Ca\textsuperscript{2+}-Transport ATPases of Vascular Smooth Muscle

Jan A. Eggermont, Matty Vrolix, Luc Raeymaekers, Frank Wuytack, and Rik Casteels

To characterize the Ca\textsuperscript{2+}-transport properties of the plasma membrane and of the endoplasmic reticulum of bovine pulmonary artery, membrane vesicles are subfractionated by a procedure of density-gradient centrifugation that takes advantage of the selective effect of digitonin on the density of plasma-membrane vesicles. The obtained endoplasmic-recticum fraction contains hardly any plasma-membrane vesicles, whereas the plasma-membrane fraction is still contaminated by a substantial amount of endoplasmic-recticum vesicles. An adenosine 5'-triphosphate (ATP) energized Ca\textsuperscript{2+}-transport system and a Ca\textsuperscript{2+}-stimulated ATPase activity are present in both subcellular fractions. The Ca\textsuperscript{2+} transport by the plasma membrane is catalyzed by a (Ca\textsuperscript{2+},Mg\textsuperscript{2+})-ATPase of M\textsubscript{r} 130,000. It binds calmodulin and it has a low steady-state phosphoprotein intermediate level. The endoplasmic-recticum vesicles contain a Ca\textsuperscript{2+}-transport ATPase of M\textsubscript{r} 100,000 that is characterized by a high steady-state phosphointermediate level. It is antigenically related to the Ca\textsuperscript{2+}-pump protein of cardiac sarcoplasmic reticulum. Phospholamban, the regulatory protein of the Ca\textsuperscript{2+}-transport enzyme of cardiac sarcoplasmic reticulum, is also present in the endoplasmic reticulum of the pulmonary artery. A comparison of these fractions with the previously characterized fractions from porcine gastric smooth muscle reveals important differences in the basal Mg\textsuperscript{2+}-ATPase activity, in the ratio of the (Ca\textsuperscript{2+},Mg\textsuperscript{2+})-ATPase of the plasma-membrane to that of the endoplasmic reticulum, and in the ratio of the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase activity to the plasmalemmal (Ca\textsuperscript{2+},Mg\textsuperscript{2+})-ATPase activity. These differences can be ascribed in part to the species and in part to the tissue. These data suggest that in the bovine pulmonary artery the Ca\textsuperscript{2+} extrusion via the ATP-dependent Ca\textsuperscript{2+} pump may have a less predominant role, and that the Ca\textsuperscript{2+} uptake by the endoplasmic reticulum, and possibly also the Ca\textsuperscript{2+} extrusion via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger could be more important in this tissue than in the porcine stomach.

(\textit{Circulation Research} 1988;62:266-278)

Changes in the intracellular Ca\textsuperscript{2+}-concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) regulate the contraction-relaxation cycle of smooth muscle. To counteract the Ca\textsuperscript{2+} influx through the plasma membrane (PM) and the Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER), the smooth muscle cell possesses two different types of Ca\textsuperscript{2+}-transport ATPases, one located at the PM, the other at the ER. However, the relative contribution of each Ca\textsuperscript{2+}-transport mechanism to the cellular Ca\textsuperscript{2+} homeostasis, their regulation by second messengers, and the role of other mechanisms such as the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger have not yet been unequivocally specified. A major obstacle in tackling these problems is the difficulty in separating the membranes containing these Ca\textsuperscript{2+}-transport systems. Although the isolation of PM from vascular smooth muscle has been reported, no purified ER has been obtained. Calcium-oxalate loading of ER vesicles has been used to obtain a purified ER fraction from stomach smooth muscle. A major disadvantage of this procedure is the interference of intravesicular calcium-oxalate crystals with assays for (Ca\textsuperscript{2+},Mg\textsuperscript{2+})-ATPase activity, Ca\textsuperscript{2+} uptake, and Ca\textsuperscript{2+} release.

We have recently developed a method to isolate a purified ER fraction from visceral smooth muscle without calcium-oxalate loading. This procedure has now been applied to vascular smooth muscle, and we report the isolation of a purified ER fraction from bovine main pulmonary artery. The separation of ER and PM vesicles has allowed us to characterize in this tissue the two types of Ca\textsuperscript{2+}-transport ATPases by means of their (Ca\textsuperscript{2+},Mg\textsuperscript{2+})-ATPase activity, their phosphointermediate, the calmodulin binding, and antigenic properties. A comparison of the phosphointermediates in different types of smooth muscle also reveals important differences of the relative amounts of both Ca\textsuperscript{2+}-transport ATPases.

Materials and Methods

Preparation of Membrane Fractions

Membrane fractions were prepared as described by Raeymaekers et al. Briefly, bovine main pulmonary arteries (the truncal part between the pulmonary valve and the bifurcation) were obtained from a local slaughterhouse about 15 minutes after killing of the animal and immediately cooled in ice. After removing the adventitial layer, the arteries were chopped and passed through a press as described by Wuytack et al. The mince (300 g) was then homogenized with an Ultra-turrax (Stanfen, FRG) (3×10 seconds) in 3 volumes of ice-cold 0.25 M sucrose containing 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride and centrifuged in a Sorvall GSA (Wilmington, Delaware) motor at 13,000g\textsubscript{max} for 30 minutes. The pellet and the floating layer were discarded and the supernatant, designated as the postmitochondrial su-
pertinent, was pooled. One hundred fifty milligrams digitonin, dissolved in 10 ml ethanol, was added to 800 ml of the supernatant. Solid sucrose and potassium chloride were added up to a final concentration of 50% (wt/wt) and 0.6 M, respectively. This material was loaded in a Beckman Ti15 (Palo Alto, California) or a Kontron T92 32 (Zürich, Switzerland) zonal rotor below 250 ml of a 15–45% sucrose gradient that was linear with respect to volume and 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 g for 20 hours at 4° C. Fixed volume fractions were collected and each fraction was assigned a percent sucrose value as determined by a Zeiss (Oberkochen, FRG) hand refractometer. The fractions were diluted three times with 0.6 M KCl containing 1 mM dithiothreitol and pelleted in a Kontron T92 45.94 rotor at 140,000 g for 1 hour at 4° C. The pellets were resuspended in 0.25 M sucrose containing 1 mM dithiothreitol and stored at –80° C.

In some preparations, no digitonin was added to the postmitochondrial supernatant. In other experiments, the potassium chloride in the postmitochondrial supernatant and in the sucrose gradient was omitted.

Membrane fractions from porcine gastric smooth muscle (antral part), porcine main pulmonary artery, or bovine thoracic aorta were prepared by the same procedure. Porcine tissues were obtained about half an hour after the killing of the animals. Inside-out plasmalemmal vesicles from pig erythrocytes and sarcoplasmic reticulum (SR) vesicles from pig skeletal muscle were prepared as described previously. Bovine cardiac sarcoplasmic reticulum vesicles were prepared according to Jones et al.

**Enzyme Assays**

All enzyme activities were assayed as described previously by continuously monitoring the reaction rate at 37° C in a Beckman DU7 spectrophotometer (Irvine, California) or in an Aminco DW2 spectrophotometer (Silver Spring, Maryland). 5′-Nucleotidase was assayed using a Sigma kit (Sigma Chemical, St. Louis, Missouri). Reduced nicotinamide dinucleotide (NADH) or reduced nicotinamide dinucleotide phosphate (NADPH) cytochrome c reductase (rotenone-insensitive) activities were measured by following the reduction of cytochrome c at 550 nm as described. Basal Mg2+-ATPase activity was measured by following the oxidation of NADH at 340 nm in the presence of a NADH coupled enzyme system. Because of its nonlinear time course, the basal Mg2+-ATPase activity was arbitrarily defined as the activity corresponding to the decrease in absorbance between time zero and 2 minutes. The (Na+,K+)-ATPase activity and the (Ca2+,Mg2+)-ATPase activity were measured in the presence of a NADH coupled enzyme system as described previously. The (Na+,K+)-ATPase activity is defined as the ATPase activity that was inhibited by 10 μM digitoxigenin. The (Ca2+,Mg2+)-ATPase activity is defined as the ATPase activity that was stimulated by the addition of Ca2+ (up to a final free concentration of 10−4 M) to a Ca2+-free, EGTA (0.5 mM) containing medium. Because of the nonlinear basal Mg2+-ATPase activity, the (Na+,K+)-ATPase and (Ca2+,Mg2+)-ATPase activities were measured with a double-beam spectrophotometer as described. In the first part of the experiment, the sample and the reference cell were identical in composition, resulting in an absorbance-difference signal that was perfectly constant with time. After recording this baseline, digitoxigenin [for measuring (Na+,K+)-ATPase] or Ca2+ [for recording (Ca2+,Mg2+)-ATPase] was added to one of the cells, and the enzyme activity was calculated from the slope of the curve. To obtain the maximal specific activity of the (Ca2+,Mg2+)-ATPase of the plasmalemma, 0.6 mM calmodulin was added. Alamethicin (0.5 μg/μg protein) was included to expose latent (Na+,K+)-ATPase and (Ca2+,Mg2+)-ATPase activities.

The distribution of the membrane markers in the sucrose gradient is presented in normalized histograms as described by Beaufay et al. The histograms are plotted on a density scale, expressed in percent sucrose, which is divided into zones corresponding to the equilibrium density of the collected fractions. The ordinate gives the frequency within the corresponding zone of the sucrose gradient. The frequency equals a/(A·D%), where "a" is the activity recovered within a zone of the gradient, "A" is the sum of the activities recovered in the whole gradient, and "D%" is the percent sucrose spanned by that zone. The surface area of each section of the diagram (frequency × D%) equals a/A and corresponds to the fraction of the total activity recovered in that zone. The total surface area equals 1.0. The results of 5 experiments were averaged by superimposing the histograms of the 5 experiments and calculating the mean frequency for every uneven percent sucrose between 17% and 39% sucrose. With these data a new histogram was plotted.

**Measurement of the Phosphointermediate Formation of the Ca2+-Transport ATPases**

Membrane vesicles were phosphorylated in a medium (50 μl total volume) containing 120 mM KCl, 30 mM imidazole (pH 6.9), 50 μM CaCl2 or 0.5 mM EGTA, alamethicin (0.5 μg/μg protein), and 1 mg/ml of membrane proteins. In some experiments, 50 μM LaCl3 was added. The reaction was started by adding 6 mM ATP containing ATP[γ-32P] at 10 μCi/nmol and was allowed to proceed for 20 seconds at 4° C. It was stopped with 150 μl stop solution containing 10% trichloric acid (TCA), 50 mM H2PO4, and 0.5 mM ATP. The samples were kept on ice for 10 minutes and then transferred to a tube containing 10 ml 0.2 M sodium acetate at pH 5.3 without or with 0.2 M hydroxyamine. In some experiments, the samples were transferred to a tube containing 10 ml stop solution instead of the sodium acetate solution. After 20 minutes at room temperature, the samples were filtered on a glass microfiber filter (Whatman GFA or GF, Maidstone, England). The filters were rinsed with 4 × 7.5 ml stop solution, dried, and counted to
determine the amount of $^{32}$P retained on the filter. The difference between the phosphate incorporation in the presence of Ca$^{2+}$ and the incorporation in the Ca$^{2+}$-free, EGTA-containing medium was taken as the Ca$^{2+}$-dependent phosphorylation.

Phosphorylation of the Ca$^{2+}$-transport ATPases and separation of the phosphorylated intermediates in acid gels were carried out as described previously. Phosphorylation with the catalytic subunit of the cAMP-dependent protein kinase were obtained from Sigma. Peroxidase and autoradiography were carried out as described previously.

Electrophoresis and Western Blotting

Laemmli-type SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 7.5% or 12% slab gels of 0.75 mm thickness. The gels were either stained with Coomassie brilliant blue or used for the electrophoretical transfer of the separated proteins on a nitrocellulose sheet by a Western blotting procedure.

Calmodulin binding to membrane fractions separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose sheet was done according to a previously described method. The 32-kDa calelectrin of porcine gastric smooth muscle was purified as described previously. Rabbit polyclonal antibodies against this protein were raised in rabbits. Antibody binding and detection of the immune complexes were carried out on membrane fractions separated by SDS-PAGE and electroblotted onto a nitrocellulose sheet. Bound anti-ribophorin I or anti-calelectrin antibodies were visualized using peroxidase-conjugated secondary antibodies.

Antibodies against the 100-kDa ATPase were visualized by Protein A labeling and autoradiography.

Other Assays

Ca$^{2+}$ uptake was measured at 37°C in a medium containing (in mM) KCl 150, Na$_2$SO$_4$ 5 (to block mitochondrial Ca$^{2+}$ uptake), imidazole-HCl (pH 6.9) 30, Tris-ATP 5, MgCl$_2$ 6, $^{45}$Ca EGTA 1, with or without 5 mM potassium oxalate or 50 mM potassium phosphate. The filters were rinsed and the $^{45}$Ca remaining on the filters was counted.

Protein was measured by the method of Lowry et al using bovine serum albumin as a standard.

Materials

Calmodulin and calmodulin were prepared as described. Digitonin was obtained from Merck, Darmstadt, FRG. Alamethicin was from Sigma. Rabbit skeletal muscle lactate dehydrogenase and pyruvate kinase were obtained from Boehringer, Mannheim, FRG. The catalytic subunit of the cAMP-dependent protein kinase was obtained from Sigma. Peroxidase-conjugated swine anti-rabbit and peroxidase-conjugated rabbit anti-mouse immunoglobulins were supplied by DAKO-Patts, Copenhagen, Denmark. ATP[$\gamma$-32P] (3,000 Ci/mmol), Protein A [123I] (30 mCi/mg) and $^{45}$Ca were obtained from Amersham, Little Chalfont, England. Mouse monoclonal antibodies against ribophorin I from canine liver were kindly provided by Dr. M. Hortsch, EMB Laboratory, Heidelberg. Rabbit polyclonal antibodies against the cardiac SR Ca$^{2+}$ pump were a gift from Dr. K. Gietzen, University of Ulm, Ulm, FRG. Rabbit polyclonal antibodies against phospholamban from canine cardiac SR were kindly provided by Dr. L.R. Jones, Indiana University, Indianapolis, Indiana.

Results

Subcellular Fractionation of the Membrane Vesicles (Figure 1 and Table 1)

Because digitonin forms an equimolar complex with cholesterol, it selectively increases the density of plasma-membrane (PM) vesicles and only slightly affects the density of other membranes. This selective digitonin effect results from the higher cholesterol content of PM vesicles as compared with internal membranes.

Density-gradient centrifugation of a postmitochondrial supernatant of bovine pulmonary artery in the presence of digitonin results in a shift of the major part of membrane proteins so that 86% of the protein is recovered between 28 and 40% sucrose (Figure 1, Panels A-C). Concomitantly, almost all the PM vesicles are recovered in this zone: 97% of the (Na$^+$,K$^+$)-ATPase activity, and 94% of the $^{5'}$-nucleotidase activity (Table 1 and Figure 1, Panels 3B and C). Because it presents the highest specific activity of PM markers, the 28–40% sucrose zone will be referred to as the PM fraction, without implying that it is a totally pure PM fraction (see below). The distribution of the basal Mg$^{2+}$-ATPase activity correlates very well with that of the (Na$^+$,K$^+$)-ATPase (Figure 1, Panels 3B and C), confirming the plasmalemmal localization of the Mg$^{2+}$-ATPase activity.

The distribution of the putative ER markers NADH cytochrome c reductase (rotenone-insensitive) is bimodal (Figure 1, Panels 2B and C). The predominant part of the total activity of these enzymes is contained within the PM zone (Table 1). This may be due to contamination with ER vesicles or to an enzyme activity associated with PM vesicles or with outer mitochondrial membranes. However, approximately one third of their total activity is situated at a density lower than 28% sucrose, and the highest specific enzyme activities of the ER markers NADH and NADPH cytochrome c reductase are observed in the 16–24% sucrose zone. The material recovered in this zone will be referred to as the ER fraction. This fraction is practically free of plasma membranes and is comprised of outer mitochondrial membranes. The predominant part of the total activity of these enzymes is situated at a density lower than the PM fraction (Table 1).

Ninety-four percent of the total enzyme activity of $^{5'}$-nucleotidase is localized in the PM fraction. The specific activity in the PM fraction of the ER markers NADH and NADPH cytochrome c reductase is observed in the 16–24% sucrose zone. The material recovered in this zone will be referred to as the ER fraction. This fraction is practically free of plasma membranes and is comprised of outer mitochondrial membranes. However, approximately one third of the total activity of the ER markers NADH and NADPH cytochrome c reductase are observed in the 16–24% sucrose zone. The material recovered in this zone will be referred to as the ER fraction. This fraction is practically free of plasma membranes and is comprised of outer mitochondrial membranes.
PM/ER ratio is noted for (Na\(^{+},K^{+}\))-ATPase and Mg\(^{2+}\)-ATPase respectively (Table 1). This difference may be explained by the presence of a small amount of 5'-nucleotidase in intracellular membrane vesicles that is not shifted by digitonin or by the adsorption of a soluble form of the enzyme to the ER membranes. The presence of an enzyme associated with intracellular membranes\(^{24}\) and of the soluble enzyme\(^{25}\) has been demonstrated in hepatocytes.

The total recovery in our fractions of the membrane markers has been calculated using the postmitochondrial supernatant as a reference material. Recoveries of 5'-nucleotidase and NADH cytochrome c reductase (rotenone-insensitive) amount only to between 16 and 28%, because a soluble form of these enzymes also occurs\(^{25}\) and because they might also be partially extracted by the treatment with high potassium chloride and digitonin. In contrast, recoveries between 65 and 100% are obtained if (Na\(^{+},K^{+}\))-ATPase activity is used as a parameter, indicating that the majority of the PM vesicles are recovered. Neither the recovery of the (Ca\(^{2+},Mg^{2+}\))-ATPase activity nor that of the Ca\(^{2+}\)-dependent phosphointermediate could be determined because in the postmitochondrial supernatant the basal Mg\(^{2+}\)-ATPase activity and the Ca\(^{2+}\)-independent, hydroxylamine-insensitive phosphoprotein level were too high to allow an accurate measurement of the (Ca\(^{2+},Mg^{2+}\))-ATPase activity or phosphointermediate level, respectively. The recovery of the oxalate-stimulated calcium uptake is only 20%. However, we have previously shown that the oxalate-stimulated calcium uptake is very sensitive to centrifugation,\(^{26}\) and, therefore, this parameter underestimates the recovery of the corresponding ER-type ATPase.

Finally, it should be mentioned that the boundary between the ER and PM zones, deduced from the distribution of the total enzyme activity of the PM markers, was always very sharp (see Figure 1, Panel 3B). However, its position in the sucrose gradient varied in different experiments from 29 to 31.5%.
This conclusion is based on a crossreaction of antibodies not only bind to the immunizing antigen of bovine pulmonary artery contain a larger amount of potassium chloride. The other polypeptides belong to the group of proteins that are associated with membranes in a Ca2+-dependent way but of which the function is still unknown. They have been named calelectrins or calcimedins. (For review, see Geisow and Walker27 and laboratories.27 The 37-kDa calelectrin is preferentially associated in the pulmonary artery with the PM fraction as observed for the calelectrin-like proteins in other cells.27 It is not clear at present whether the differences in calelectrin content of membranes of pig stomach and bovine pulmonary artery reflect differences in the amount of calelectrin in vivo, or whether it is due to a difference in the extraction of these proteins by a medium of high ionic strength.

Ribophorin I is a membrane protein that binds the protein synthesizing ribosomal complex to the rough ER membrane and is exclusively localized in the rough ER.29 A monoclonal antibody directed against this protein selectively recognizes one protein in membranes from porcine gastric smooth muscle and bovine pulmonary artery. This protein is mainly present in the ER-enriched fraction. Omission of potassium chloride from the preparative solutions results in a more equal distribution over the whole sucrose gradient (Figure 2B).

Phospholamban, a 22-kDa protein that regulates the Ca2+-transport ATPase of cardiac SR,30 has recently also been demonstrated in smooth muscle ER. This 22-kDa protein can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and is decomposed by boiling in SDS. Figure 3 shows such a protein, localized predominantly in the ER-enriched fractions, suggesting the presence of phospholamban in the ER of bovine pulmonary artery. Besides phospholamban, various other polypeptides of the ER of vascular smooth muscle are phosphorylated by cAMP-dependent protein kinase, while under identical conditions very little phosphorylation of plasma-membrane proteins is observed (Figure 3).

Subcellular Localization of Ca2+-Transport ATPases Ca2+ uptake. Both ER and PM vesicles contain an ATP-dependent, azide-insensitive Ca2+-uptake system (Figure 4). Ca2+-precipitating anions, such as oxalate

\[
\text{Table 1. Distribution of Protein Content and Total Activities of Membrane Markers, (Ca}^{2+}, \text{ Mg}^{2+}\)-ATPase and Phospholipid Intermediate in the Sucrose Gradient and Specific Activities of These Enzymes in the Endoplasmic Reticulum and Plasma Membrane Fraction}
\]

<table>
<thead>
<tr>
<th>% of total activity*</th>
<th>16–28% Sucrose</th>
<th>28–40% Sucrose</th>
<th>Specific activities†</th>
<th>ER</th>
<th>PM</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>14.1 ± 5.2</td>
<td>85.9 ± 5.2</td>
<td>61.2 ± 13.4</td>
<td>14.2 ± 4.0</td>
<td>4.3 (ER/PM)</td>
<td></td>
</tr>
<tr>
<td>NADPH cyt c reductase</td>
<td>30.9 ± 10.2</td>
<td>69.1 ± 10.2</td>
<td>9,502 ± 2,914</td>
<td>1,379 ± 169</td>
<td>6.9 (ER/PM)</td>
<td></td>
</tr>
<tr>
<td>NADH cyt c reductase</td>
<td>37.6 ± 11.6</td>
<td>62.4 ± 11.6</td>
<td>235 ± 79</td>
<td>932 ± 399</td>
<td>4.0 (PM/ER)</td>
<td></td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>5.9 ± 1.9</td>
<td>94.1 ± 1.9</td>
<td>359 ± 134</td>
<td>4,218 ± 1,457</td>
<td>11.7 (PM/ER)</td>
<td></td>
</tr>
<tr>
<td>Mg2+-ATPase</td>
<td>3.2 ± 0.8</td>
<td>96.8 ± 0.8</td>
<td>28.6 ± 16</td>
<td>445 ± 245</td>
<td>17.1 (PM/ER)</td>
<td></td>
</tr>
<tr>
<td>(Na+, K+)-ATPase</td>
<td>2.8 ± 1.0</td>
<td>97.2 ± 1.0</td>
<td>358 ± 87.0</td>
<td>282.5 ± 84.0</td>
<td>1.3 (ER/PM)</td>
<td></td>
</tr>
<tr>
<td>(Ca2+, Mg2+)-ATPase</td>
<td>14.3 ± 3.1</td>
<td>85.7 ± 3.1</td>
<td>63.5 ± 2.5</td>
<td>13.0 ± 2.9</td>
<td>4.9 (ER/PM)</td>
<td></td>
</tr>
<tr>
<td>Phosphoprotein</td>
<td>29.3 ± 8.1</td>
<td>70.7 ± 8.1</td>
<td>97.2 ± 1.0</td>
<td>61.2 ± 15.4</td>
<td>4.3 (ER/PM)</td>
<td></td>
</tr>
</tbody>
</table>

*Given values represent percent of total activity recovered in endoplasmic reticulum (ER) fraction (16–28% sucrose) and in plasma membrane (PM) fraction (28–40% sucrose). Values are mean ± SEM of five experiments.

†Specific activities are expressed in pmol/min/mg protein except for phosphoprotein formation, which is expressed in pmol phosphate/mg protein. Values are mean ± SEM of five experiments.
FIGURE 2. SDS-PAGE and immunoblotting of different membrane fractions from bovine main pulmonary artery and porcine stomach. All fractions were prepared with digitonin with or without 0.6 M KCl as described in "Materials and Methods." Endoplasmic reticulum (ER) and plasma membrane (PM)-enriched fractions are indicated. A, Coomassie blue staining pattern of SDS-PAGE gel; B, immunoblot of a gel identical to that shown in A, but sequentially treated with monoclonal antibody to ribophorin I and with antiserum to the 32-kDa calelectrin. The antibody to ribophorin I stains at a 68-kDa band and the antiserum against calelectrin at 32, 35, and 37 kDa.

at 5 mM and phosphate at 50 mM, markedly enhance the Ca\textsuperscript{2+} uptake in the ER fraction, whereas their stimulatory effect is less pronounced in the PM fraction, as previously observed in pig stomach smooth muscle.\textsuperscript{36} Oxalate and phosphate are equally potent stimulators of the Ca\textsuperscript{2+} uptake in the PM fraction of the bovine pulmonary artery. This is in contrast to our previous findings on the PM fraction of porcine gastric smooth muscle where oxalate was less effective than phosphate.\textsuperscript{3} It is therefore possible that the bovine pulmonary artery PM fraction contains a relatively larger amount of the oxalate-stimulated Ca\textsuperscript{2+}-trans-
transport ATPase. To measure the maximal (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase activity, we routinely added calmodulin (10 \(\mu\)g/ml) because the (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase of the PM is a calmodulin-dependent enzyme. To unmask latent enzyme activities, we also included alamethicin, a channel-forming antibiotic. This substance makes the membranes permeable for ATP, exposes the intravesicular membrane side of closed vesicles to the reaction medium, and prevents the accumulation of Ca\(^{2+}\) inside the vesicles.\(^{12}\) Such intravesicular Ca\(^{2+}\) concentration would inhibit the (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase activity.

Fourteen percent of the total (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase activity is found in the ER zone of the density gradient (Table 1). The distribution of the total (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase activity (Figure 1, Panels 4B and C) is intermediary between that of the PM markers (Figure 1, Panels 3B and C) and that of the ER markers (Figure 1, Panels 2B and C). The specific (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase activity of the PM and ER fraction does not differ appreciably (Table 1). These findings are compatible with the presence of two types of Ca\(^{2+}\)-transport ATPase that are differentially distributed (see below).

**Phosphorylation experiments.** DISTRIBUTION OF THE PHOSPHORYLATED INTERMEDIATES IN THE SUCROSE GRADI-

**Visualiation of two different phosphoprotein intermediates by gel electrophoresis.** The phosphoprotein that is formed after phosphorylation and gel electrophoresis that show only one phosphointermediate of 100 kDa, which is predominantly localized in the ER (see p. 273).
The time course of the Ca\(^{2+}\) uptake in a fraction enriched in endoplasmic reticulum (ER) and in plasma membrane (PM) vesicles. Ca\(^{2+}\) uptake was performed in the absence of ATP (○), in the presence of 5 mM ATP without precipitating anions (□), in the presence of 5 mM ATP and 50 mM phosphate (●) and in the presence of 5 mM ATP and 5 mM oxalate (■).

**Figure 5.** Autoradiogram of a dried polyacrylamide gradient gel (5–15%) showing the phosphointermediates of the Ca\(^{2+}\)-transport ATPase in different subcellular fractions of bovine pulmonary artery. Phosphorylation was performed in the presence of ATP[y-\(^{32}\)P] and 50 μM Ca\(^{2+}\) without La\(^{3+}\). The phosphointermediates of SR of porcine skeletal muscle (SR) and of inside out vesicles of porcine red blood cells (RBC) are also shown. % sucrose refers to the zone in the sucrose gradients from which the samples were taken. Under the autoradiogram, the phosphointermediate level (pmol P/mg), the (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase activity (nmol/mg/min), and the ratio of (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase to phosphointermediate level (10^15/min) are shown for each fraction. The underlined ratios were used to calculate the (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase activity catalyzed by the 100-kDa enzyme in the other fractions, as explained in the text.
Further characterization of the Ca\(^{2+}\)-transport ATPases. Rabbit polyclonal antibodies elicited against the 100-kDa Ca\(^{2+}\)-transport ATPase of porcine cardiac SR bind in a highly specific way to a polypeptide of the same Mr, that is mainly associated with the ER-enriched fractions (Figure 6).

Binding of calmodulin\([^{125}\text{I}]\) can be studied on nitrocellulose blots of membrane fractions separated by SDS-PAGE. Figure 7 shows the binding of calmodulin \([^{125}\text{I}]\) to the plasmalemmal (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase. As previously observed on stomach smooth muscle, most binding occurs with 3 polypeptides presenting a Mr of 128–124 kDa. At least one of them corresponds to the Ca\(^{2+}\)-transporting enzyme. An 86-kDa calmodulin-binding polypeptide is also associated with the PM fraction, while a 62-kDa polypeptide can be detected in the ER fraction. No binding occurs at the 100-kDa (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase.

The visualization of the phosphorylated intermediates by gel electrophoresis and autoradiography (Figure 5) reveals only one phosphoprotein of 100 kDa in the membrane fractions of the bovine pulmonary artery. This result clearly differs from our previous observations on membranes prepared from porcine stomach following the same method, in which a 130-kDa phosphoprotein in addition to the one of 100 kDa could be easily visualized. The isolated membrane fractions from both tissues are compared in parallel experiments in Figure 8. To differentiate further the two ATPases in addition to their molecular mass and to facilitate the detection of the 130-kDa phosphointermediate, phosphorylation was also carried out in the presence of La\(^{3+}\). La\(^{3+}\) suppresses the phosphoprotein formation of the 100-kDa ATPase but enhances that of the 130-kDa ATPase. Figure 8 clearly shows that in the ER and PM fractions of porcine stomach, the ratio of the 130-kDa to the 100-kDa phosphointermediate is much higher than in the corresponding fractions from bovine pulmonary artery. Consequently, these data suggest that in bovine pulmonary artery and aorta, the contribution of the Ca\(^{2+}\)-transport ATPase to the total (Ca\(^{2+}\),Mg\(^{2+}\))-ATPase activity is larger than it is in porcine gastric smooth muscle. Even in the presence of La\(^{3+}\), the PM-enriched fraction from bovine pulmonary artery presents more 100 kDa phosphorylation than 130 kDa, whereas in porcine stomach, relatively much more phosphorylation is seen at 130 kDa. Figure 8 also
shows that the bovine aorta presents a pattern of the other extreme. Its PM fraction, although enriched to a similar degree in (Na⁺,K⁺)-ATPase activity (data not shown), presents only the 100-kDa ATPase and the 130-kDa ATPase cannot be detected, even if La³⁺ is included. To find out whether the difference observed between the bovine vascular tissues and the porcine stomach is due to species or tissue differences, membrane fractions isolated by the same procedure from porcine pulmonary artery were included. They presented an intermediate distribution pattern of 100- and 130-kDa phosphoprotein intermediates (Figure 8), suggesting that tissue was well as species differences are contributing. These different phosphorylation patterns cannot be explained by the presence of factors influencing the phosphorylation such as a difference in the basal Mg²⁺-ATPase activity between bovine and porcine PM fractions. This possibility could be ruled out because mixing of plasma membranes of porcine gastric smooth muscle with PM vesicles of bovine pulmonary artery did not affect the phosphointermediate formation (data not shown).

Discussion

We have previously shown that a combination of potassium chloride extraction, addition of digitonin, and application of the sample underneath a sucrose density gradient is an efficient procedure to obtain from porcine gastric smooth muscle a membrane fraction enriched in ER and to eliminate contaminating cytoskeletal and contractile proteins. We have now applied this method to a vascular smooth muscle to characterize its Ca²⁺-transporting enzymes. This work confirms our previous results on gastric smooth muscle but also reveals important differences between various types of smooth muscles.

The material recovered between 16 and 24% sucrose is considered as a purified ER fraction. However, because of the diversity of the intracellular organelles the term “ER fraction” may be a misnomer and the relative contribution of rough and smooth ER, Golgi and coated vesicles or lysosomes to this fraction remains to be determined. This low density fraction definitely contains membranes derived from the rough ER, as indicated by the presence of ribophorin I. This is not unexpected since treatment with high potassium chloride is known to reduce the number of bound ribosomes. The selective enrichment in the ER-type 100-kDa Ca²⁺-transport ATPase and in phospholamban indicates that this fraction also contains membranes involved in the Ca²⁺ storage. The very low equilibrium density of these membrane vesicles may be an intrinsic feature or it may be due to the loss of some associated proteins by the treatment with high potassium chloride. However, because of its good separation from plasma membrane vesicles, this fraction is at present the most suitable for the study of the 100-kDa transport ATPase.

The PM-enriched fraction at 28–40% sucrose still contains an appreciable amount of putative ER markers and of the 100-kDa Ca²⁺-transport ATPase of the ER type. This contamination may be explained by the presence of cholesterol-containing intracellular membranes that are also shifted by digitonin or by the presence of ER vesicles that are somehow connected to PM vesicles. This association may be an artifact induced by the homogenization or it may have a morphological correlate because junctional ER vesicles have been observed to be linked to the plasma membrane by means of “bridging structures.”

An interesting difference between the PM vesicles from bovine pulmonary artery and porcine gastric smooth muscle is that the (Na⁺,K⁺)-ATPase activity is higher and that the (Ca²⁺,Mg²⁺)-ATPase activity is lower in the pulmonary artery (present results) than in gastric smooth muscle. Because the activity of the (Na⁺,K⁺)-ATPase sets an upper limit to the Ca²⁺ efflux that can be mediated by a Na⁺-Ca²⁺ exchanger, the bovine pulmonary artery may, in contrast to the pig antrum, present the proper conditions for Na⁺-Ca²⁺ exchange playing an important role in Ca²⁺ extrusion. However, it remains to be determined whether the...
Na\textsuperscript+-Ca\textsuperscript{2+} exchanger plays a more important role in the regulation of the intracellular Ca\textsuperscript{2+} concentration in bovine pulmonary artery than it does in porcine antrum.

Our results demonstrate the presence of two different types of Ca\textsuperscript{2+}-transport ATPase in bovine main pulmonary artery, which is in agreement with our previous observations in porcine gastric smooth muscle.\textsuperscript{1} A 130-kDa, calmodulin\textsuperscript{[22]}-binding protein that is associated with the PM-enriched fraction represents the plasmalemmal type of Ca\textsuperscript{2+}-transport ATPase, whereas the 100-kDa protein that reacts with polyclonal antibodies against the (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase of porcine cardiac SR, that does not bind calmodulin\textsuperscript{[22]} and that occurs mainly in the ER fraction corresponds to the ER Ca\textsuperscript{2+} pump. Phospholamban is also present in the ER fraction and may have a similar function as in cardiac SR\textsuperscript{30} being a cAMP-dependent and or a Ca\textsuperscript{2+} + calmodulin-dependent regulator of the ER Ca\textsuperscript{2+} pump.

Whereas the 100-kDa phosphointermediate is easily visualized in the bovine pulmonary artery and aorta, the 130-kDa phosphointermediate can only be observed in bovine pulmonary artery if La\textsuperscript{3+} is included in the phosphorylation medium and it cannot be visualized in the bovine aorta even if La\textsuperscript{3+} is included. The distribution and the relative enrichment of the phosphointermediate levels in the isolated fractions do not coincide with those of the (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase activity. This inconsistency can be explained by assuming that the phosphointermediate level of the 130-kDa Ca\textsuperscript{2+}-transport ATPase of the plasmalemma is, for the same (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase activity, much lower than that of the 100-kDa ATPase. The observation that the ratio of ATPase activity to phosphointermediate level is much higher for the 130-kDa ATPase than for the 100-kDa ATPase is not surprising in view of the known kinetic behavior of the 100-kDa ATPase of skeletal muscle SR\textsuperscript{33} and of the 130-kDa enzyme of erythrocytes.\textsuperscript{34} The phosphointermediate of the 100-kDa enzyme can reach a level close to the maximal number of catalytic sites\textsuperscript{33} whereas the phosphointermediate of the 130-kDa enzyme amounts at most to 10\% of the enzyme.\textsuperscript{34} For this reason, the 130-kDa phosphointermediate is not a suitable PM marker, e.g., for assessing the contamination of ER vesicles by PM vesicles, although it has been used by several authors for that purpose. Because in bovine aortic smooth muscle no 130-kDa phosphoprotein was observed, Sumida et al proposed that the mechanism of regulation of cytoplasmic Ca\textsuperscript{2+} in aortic smooth muscle might be analogous to that in skeletal and cardiac muscle.\textsuperscript{35} Also, Chiesi et al\textsuperscript{36} did not observe a phosphoprotein of 130 kDa in bovine aortic microsomes and therefore concluded that these microsomal preparations were enriched in ER vesicles. However, in view of our findings, these results have to be reinterpreted.

We have made a semiquantitative measurement of the (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase activity of the 130-kDa enzyme by calculating the activity of the 100-kDa ATPase that contaminates the PM fraction, and by subtracting this activity from the actually measured Ca\textsuperscript{2+}-stimulated ATPase activity. According to this calculation the 100-kDa ER-type ATPase would represent 43\% of the total (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase activity, and the remaining 57\% would be catalyzed by the 130-kDa PM-type (Ca\textsuperscript{2+}, Mg\textsuperscript{2+})-ATPase.

It should be pointed out that the calculated ratio of the 100-kDa to the 130-kDa Ca\textsuperscript{2+}-transport ATPase may not represent the Ca\textsuperscript{2+}-transporting capacity of the ER and PM membranes in the living tissue. For instance, the 100-kDa Ca\textsuperscript{2+}-transport ATPase of the SR of skeletal muscle has a stoichiometric ratio (Ca\textsuperscript{2+}-transported/ATP hydrolyzed) of 2,\textsuperscript{32} whereas this ratio equals 1 for the 130-kDa enzyme of smooth muscle.\textsuperscript{35} If these values are applied to the smooth muscle enzymes, the contribution of the 100-kDa enzyme would increase from 43\% to 57\%. In addition, the relative importance of the 100-kDa to the 130-kDa enzyme will depend on many factors, such as their stimulation or inhibition by second messengers and on the kinetics of activation of the 130-kDa ATPase by calmodulin.\textsuperscript{18} The Ca\textsuperscript{2+}-transport capacity of the ER may also be influenced by the presence of intraluminal Ca\textsuperscript{2+}-binding proteins, such as calsequestrin.\textsuperscript{30} In addition, definite conclusions about the relative importance of both Ca\textsuperscript{2+}-transport enzymes cannot be drawn without a precise knowledge of their recovery in the isolated fractions.

Differences in the ratio of the ER-type ATPase to the PM-type ATPase between different smooth muscles can be demonstrated by comparing the relative amount of the two phosphorylated transport intermediates. In both ER and PM membrane fractions of bovine pulmonary artery, the ratio of the 100- to the 130-kDa phosphointermediate is much higher than in the corresponding porcine gastric smooth muscle membrane fractions. The relative contribution of the 100-kDa Ca\textsuperscript{2+} transport ATPase to the cellular Ca\textsuperscript{2+} transport capacity may, therefore, be appreciably larger in bovine pulmonary artery than in gastric smooth muscle. This difference is partly species-dependent and partly tissue-dependent because porcine pulmonary artery presents an intermediate pattern of phosphorylated ATPases. In bovine aorta, no 130-kDa phosphoprotein intermediate could be detected, although its presence has been demonstrated previously by calmodulin affinity chromatography.\textsuperscript{40} In this tissue, calcium transport in the ER may be even more important than in the pulmonary artery. The way in which the intracellular Ca\textsuperscript{2+} homeostasis is realized may, therefore, differ according to the type of smooth muscle, and the contribution of the ER may be relatively more important in vascular smooth muscle than in visceral smooth muscle. This hypothesis is in agreement with morphological data showing a well-developed ER system (up to 7.5\% of the nucleus- and mitochondria-free cell volume) in large elastic arteries such as the rabbit main pulmonary artery, whereas in visceral smooth muscle, only 2\% of the cell volume is occupied by the ER.\textsuperscript{44} However, even between smooth muscle presenting a similar ER content, such as the guinea pig portal vein (2\% of cell volume\textsuperscript{45}) and the guinea pig taenia coli
Eggermont et al. *Cal* **Ca**++ Transport in Vascular Smooth Muscle

(1.5% of cell volume**), differences in Ca**++-handling properties may exist.**

Acknowledgments
We thank Dr. K. Gietzen (Department of Pharmacology and Toxicology, University of Ulm) for the gift of antiserum to the Ca**++** pump of cardiac SR and Dr. M. Hortsch (EMBL, Heidelberg) for the gift of monoclonal antibodies to ribophorin I.

References
23. Raeymaekers L, Casteels R: The calcium uptake in smooth muscle microsomal vesicles is reduced by centrifugation. *Cell Calcium* 1984;5:205–210
Ca²⁺ pump revealed by calcium transients in human red cells. Biochim Biophys Acta 1983;730:295–305

Key Words: vascular smooth muscle • Ca²⁺-transport ATPase • endoplasmic reticulum • plasma membrane