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The Ca^{2+}-transport ATPases in smooth muscle

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Summary. A calmodulin stimulated Ca^{2+}-transport ATPase which has many of the characteristics of the erythrocyte type Ca^{2+}-transport ATPase has been purified from smooth muscle. In particular, the effect of calmodulin on these transport enzymes is mimiced by partial proteolysis and antibodies against erythrocyte Ca^{2+}-transport ATPase also bind to the smooth muscle $(Ca^{2+} + Mg^{2+})$ATPase. A correlation between the distribution of the calmodulin stimulated $(Ca^{2+} + Mg^{2+})$ATPase and $(Na^{+} + K^{+})$ATPase activities in smooth muscle membranes separated by density gradient centrifugation suggests a plasmalemmal distribution of this $(Ca^{2+} + Mg^{2+})$ATPase. A phosphoprotein intermediate in smooth muscle which strongly resembles the corresponding phosphoprotein in sarcoplasmic reticulum of skeletal muscle may indicate the presence in smooth muscle of a similar type of Ca^{2+}-transport ATPase.
Key words. Smooth muscle; calmodulin overlay; Ca^{2+}-transport ATPase phosphorylated intermediate.

1. Introduction

There is now ample experimental evidence for the existence in smooth muscle not only of an ATP-dependent Ca^{2+}-extrusion system in the plasma membranes, but also of an ATP-dependent Ca^{2+}-accumulation system in endo-(sarco)plasmic reticulum.

The relative contribution of both Ca^{2+}-transport systems to the removal of Ca^{2+} from the cytoplasm during relaxation remains unknown.

It has been known for some time[1,3,6,8,18] and it can be easily demonstrated, that membrane vesicle fractions prepared from different types of smooth muscle present a Ca^{2+} uptake with high affinity in the presence of MgATP. However, it proved to be more difficult to demonstrate the presence of a corresponding (Ca^{2+} + Mg^{2+})-dependent ATPase (CaMg ATPase) activity. This is mainly due to the presence in smooth muscle of the high activity of a Mg ATPase which masks the CaMg ATPase activity. Moreover a progressive decrease in activity of this Mg ATPase occurring in microsomes from some smooth muscles during the ATPase assay has further complicated the determination of the CaMg ATPase activity.

The present paper summarizes some of our recent results concerning the purification, characterization and subcellular localization of a calmodulin stimulated Ca^{2+}-transport ATPase from smooth muscle. This CaMg ATPase has many characteristics in common with the Ca^{2+}-transport ATPase from the plasma membranes of human erythrocytes. Because of its resemblance to the erythrocyte-type ATPase, one might hypothesize that also this ATPase of smooth muscle is a plasma membrane component. The question also arises whether the ATP-dependent Ca^{2+}-transport system in endoplasmic reticulum is similar to this presumed plasmalemmal CaMg ATPase or whether it would rather resemble the CaMg ATPase of sarcoplasmic reticulum of skeletal muscle.

2. Methods

Calmodulin-stimulated CaMg ATPase from smooth muscle was prepared as described earlier[20].

Antibodies were raised against sarcoplasmic reticulum from porcine skeletal muscle and against calmodulin stimulated CaMg ATPase from porcine erythrocytes purified by calmodulin affinity chromatography and the binding of antibodies was studied by immunoblotting techniques[22]. For the activation of the smooth muscle ATPase by proteolysis, the CaMg ATPase purified from smooth muscle was first reconstituted in phosphatidyl choline by cholate dialysis[17]. The reconstituted vesicles containing a total of approximately 50 µg CaMg ATPase in 130 mM KCl, 20 mM K HEPES (pH 7.4), 50 µM $MgCl_2$ and 1 mM dithiotreitol, were then incubated with 5 µg/ml trypsin (Boehringer, Mannheim) on ice for the times as indicated and the reaction was stopped by 50 µg soy bean trypsin-inhibitor (Boehringer, Mannheim). ATPase was measured in the presence of 0.1 µg/ml A23187.

For fractionation of the microsomes by density-gradient centrifugation, the homogenate was centrifuged in a Sorvall GSA rotor at 9000 rev/min for 30 min to remove large fragments and mitochondria. The supernatant was brought to 0.6 M KCl and 45% sucrose, and applied below a 15 to 40% sucrose gradient in a Beckman Ti15 zonal rotor. The sucrose gradient also contained 0.6 M KCl. Centrifugation was carried out at 30,000 rev/min for 20 h. Ca^{2+}-uptake and enzyme activities were determined as described previously[13], except that saponin (200 µg/ml) was included in the medium used for the determination of the NaK ATPase and CaMg ATPase activities.

3. Results

A membrane vesicle fraction prepared from smooth muscle of porcine coronary artery[19] or porcine stomach[12] contains a CaMg ATPase activity with a high affinity for Ca^{2+}. This enzyme activity indicates the presence of an ATP driven Ca^{2+}-transport system. Both this CaMg ATPase activity and the Ca^{2+}-transport, measured in the absence of oxalate, were found to be stimulated by calmodulin[13,20].

Starting from 100 g of frozen porcine antral smooth muscle we can obtain approximately 110 mg of protein in the KCl-extracted microsomal fraction. This membrane fraction is a mixture of membranes from different subcellular origin. The membrane vesicles are either leaky or sealed for substrates (e.g. ATP and Ca^{2+}) and they present an orientation which is either the same or opposite to that present in the cell. In order to estimate the total transport enzyme activity in our microsomes we found that the optimal procedure consisted in an unmasking of CaMg ATPase- and NaK ATPase activities by addition of 0.2 mg/ml saponin. The following values were obtained (nmoles/mg protein/min at 37 °C, given as mean ± SE of mean, the number of observations between parenthesis): Mg ATPase: 92 ± 6.7 (5), NaK ATPase 25 ± 5.5 (2), CaMg ATPase 44 ± 5.9 (5). In the presence of saturating concentrations of calmodulin (0.6 µM) the CaMg ATPase activity was increased by a factor of 2.9 ± 0.25 to a value of 120 ± 2.0 nmoles/mg protein/min. It is noteworthy that we observed in human erythrocyte vesicles a twofold higher calmodulin stimulation under the same experimental conditions. Several explanations can be given for this discrepancy between the two tissues, and at present none of them can be ruled out on the basis of the experimental evidence: 1) The CaMg ATPase in the microsomes from smooth muscles would already be partially activated by contaminating calmodulin. 2) The CaMg ATPase in the vesicles is partially activated by a mechanism which does not depend on calmodulin such as by negatively charge amphiphiles[5] or by limited proteolysis[15]. 3) The CaMg ATPase from smooth muscle differs from that of human erythrocytes by its intrinsic potentiality to be stimulated by calmodulin. 4) Smooth muscle microsomes contain different types of CaMg ATPases and not all of them can be stimulated by calmodulin.

Affinity chromatography on a calmodulin-Sepharose 4B gel, a technique originally designed for the purification of CaMg ATPase from human erythrocytes[4,10], was used to purify also the smooth muscle enzyme[20]. Proteins were first solubilized from the membranes with Triton X-100 and then incubated with the calmodulin-Sepharose gel in the presence of Ca^{2+}. After extensive washing of the gel, those proteins that were bound to the calmodulin gel in a Ca^{2+}-dependent way were released by reducing the concentration of ionised calcium by adding 2 mM EGTA. The continuous presence of phospholipids during purification was necessary in order to preserve enzyme activity. The enzyme preparation obtained in this way consisted mainly of an ATPase with M_R of 140–150 kDa as estimated from Laemmli-type sodium dodecyl-polyacrylamide gel electrophoresis. It was often observed that this protein band was composed of two closely spaced bands. Besides the ATPase, some contaminant polypeptide

bands were also present but in a variable amount which could be further decreased by more extensive washing of the calmodulin gel. The specific activity of the purified CaMg ATPase preparation amounts to 11.9 μmoles Pi/mg protein/min at 37 °C and in the presence of 10^{-5} M Ca^{2+} and 0.6 μM calmodulin. This value is comparable to the one obtained for CaMg ATPase purified from human erythrocytes[4,10] and approximately half of the value obtained for pure CaMg ATPase from sarcoplasmic reticulum of skeletal muscle and NaK ATPase from kidney[9].

The purified CaMg ATPase can be stimulated by calmodulin, but the level of stimulation depends on the lipid environment of the enzyme as well as on its structural integrity. When the CaMg ATPase is eluted from the calmodulin affinity gel in the presence of 0.4% Triton X 100/0.05% asolectin (a crude soy bean phospholipid mixture consisting for about one fifth of negatively charged phospholipids[11]) the CaMg ATPase from smooth muscle is stimulated by 0.60 μM calmodulin (at 10^{-5} M Ca^{2+}) only by a factor of 1.42 ± 0.03 (45). When on the other hand the elution medium contains a neutral phospholipid like phosphatidyl choline the calmodulin stimulation increases by a factor of 2.27 ± 0.13 (15).

Limited trypsin digestion of the CaMg ATPase purified from smooth muscle increases the CaMg ATPase activity obtained in the absence of calmodulin while at the same time it reduces the calmodulin stimulation (fig. 1). This increase of the enzyme activity by limited tryptic digestion has first been described for erythrocyte Ca^{2+}-transport ATPase[5,15,16] and also by this property both transport enzymes resemble each other.

We have demonstrated that the purified CaMg ATPase activity is a genuine Ca^{2+} transport ATPase by reconstituting the enzyme in artificial lipid vesicles. These vesicles thereupon present an ATP dependent Ca^{2+} accumulation with a Ca^{2+}/ATP ratio of 1[17].

In these vesicles the influence of the phospholipid environment of the CaMg ATPase on its calmodulin activation is demonstrated even more clearly than in the case of the solubilized enzyme as described above. Whereas the stimulation factor of the CaMg ATPase by calmodulin in asolectin vesicles amounts to 1.25 ± 0.09 (24), it reaches a value of 4.05 ± 0.63 (12) in phosphatidyl choline vesicles.

The reaction between antibodies raised against CaMg ATPase from porcine erythrocytes or against fragmented sarcoplasmic reticulum from porcine skeletal muscle and the CaMg ATPase of smooth muscle has also been examined (fig. 2). IgG against the erythrocyte ATPase bind to the ATPase purified either from erythrocytes or from smooth muscle and these antibodies also bind to a protein of a similar M_R in the smooth muscle microsomes. However, IgG raised to skeletal muscle CaMg ATPase bind only to the ATPase of skeletal muscle sarcoplasmic reticulum and not to the CaMg ATPase purified from erythrocytes or smooth muscle. Neither is there any reaction with a band at 100 kDa in the smooth muscle micro-

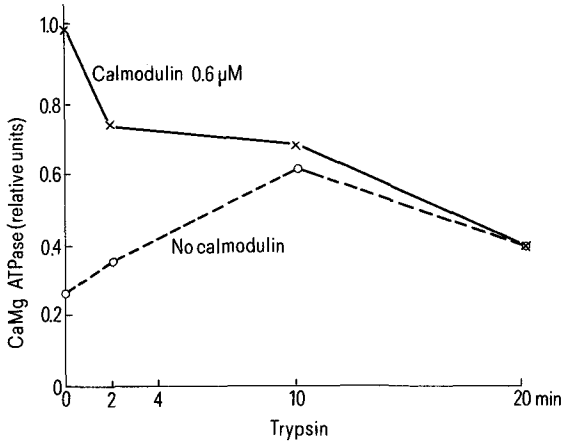

Figure 1. The effect of partial proteolysis of the CaMg ATPase from smooth muscle on its ATPase activity. CaMg ATPase purified from antral smooth muscle by means of calmodulin affinity chromatography was reconstituted in egg yolk phosphatidyl choline by the cholate dialysis method. The vesicles containing about 50 μg CaMg ATPase were incubated on ice in the presence of 5 μg/ml trypsin. The reaction was stopped by 50 μg soy bean trypsin inhibitor and the CaMg ATPase activity was measured in the presence of 0.1 μg/ml A23187 at 10^{-5} M [Ca^{2+}] both in presence and absence of 0.6 μM calmodulin.

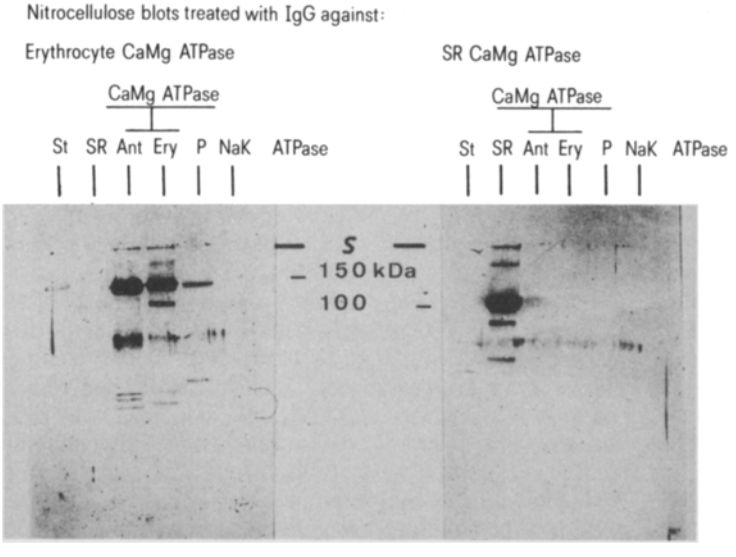

Figure 2. The binding of antibodies (IgG) against erythrocyte CaMg ATPase and against the CaMg ATPase from skeletal muscle to different antigens. An immunoblotting procedure was used as described earlier[22]. On each gel were applied from left to right St, M_R standards; SR, sarcoplasmic reticulum from porcine skeletal muscle; Ant, CaMg ATPase purified by means of calmodulin affinity chromatography from porcine antrum smooth muscle; Ery, a similar CaMg ATPase obtained from porcine erythrocytes; P, a microsomal fraction from porcine antral smooth muscle; NaK ATPase, NaK ATPase from porcine kidney purified according to De Smedt et al.[2].

somes. We therefore propose that if an ATPase of the sarcoplasmic reticulum type is present in smooth muscle microsomes, its binding with antibodies against skeletal muscle sarcoplasmic reticulum remains below the detection limit.

It was clearly demonstrated in the preceding sections that smooth muscle cells contain a Ca^{2+}-transport ATPase which is very similar to the CaMg ATPase of erythrocytes. It is likely that also in smooth muscle cells this enzyme is confined to the sarcolemma. We have further tested this hypothesis by separating membrane fractions by density gradient centrifugation and by studying the distribution of the CaMg ATPase activity and its relation to the NaK ATPase activity. The latter enzyme is generally considered to be the most reliable plasma membrane marker. The Ca^{2+} uptake by vesicles does not only depend on Ca^{2+} pumping but also on the integrity of the vesicular membrane, which might loose its function as a permeability barrier and allow the transported molecules to leak in the opposite direction. We conclude therefore that the CaMg ATPase activity may be a better quantitative index of Ca^{2+} transport than the rate of Ca^{2+} transport itself.

We have also studied the distribution of the rate of oxalate stimulated Ca^{2+} uptake, because we have recently observed that in previous studies from our and other laboratories the oxalate stimulated Ca^{2+} uptake might have been underestimated. It was found that the oxalate stimulated Ca^{2+} uptake was very sensitive to mechanical damage induced by pelleting[14]. Since under our conditions of centrifugation (see methods section) pelleting is avoided, the oxalate stimulated Ca^{2+} uptake could be largely preserved. The main features of our present centrifugation method are the avoidance of pelleting by immediate application of the post-mitochondrial supernatant below a density gradient, together with the inclusion of 0.6 M KCl both in the microsomal suspension and in the density gradient. In addition to preserving the oxalate stimulated Ca uptake, this flotation rather than sedimentation procedure combined with the inclusion of 0.6 M KCl also resulted in an effective extraction and separation of extrinsic proteins from the membranes, as was shown by sodium-dodecyl polyacrylamide gel electrophoresis of the different fractions obtained from the gradient. Particularly the content of protein bands at positions corresponding to those of the contractile proteins actin, (42 kD) and myosin (200 kD) was decreased appreciably by this procedure. As could be expected, the extraction of extrinsic proteins induced a shift to lower densities of the marker enzyme activities and concomitantly increased their specific activities (data not shown). The distribution of marker enzymes in the gradient fractions is shown in figure 3. It is clear that the activities of NaK ATPase and of CaMg ATPase measured in the presence of 0.6 μM calmodulin do correlate quite well. Both enzymes are found over a wide density range with a maximum between densities of 25% and 30% sucrose. Assuming that NaK ATPase is a good marker for plasma membranes, this finding would indicate that the largest fraction of the Ca^{2+} transport activity is located in this organelle.

Under the present conditions of preparation, the rate of oxalate-stimulated Ca^{2+} uptake in the gradient fractions is several fold higher than in membranes prepared by the conventional pelleting procedure, reaching a maximum of about 80 nmoles/mg protein/min at a density of about 24% sucrose. In contrast to the CaMg ATPase activity, this Ca^{2+} uptake presents a distribution which is different from that of the NaK ATPase activity. The distribution of NADH cytochrome c reductase (rotenone insensitive) a putative marker for endoplasmic reticulum, is more similar to that of the rate of oxalate-stimulated Ca^{2+} uptake. A link between both activities in microsomes of stomach smooth muscle was also suggested by earlier experiments in which the NADH cytochrome c reductase activity was found to be enriched in vesicles loaded with calcium oxalate[13].

The low density gradient fractions might be very suitable as a starting material for the isolation of internal membranes by loading them with calcium oxalate. Preliminary experiments indicate that this is indeed the case, and that the yield and purity of the oxalate-loaded vesicles are significantly improved. The study of this fraction may help to characterize the CaMg ATPase responsible for the oxalate-stimulated Ca^{2+} transport.

4. Discussion

Smooth muscle microsomes of the antrum present a calmodulin-stimulated CaMg ATPase activity which is the enzymatic correlate of Ca^{2+}-transport in these membranes. The transport function was clearly demonstrated by purification of the enzyme followed by reconstitution in artificial lipid vesicles. This calmodulin dependent enzyme has many characteristics in common with the erythrocyte ATPase: 1) the M_R of 140–150 kDa, 2) stimulation by calmodulin or anionic amphiphiles, 3) resemblance between the effect of the calmodulin stimulation and that

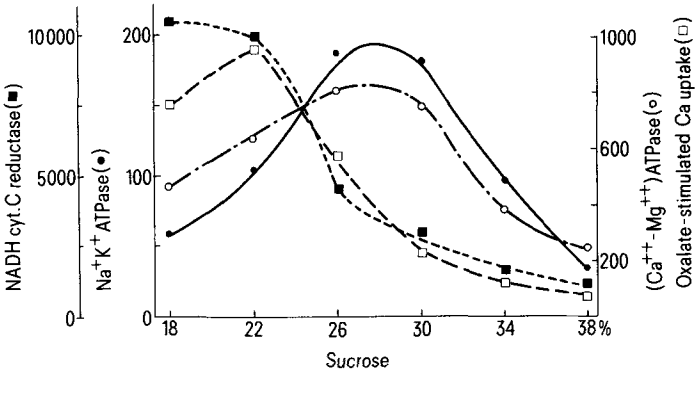

Figure 3. The distribution of specific activities of two transport ATPases (NaK ATPase and CaMg ATPase), of the putative endoplasmic reticulum marker NADH cytochrome c reductase (rotenone-insentive), and of the rate of oxalate-dependent Ca uptake. The enzyme activities are expressed in nmol/mg per min, the rate of Ca uptake in nmol/mg per 20 min. The membrane fractions were prepared in a zonal rotor on a sucrose density gradient containing 0.6 M KCl.

of partial proteolysis, 4) the increase of steady state levels of the phosphoprotein intermediates by La^{3+}, 5) the reaction of antibodies against the smooth muscle ATPase with the ATPase from erythrocytes and conversely of antibodies against the erythrocyte ATPase with the smooth muscle ATPase. By all these characteristics the CaMg ATPase purified from smooth muscle differs from the Ca^{2+}-transport enzyme in fragmented sarcoplasmic reticulum of skeletal muscle. This enzyme has a M_R of 100,000, it is not stimulated by calmodulin nor by partial proteolysis, its steady state level of phosphoprotein intermediate is decreased and not increased by La^{3+}, and it does not react with the antibodies against the smooth muscle ATPase. In addition antibodies against the CaMg ATPase of sarcoplasmic reticulum from skeletal muscle do not recognize the calmodulin stimulated ATPase.

The correlation between the distribution of the NaK ATPase- and calmodulin-stimulated CaMg ATPase activities suggest that this Ca^{2+}-transport enzyme is also in smooth muscle confined to the plasma membrane. There is some experimental evidence in favor of the existence in the microsomal fraction of another type of CaMg ATPase which could correspond to an endoplasmic ATPase. 1) Our phosphorylation experiments show that in the vesicles a Ca^{2+}-dependent acyl phosphate bond is formed with a M_R similar to that in sarcoplasmic reticulum of skeletal muscle (100 kDa)[21]. Furthermore, similarly to the sarcoplasmic reticulum CaMg ATPase from skeletal muscle, this phosphoprotein level decreases by addition of La^{3+} instead of increasing as the erythrocyte CaMg ATPase does. We consider it unlikely that this 100 kDa ATPase is a proteolytic product of the 140 kDa calmodulin binding ATPase, because the Ca^{2+}-dependent phosphorylation of the 130 kDa protein is stimulated by 100 μM La^{3+} whereas this of the 100 kDa protein is inhibited. Moreover we found that the Ca^{2+}-dependent phosphorylation which is stimulated by La^{3+} in smooth muscle membranes is more resistant to limited trypsin digestion than the La^{3+} unstimulated one (unpublished results). We must point out that our antibodies directed against the sarcoplasmic reticulum ATPase from skeletal muscle do not recognize a band of M_R 100 kDa in smooth muscle microsomes. This finding indicates that any CaMg ATPase in smooth muscle related to the Ca^{2+}-transport ATPase in sarcoplasmic reticulum of skeletal muscle is probably sufficiently different from it to prevent any cross-reaction with the antibodies.

Another indication for the presence in smooth muscle of a different type of Ca^{2+} transport is that only the ATP driven Ca^{2+} accumulation in the absence of oxalate is stimulated by calmodulin. Ca^{2+} accumulation measured in the presence of oxalate which for a large part can be ascribed to vesicles of fragmented endoplasmic reticulum is not stimulated by calmodulin, suggesting that the endoplasmic reticulum contains a calmodulin-insensitive ATPase. We cannot exclude the possibility that the specific lipid environment of the CaMg ATPase in endoplasmic reticulum might prevent the activation of the ATPase by calmodulin.

^{125}I-Calmodulin binding studies in membrane fractions enriched in endoplasmic reticulum obtained by means of calcium oxalate loading density perturbation techniques will be helpfull to discriminate between these possibilities.

It is interesting to note that the specific activity of the NaK ATPase is lower than this of the CaMg ATPase in each of the density gradient fractions. This is a further indication that the contribution of a Na-Ca exchange mechanism which has been proposed as an alternative way for reduction of intracellular Ca^{2+}[7] might be less important than that of the CaMg ATPase. The extrusion of the Na^+ ions that had entered the cell in exchange for Ca^{2+} would require extra ATP splitting by the NaK ATPase and is therefore limited by the maximal activity of the latter enzyme.

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The use of subcellular membrane fractions in analysis of control of smooth muscle function

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Introduction

Analysis of control of smooth muscle function has always been complicated by the diversity of the types and functions of smooth muscle. Thus the occurrence of smooth muscle primarily innervated by excitatory sympathetic nerves (e.g. some blood vessels and vas deferens), others primarily innervated by excitatory parasympathetic and inhibitory nonadrenergic, noncholinergic nerves (e.g. circular muscle of the gut), and yet others (e.g. urinary bladder detrusor) innervated by excitatory cholinergic nerves as well as by excitatory noncholinergic, nonadrenergic nerves[5,9,10,11,22] makes it clear that the postsynaptic receptors and their coupling via conductance channels and receptor-operated-Ca stores must differ vastly, as between various smooth muscles. Diversity in smooth muscle is not limited to its innervation and the effector mechanisms operated by neurotransmitters, it also occurs in the nature of the excitation-contraction coupling mechanisms and in the methods whereby relaxation from contraction is achieved. Muscles may be activated to contract by electromechanical means (opening of voltage-dependent Ca^{2+} channels), inhibited from contraction by opening of a variety of voltage- or Ca-dependent K^+-channels, excited by pharmacomechanical means (release of intracellular Ca-stores) or relaxed by other post- or presynaptic mechanisms. There is controversy about whether relaxation by postsynaptic mechanisms occurs by resequestration of internal calcium in endoplasmic reticulum or mitochondria or whether it is by pumping of calcium out of cells. This controversy will be considered further below in so far as evidence from isolated subcellular fractions bears on it. There is further controversy about the location of the internal stores of calcium which couple excitation to those contractions which occur (in some cases) without any external calcium. It is usually assumed that they are located in the endoplasmic reticulum, but a possible locus in the plasma membrane has not been excluded. Since studies with subcellular membranes do not provide much evidence about this question, it will be discussed only briefly.

The question arises: 'Are there levels of organization of smooth muscle at which common mechanisms operate to control contractile function?' Possibly one such level is the contractile apparatus itself, a matter not the focus of this chapter, but even here there appears to be diversity[23]. Another such level where common features of control may exist is the set of mechanisms for removal of elevated intracellular Ca^{2+}. Sites of Ca^{2+}-removal may also be sites of release of Ca^{2+} by pharmacomechanical means, but this turns out to be not necessarily or commonly the case. However, the question of how Ca is removed from the cell interior (transport to the extracellular space or resequestration internally in a cytoplasmic organelle or both) has also proved to be difficult to resolve, and it is unclear whether this is due to real diversity of smooth muscles, technical difficulties, conceptual limitations or other reasons.

The chapter will focus on the studies carried out in our laboratories on subcellular fractions in study of Ca-control systems. It is appropriate to point out in a volume dedicated to Dr Bülbring that findings she made or inspired using electrophysiological techniques about the function calcium in excitation of smooth muscle have provoked these as well as many other studies. There are many other ways to approach analysis of how smooth muscle cells control their internal calcium and the value, and limitations of study of membrane fractions need to be considered. Some general features will emerge: 1) from microsomal fractions of smooth muscles, it is always possible to obtain subfractions which are highly enriched in plasma membranes; 2) these plasma-membrane en-