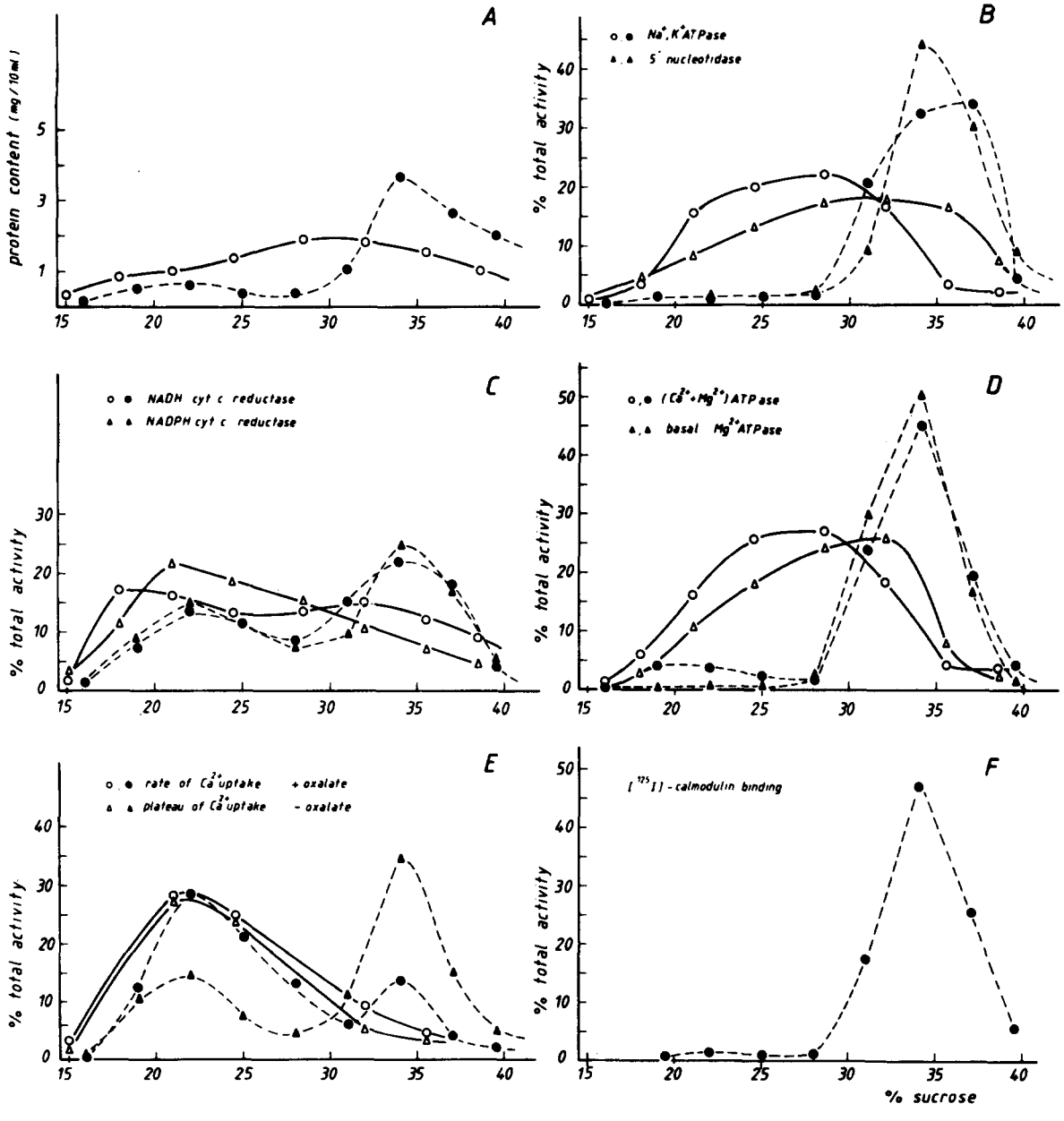


Fig. 2. Density distributions of protein content, enzyme activities, Ca^{2+} uptake, and ^{125}I-calmodulin binding in membrane fractions subfractionated on a sucrose density gradient containing 0.6 M KCl. Solid lines and open symbols: control. Broken lines and closed symbols: sample treated with digitonin. The results of a typical experiment are shown. Similar results were obtained in four other experiments.

clear, but it could be due to the existence of different subdomains in the plasma membrane [19] differing in enzyme content.

In the presence of digitonin the largest portion of the protein content was shifted to higher densities (Fig. 2A). The plasma membrane markers were shifted similarly and very little activity remained below a density of 25% sucrose (Fig. 2B).

The distribution of the basal Mg^{2+}-ATPase activity resembled that of the activity of $(Na^+ + K^+)$-ATPase and 5′-nucleotidase and it was similarly shifted by digitonin (Fig. 2D), confirming the contention that the Mg^{2+}-ATPase is predominantly localized in the plasma membrane [20].

Rotenone-insensitive NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase presented two maxima when digitonin was added to the sample. One peak was seen at the same sucrose concentration as that of the plasma membrane markers while a second peak remained at about 20% sucrose without being affected by digitonin. It can be assumed that this latter fraction could represent endoplasmic reticulum on the basis of several observations: (1) It has been shown for liver cells that the cholesterol content of endoplasmic reticulum membranes is low and hence their density is little affected by digitonin [21]. (2) The specific activity of the plasma membrane markers $(Na^+ + K^+)$-ATPase and 5′-nucleotidase in this fraction was very low (Table II). (3) This fraction contained the highest specific activities of NADH- and NADPH-cytochrome *c* reductase (Table II). (4) This fraction contained most of the oxalate-stimulated Ca^{2+} uptake (see below and Fig. 2D), as has been observed for endoplasmic reticulum vesicles [7].

The contamination of the endoplasmic reticulum fraction with plasma membranes or vice versa can be estimated from the observed specific activities of the marker enzymes in the different fractions (Table III). Depending on which marker is used in the calculation, the endoplasmic reticulum would contain 10–20% plasma membranes, the plasma membrane fraction 20–25% endoplasmic reticulum. This latter value is based on the assumption that all the NADH- and NADPH-cytochrome *c* activity is present in the internal membranes, and that none occurs in the plasma membranes. This is not necessarily true, because it has been shown that plasma membranes of liver cells do contain these enzymes, be it in smaller amounts than the endoplasmic reticulum [22]. If this were also the case for smooth muscle, the proposed value of 75% for the purity of the plasma membrane fraction would be an underestimation. If, however, these markers are confined to the endoplasmic reticulum, the shift of the activities of NADH- and NADPH-cytochrome *c* reductase by digitonin could suggest that some endoplasmic reticulum membranes remain associated with the plasma membranes. A further argument for this contention is the observation that the fractions shifted by digitonin still contain some 100 kDa $(Ca^{2+} + Mg^{2+})$-ATPase, the enzyme which is most enriched in the purified endoplasmic reticulum (see below). The relative amount of purified and plasmalemma-associated endoplasmic reticulum can be estimated from the area under the two peaks of Fig. 2C (broken line), provided the specific

TABLE II

SPECIFIC ACTIVITIES OF MARKER ENZYMES, Ca^{2+} UPTAKE AND ^{125}I-CALMODULIN BINDING IN MEMBRANE FRACTIONS SEPARATED BY USING DIGITONIN

Fractions containing either the highest specific activity of $(Na^+ + K^+)$-ATPase and 5′-nucleotidase (plasma membrane fraction VII, recovered at 34% sucrose) or the highest specific activity of NADPH-cytochrome *c* reductase and rate of oxalate-stimulated Ca^{2+} uptake (endoplasmic reticulum fraction III, recovered at 22% sucrose) were selected from the same gradient analysed in Fig. 2. The units are nmol substrate · mg^{-1} · min^{-1} at 37°C, except when indicated otherwise. The fraction numbers are defined in the legend of Fig. 4.

	Endoplasmic reticulum	Plasma membranes
Rate of oxalate stimulated Ca^{2+} uptake	75	10
Plateau value of Ca^{2+} uptake in the absence of oxalate (nmol · mg^{-1})	94	57
$(Ca^{2+} + Mg^{2+})$-ATPase	301	645
5′-Nucleotidase	176	692
$(Na^+ + K^+)$-ATPase	19	150
NADH-cyt. *c* reductase (rotenone insensitive)	11300	3650
NADPH-cyt. *c* reductase	213	58
^{125}I-calmodulin binding (cpm/mg)	17500	107000

TABLE III

CALCULATED VALUES OF THE CONTAMINATION OF THE ENDOPLASMIC RETICULUM (ER) BY PLASMA MEMBRANES (PM), OR VICE VERSA

The purity of the endoplasmic reticulum and plasma membrane-enriched fractions analysed in Table II, was calculated from the specific activities of marker enzymes or from the binding of calmodulin to the 130 kDa ATPase in each fraction. The equations used are $a_{11}/x + a_{12}/y = 1$ and $a_{21}/x + a_{22}/y = 1$, in which x and y represent the activity of endoplasmic reticulum and plasma membrane markers in 100% pure endoplasmic reticulum and plasma membrane fractions, respectively. a_{11} and a_{12} are the specific activities, measured in the endoplasmic reticulum fraction, of the endoplasmic reticulum and plasma membrane markers, respectively. a_{21} and a_{22} represent the specific activities, measured in the plasma membrane fraction, of the endoplasmic reticulum and plasma membrane markers, respectively. The values shown below equal 100 a_{11}/x for ER, and 100 a_{22}/y for PM. The calculation does not take into account contamination by other membranes such as mitochondria or non-membrane protein.

Marker enzyme		% purity calculated from specific activities of different marker enzymes		
		$(Na^+ + K^+)$-ATPase	5'-Nucleotidase	Calmodulin binding to 130 kDa protein
NADPH-cyt. *c* reductase	ER	89	79	87
	PM	75	77	75
NADH-cyt. *c* reductase	ER	90	80	87
	PM	70	73	71

activity of the marker enzymes in both subfractions is similar. Values of 40% and 60%, respectively, were obtained.

The low density fraction, recovered between 18 and 25% sucrose from a digitonin-treated sample, will hereafter, unless stated otherwise, be designated as 'endoplasmic reticulum fraction', and the high density fraction between about 32 and 37% sucrose as 'plasma membrane fraction'. These designations are introduced for convenience, without implying that these fractions are pure.

Fig. 3 shows electron micrographs of negatively stained samples of some membrane fractions. A plasma membrane fraction treated with digitonin (Fig. 3A) and a membrane mixture containing mainly plasma membranes (Fig. 3B) contain many large vesicles. Most vesicles in the endoplasmic reticulum fraction are small (Fig. 3C). Many large vesicles present 4 nm particles at the surface. Most membranes in the fraction which was shifted to a higher density by digitonin acquire a typical morphology of long cylindrical structures [23,24] and have smooth surfaces. It is not clear whether the absence of surface particles is an intrinsic property of these membranes or whether the surface particles disappear under the influence of digitonin. Intact mitochondria or fragments of mitochondrial inner membranes were seldom observed, demonstrating that contamination by these organelles is low. The low density fractions (less than 28% sucrose) were practically devoid of mitochondrial fragments.

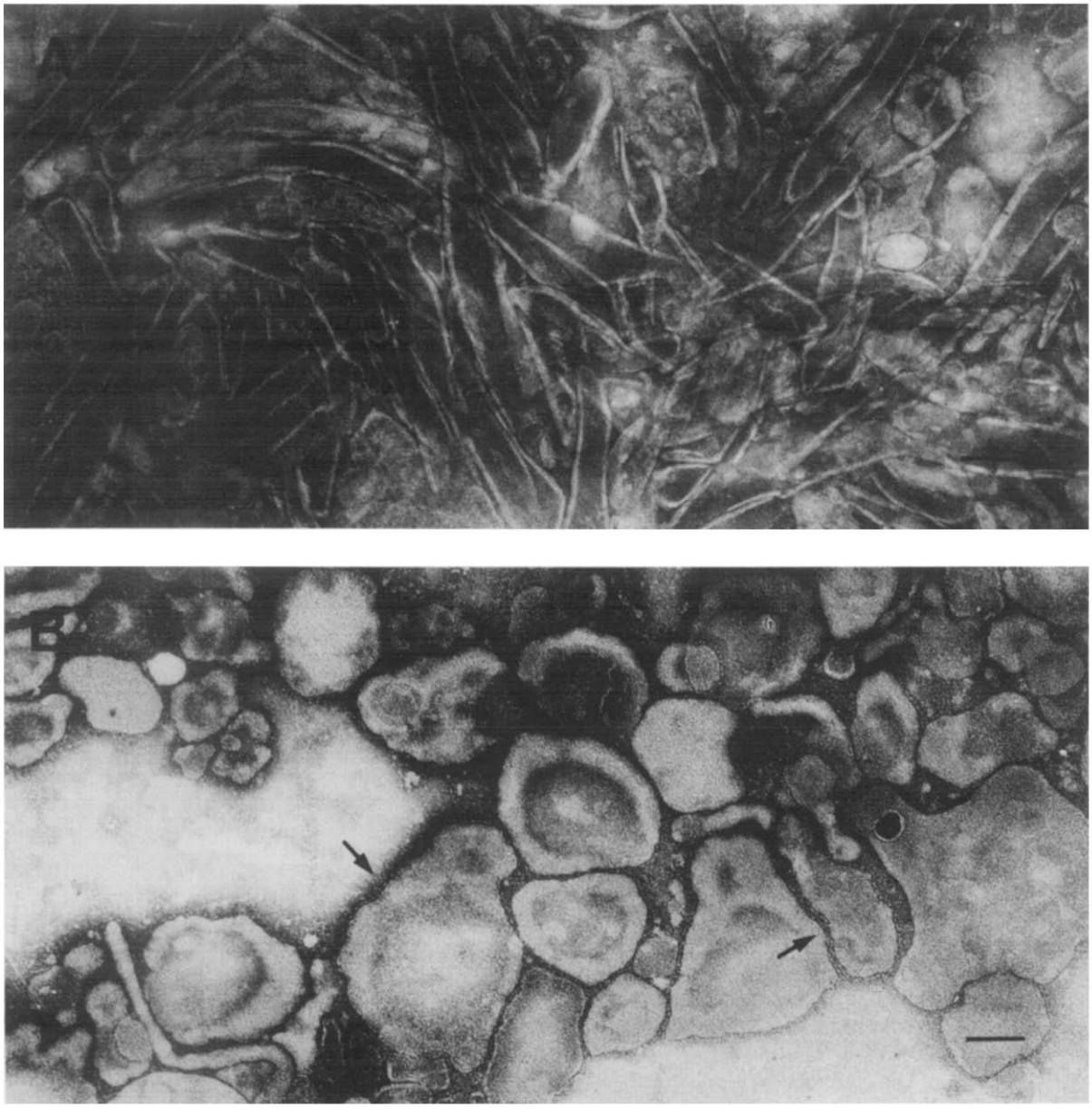
The difference in the polypeptide composition between the endoplasmic reticulum (fractions I–IV) and plasma membrane (fractions VI–VIII) can be seen in Fig. 4. Major bands of either fraction are only present in very limited amounts in the other fraction suggesting that there is no gross cross-contamination between both fractions.

Fig. 5 compares the protein patterns of endoplasmic reticulum vesicles isolated by the present density gradient procedure and of vesicles isolated by calcium oxalate loading. The protein composition is very similar, demonstrating that the two populations of vesicles are identical.

The binding of wheat germ agglutinin is shown in Fig. 6. This binding is confined to the plasma membrane fractions and mainly to glycoproteins of a molecular mass of over 100 kDa.

The distribution of the Ca^{2+} uptake and the $(Ca^{2+} + Mg^{2+})$-ATPase activity

The pattern of $(Ca^{2+} + Mg^{2+})$-ATPase activity closely followed that of the distribution of the $(Na^+ + K^+)$-ATPase (Fig. 2D), suggesting that most of the Ca^{2+} transport ATPase of the post-mitochondrial supernatant is located in the plasma membrane. This is confirmed by the effect of digitonin, which induced a shift of the largest portion of the $(Ca^{2+} + Mg^{2+})$-ATPase activity in parallel with the plasma membrane markers, while only a small fraction remained in the lower density zone containing the endoplasmic reticulum vesicles. The total $(Ca^{2+} + Mg^{2+})$-ATPase activity recovered from 100 g of antrum smooth muscle amounted to 9.0 ± 1.3 ($n = 5$) $\mu mol \cdot min^{-1}$, which



is close to the value of 12.0 reported by De Schutter et al. [26].

When no digitonin was added before centrifugation, the Ca^{2+} uptake was recovered at lower densities than the $(Ca^{2+} + Mg^{2+})$-ATPase activity, suggesting that the endoplasmic reticulum significantly contributes to the Ca^{2+} uptake (Fig. 2E). The digitonin-shift confirms this hypothesis. The digitonin-treated membranes present a distribution of Ca^{2+} uptake with two maxima, one in the endoplasmic reticulum and the other in the plasma membrane fraction. The oxalate-stimulated Ca^{2+} uptake is mainly located in the endoplasmic reticulum fraction, whereas the total amount of the Ca^{2+} uptake in the absence of oxalate is largest in the plasma membrane fraction. However, because the total activity of oxalate-independent Ca^{2+} uptake which is shifted by digitonin is much smaller

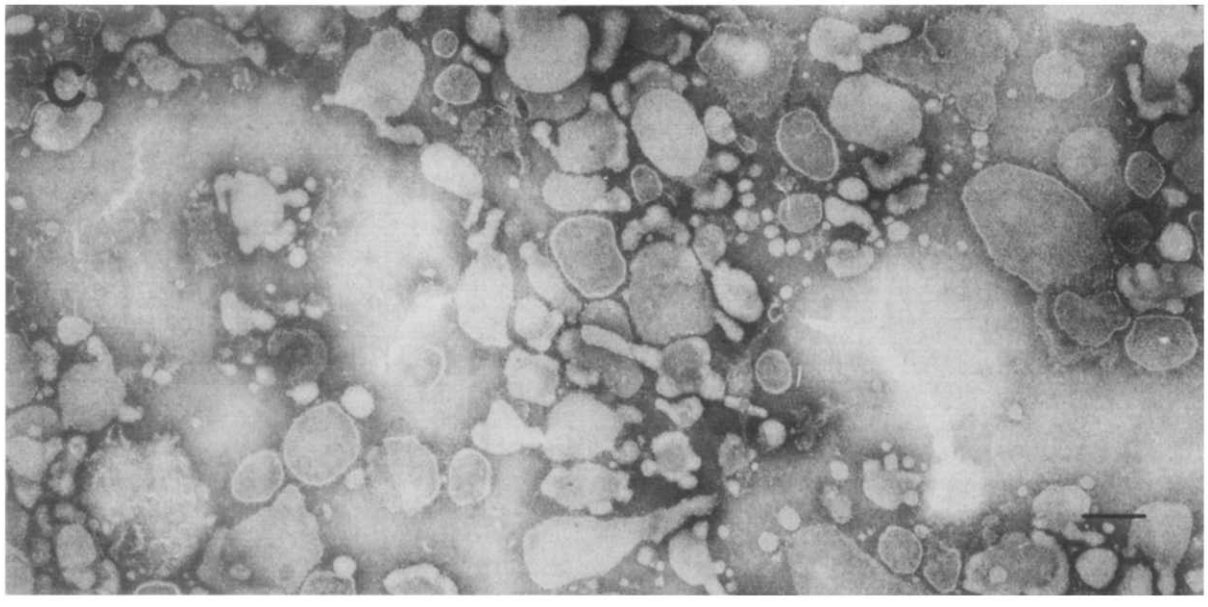


Fig. 3. Electron micrographs of the same membrane fractions used for the enzymatic analysis of Fig. 2. Negative staining with 1% phospotungstic acid. (A) Digitonin-treated plasma membrane fraction recovered at 34% sucrose. (B) Plasma membrane-enriched fraction without digitonin-treatment, recovered at 28.5% sucrose. (C) Digitonin-treated endoplasmic reticulum recovered at 22% sucrose. In B, many vesicles present 4-nm particles at the surface (arrows). The bar represents 100 nm. See text for description.

than the total amount of shifted protein, this results in a higher specific activity of oxalate-independent Ca^{2+} uptake in the endoplasmic reticulum fraction than in the plasma membrane enriched fraction (Table II and Fig. 7). This could be due to a difference between the endoplasmic reticulum

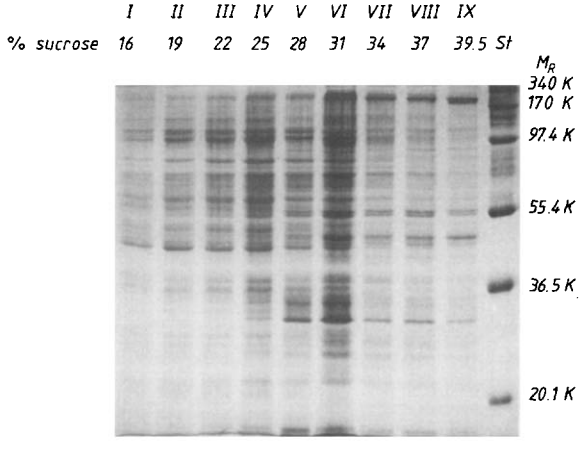


Fig. 4. Gel electrophoresis (10% gel) of the same digitonin-treated fractions which have been described in Fig. 2. Between 15 and 25 μg of protein was applied per lane. Fractions I to IV corrrespond to the presumed endoplasmic reticulum, while fractions VI to IX can be considered as plasma membranes. Molecular mass markers were run in the left lane.

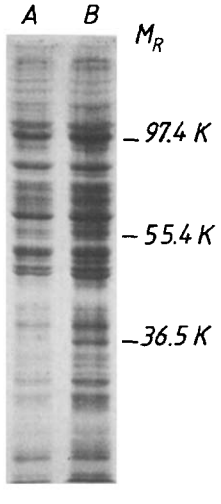


Fig. 5. Comparison of the protein composition of vesicles isolated by calcium oxalate loading (A) and of an endoplasmic reticulum fraction obtained by digitonin-treatment (fraction III recovered at 22% sucrose) (B).

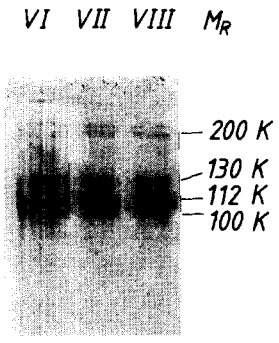


Fig. 6. Binding of wheat germ agglutinin to a blot from a parallel electrophoresis to that of Fig. 4. Binding was virtually absent in the endoplasmic reticulum fractions (II to IV). These lanes are not shown. In the plasma membranes the binding was confined to high molecular mass proteins (mainly 100, 112, 130 and 200 kDa). Therefore, the lower part of the gel was omitted from the figure. The specificity of the binding was demonstrated by its complete inhibition by 0.1 M *N*-acetylglucosamine (not shown). Molecular weights were determined by comparison with standards on the same blot and visualised by staining with Amido Black (not shown) [25].

and plasma membrane fractions in the amount of sealed and properly oriented vesicles (see below). The time-course of the Ca^{2+} uptake in both fractions is represented in Fig. 7.

The total recovery of the marker enzymes has been calculated using as reference material the crude membrane fraction pelleted from a sample of the post-mitochondrial supernatant. Several activities, particularly $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase were too low to be measured accurately in the supernatant. It was observed that keeping the postmitochondrial supernatant for 24 h in the cold room did not influence the recovery of the membrane markers in the crude membrane fraction pelleted from it, indicating that the long centrifugation time did not adversely affect the enzyme activities. Also the presence of 0.6 M KCl during this period did not influence these recoveries. The recoveries were: $(Na^+ + K^+)$-ATPase, $(Ca^{2+} + Mg^{2+})$-ATPase and NADPH-cytochrome *c* reductase, 80–95%; 5′-nucleotidase 42%; NADH-cytochrome *c* reductase, 55%. Digitonin did not significantly affect the recoveries of the enzyme activities. The total yield of the rate of Ca^{2+} uptake in the presence of oxalate was decreased by digitonin to about 90% of the control. Digitonin reduced the yield of the plateau value of the Ca^{2+} uptake in the absence of oxalate to about 70% of the control.

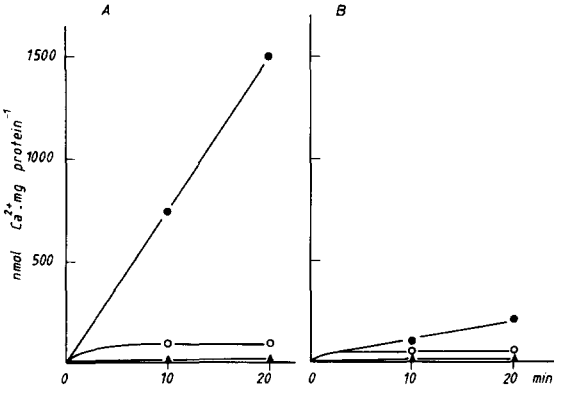


Fig. 7. The time-course of the Ca^{2+} uptake by a fraction enriched in endoplasmic reticulum (A) and in plasma membrane (B); ●, in the presence of 5 mM oxalate; ○, in the absence of oxalate; ▲, in the absence of ATP. The same fractions were used as those described in table II.

The relation of Ca^{2+} uptake to $(Ca^{2+} + Mg^{2+})$-ATPase activity

Ca^{2+} uptake only occurs in properly oriented and sealed vesicles, i.e. plasma membrane vesicles should be inside out while endoplasmic reticulum vesicles should have the same orientation as in the cell. Several characteristics of plasmalemmal enzyme activities suggest that the percentage of sealed inside-out plasma membrane vesicles is low. This situation may explain the relatively small portion of the Ca^{2+} uptake by the plasma-membrane fraction compared with its much higher content of membrane protein and of $(Ca^{2+} + Mg^{2+})$-ATPase activity. The following observations were made on both control fractions and on digitonin-treated ones: (1) $(Na^+ + K^+)$-ATPase can be measured in leaky vesicles only, because the ATP- and the ouabain-binding sites are at opposite sides of the membrane. However, also the activity in sealed inside-out vesicles should be detected when the lipid-soluble and membrane permeable inhibitor digitoxigenin is used. It was observed in our preparation that the activity measured in the presence of 50 μM digitoxigenin was at most 10% higher than that measured in the presence of 10 μM ouabain, indicating that the percentage of sealed inside-out vesicles is not higher than 10%. (2) The

activity of the ecto-enzyme 5'-nucleotidase was not significantly stimulated by treatment with Triton X-100. (3) The pore-forming antibiotic alamethicin (alamethicin/protein (w/w) ratio = 0.7) which under the conditions used allows the entry of ATP into the vesicles [13], stimulated the $(Na^+ + K^+)$-ATPase activity measured from the inhibition by ouabain only by about 20%, suggesting that the sum of sealed inside-out and outside-out vesicles, reaches at the most a value of 20%. (4) Pretreatment of plasma membrane fractions with Triton X-100 or deoxycholate over a wide range of detergent/protein ratios increased the $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase activities at the most by 20%.

Besides the number of sealed and properly oriented vesicles, other factors may contribute to the difference in the Ca^{2+} uptake capacity between different vesicle populations, as e.g. the presence or absence of intravesicular Ca^{2+} binding proteins [27].

Characterization of the $(Ca^{2+} + Mg^{2})$-ATPases by phosphorylation and calmodulin-binding

In crude membranes from stomach smooth muscle, two different $(Ca^{2+} + Mg^{2+})$-ATPases have been identified [12,15]. Phosphorylation by [γ-^{32}P]ATP and electrophoresis in acid medium in order to preserve the alkali-labile phosphoproteins, reveal two Ca^{2+}-dependent phosphoproteins, one at 130 kDa and one at 100 kDa. The addition of La^{3+} during phosphorylation increases the phosphorylation level in the first protein, but inhibits it in the second type of ATPase [12]. As shown in Fig. 8, the endoplasmic reticulum fractions contain a larger amount of 100 kD ATPase than of 130 kDa ATPase, while in the plasma membranes the 130 kDa ATPase is predominant. However, the relative amount of 100 kDa and 130 kDa $(Ca^{2+} + Mg^{2+})$-ATPase cannot be determined with certainty from these experiments because the efficiency of phosphorylation and the stability of the phosphoproteins during electrophoresis may be different for both enzymes.

It has been demonstrated by Wuytack et al. [28] that in analogy with the enzyme of erythrocyte membranes, also the $(Ca^{2+} + Mg^{2+})$-ATPase of 130 kDa requires calmodulin for full activity. Concomitantly, the smooth muscle enzyme binds ^{125}I-

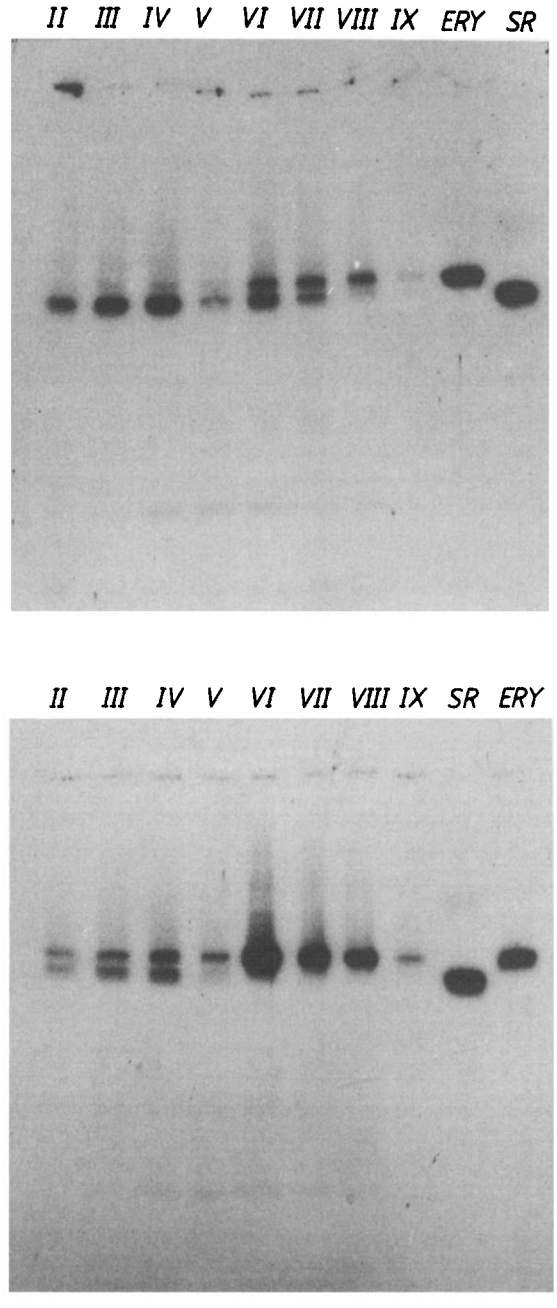


Fig. 8. Autoradiogram of a dried polyacrylamide slab gel showing the Ca^{2+}-dependent phosphorylated intermediates of the Ca^{2+}-transport ATPases of subcellular fractions separated by digitonin-treatment. Phosphorylation was carried out in the presence of 50 μM Ca^{2+}, with (bottom) or without (top) 50 μM La^{3+}. The density gradient fractions are indicated as in Fig. 4. For comparison, inside-out vesicles of pig erythrocyte membranes (ERY) and sarcoplasmic reticulum membranes from pig skeletal muscle (SR) were treated in parallel.

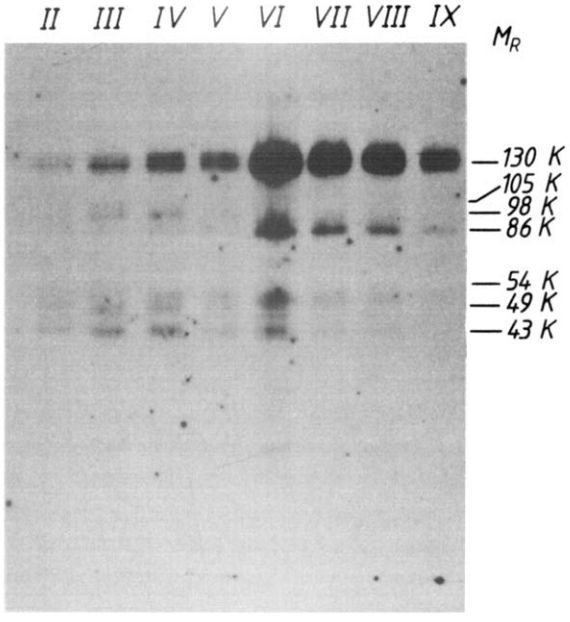


Fig. 9. ^{125}I-Calmodulin binding to nitrocellulose blots of the membrane fractions as analysed in Figs. 2 and 4. Electrophoresis was in 7.5% gels. The lanes are labeled as in Fig. 4. ^{125}I-Calmodulin mainly binds at the 130 kDa ATPase, which is predominant in the plasma membrane-enriched fractions (V to IX). Minor calmodulin-binding bands have a M_r of 86 kDa in the plasma membrane fraction, whereas in the endoplasmic reticulum, bands at 105, 98, 54, 49 and 43 kDa are found.

calmodulin after electrophoresis and blotting on nitrocellulose paper [15]. Fig. 9 shows the binding of ^{125}I-calmodulin to a nitrocellulose blot of the endoplasmic reticulum and of the plasma membrane fractions. The 130 kDa polypeptide of the plasma membranes was the main calmodulin-binding protein. Binding was very low in the endoplasmic reticulum fractions (see also Table II). The main calmodulin-binding peptide was in all of the membrane fractions accompanied by two peptides binding less calmodulin, one of a higher and one of a lower molecular mass but both differing by less than 10 kDa from the main band. The nature of these proteins remains unknown, but it appears from Fig. 9 that these three peptides are always present in the same ratio. The values for calmodulin-binding given in Table II and Fig. 2F always represent the total radioactivity in the three bands. Interestingly, a calmodulin-binding peptide with M_r 86 kDa is also associated with the plasma membranes, whereas a number of peptides binding relatively lower amounts of calmodulin were found to be associated with the endoplasmic reticulum fraction.

Discussion

Membrane fractions of smooth muscle contain variable, but often appreciable amounts of contractile and cytoskeletal proteins. We have observed that extraction with 0.6 M KCl, with or without Ca^{2+} chelators, or density gradient centrifugation resulted only in a partial extraction of these proteins. However, a combination of both procedures was very efficient if the sample was applied below the gradient, while application of the sample on top of the gradient resulted in the penetration of contaminating proteins into the gradient. The membrane fractions obtained by the present procedure presented higher activities of marker enzymes and of Ca^{2+} uptake than control fractions prepared without addition of 0.6 M KCl.

Although it is difficult to identify with certainty endoplasmic reticulum membranes because very specific markers are not known, it is reasonable to conclude that the low density fraction, obtained by using digitonin-treatment in combination with the present centrifugation method, yields vesicles derived from the internal membranes. This fraction is not shifted by digitonin, and presents very low wheat germ agglutinin binding and low activities of plasma membrane markers. However, it contains the highest specific activities of NADH and NADPH reductase. The ATP-dependent Ca^{2+} uptake in this fraction strongly depends on oxalate (a stimulation by a factor of 16-times after 20 min of uptake) and the rate of oxalate-dependent Ca^{2+} uptake (75 nmol $Ca^{2+} \cdot mg^{-1} \cdot min^{-1}$) is high compared with values reported in the literature for other, less purified, vesicular preparations from smooth muscle. The rate of Ca^{2+} uptake is low compared with that in the sarcoplasmic reticulum of skeletal and heart muscle. This difference could be related to the slower rate of relaxation of smooth muscle. It also agrees with results obtained on skinned smooth muscle preparations, in which the oxalate-stimulated Ca^{2+} uptake proceeds linearly with time for at least 30 min [7], indicating that the rate of Ca^{2+} uptake is much lower than

that in sarcoplasmic reticulum of skeletal muscle, which becomes fully loaded within a few minutes [29].

The protein composition of the endoplasmic reticulum was very similar to that of vesicles isolated by loading with calcium oxalate, suggesting that both fractions are identical. However, the availability of the present reticulum fraction should make it possible to carry out studies which could not be performed on vesicles isolated by calcium oxalate loading. One example is the study of the reaction of the $(Ca^{2+} + Mg^{2+})$-ATPase with La^{3+} as described in the results, because La^{3+} would form insoluble salts in the presence of oxalate. The absence of calcium oxalate deposits is also essential for the study of the mechanism of the Ca^{2+} release from the endoplasmic reticulum.

Our analysis of these gradient fractions includes for the first time a description of the distribution of the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase. This enzyme is a better index for Ca^{2+} transport activity than the determination of Ca^{2+} transport itself, because the latter not only depends on the activity of the Ca^{2+} pump, but also on the sidedness, the size, the Ca^{2+}+ binding capacity and the permeability of the membrane vesicles. Our analysis has also revealed a close correlation between $(Na^+ + K^+)$-ATPase and the largest fraction of the $(Ca^{2+} + Mg^{2+})$-ATPase. Both these enzyme activities are shifted by digitonin, a substance that mainly interacts with the cholesterol-rich plasma membranes [21]. These results also support the hypothesis that more ATP-dependent Ca^{2+} transport is associated with the plasma membrane than with the endoplasmic reticulum, as had already been proposed on the basis of measurements of the Ca^{2+} pump activity by determining the Ca^{2+} uptake [30,31].

The total $(Ca^{2+} + Mg^{2+})$-ATPase activity associated with the purified endoplasmic reticulum fraction was much smaller than the $(Ca^{2+} + Mg^{2+})$-ATPase shifted by digitonin. An estimate of the areas under the curve of Fig. 2D yields a value of about 0.1 for the ratio of the total amount of the $(Ca^{2+} + Mg^{2+})$-ATPase not shifted by digitonin to the amount of enzyme displaced by digitonin. However, according to our findings the plasma membranes isolated by digitonin treatment could be contaminated with up to 25–30% of endoplasmic reticulum membranes, assuming that the putative marker enzymes for endoplasmic reticulum are absent from the plasma membrane. Therefore, the value 0.1 probably underestimates the true ratio between $(Ca^{2+} + Mg^{2+})$-ATPase of the endoplasmic reticulum and that of the plasma membrane. We have also calculated that up to 60% of the total endoplasmic reticulum could be associated with the plasma membrane fraction. Taking into account this endoplasmic reticulum associated with plasma membrane, an upper limit for the ratio between the $(Ca^{2+} + Mg^{2+})$-ATPase of the endoplasmic reticulum and that present in the plasma membrane would be about 0.3.

It is not clear whether a connection of some of the endoplasmic reticulum with the plasma membrane vesicles would be due to a non-specific association of these organelles following homogenisation, or whether it would reflect an endoplasmic reticulum-plasmalemma junction as observed by electron microscopy of intact cells [32]. An association between endoplasmic reticulum and plasmalemma would mean that a density shift induced by digitonin is not a sufficient argument to conclude that certain membrane characteristics are typical for the plasmalemma. However, it is very unlikely that the large fraction of the $(Ca^{2+} + Mg^{2+})$-ATPase shifted by digitonin in the present experiments represents $(Ca^{2+} + Mg^{2+})$-ATPase activity in the endoplasmic reticulum, since the shifted $(Ca^{2+} + Mg^{2+})$-ATPase binds calmodulin, has a molecular mass of 130 kDa and since the steady-state level of its phosphoprotein intermediate is increased by La^{3+}. These characteristics are typical for a plasma membrane enzyme [15].

The Ca^{2+} transport enzyme of about 100 kDa is most likely confined to the endoplasmic reticulum. The steady-state level of its phosporylated intermediate is decreased by La^{3+} and it does not bind calmodulin when transferred from sodium dodecylsulphate polyacrylamide gels to nitrocellulose paper. In this respect it resembles the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum of skeletal muscle [12].

An additional aspect of our analysis is that it throws some light on the relative importance of Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+} transport for Ca^{2+} extrusion across the cell membrane of smooth muscle cells. This problem re-

mains controversial since many years [33]. Because extrusion of Ca^{2+} in exchange for Na^+ depends on the Na^+ gradient, and since the latter depends on the activity of the $(Na^+ + K^+)$-ATPase, Na^+-dependent Ca^{2+} extrusion would ultimately be limited by the $(Na^+ + K^+)$-ATPase activity. The Ca/ATP stoichiometry is probably about 1 for both the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane [34] and the Na^+/Ca^{2+} exchanger, since the Na^+/Ca^{2+} exchanger exchanges 3 Na^+ for 1 Ca^{2+} [35], while the $(Na^+ + K^+)$-ATPase consumes 1 ATP for 3 transported Na^+ [36]. In the plasma membrane-enriched fraction of antral smooth muscle cells the $(Ca^{2+} + Mg^{2+})$-ATPase activity was found to be about 4-times higher than the activity of $(Na^+ + K^+)$-ATPase. This high activity could not be accounted for by contaminating endoplasmic reticulum. This observation therefore strongly suggests that in stomach smooth muscle the plasmalemmal $(Ca^{2+} + Mg^{2+})$-ATPase plays a more important role in Ca^{2+} extrusion from the cells than Na^+-Ca^{2+} exchange.

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