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A CALMODULIN-STIMULATED CA^{2+} PUMP IN RAT AORTA PLASMA MEMBRANES

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An ATP-driven Ca^{2+}-transport system has been characterized in a microsomal fraction from rat aorta. Calmodulin enhanced 2.5-fold ^{45}Ca accumulation by EGTA-treated microsomes incubated with 10 μM Ca^{2+} (in the absence of oxalate) by increasing markedly the apparent affinity of the transport system for Ca^{2+}. The ionophore A23187 induced a rapid release of the sequestered ^{45}Ca. The vesicles that took up ^{45}Ca were distributed like plasmalemmal marker enzymes when the microsomal fraction was subfractionated by density gradient centrifugation. In particular, these vesicles were markedly shifted towards higher equilibrium densities after addition to the microsomes of 0.25 mg digitonin/mg protein before isopycnic centrifugation. We conclude that the calmodulin-stimulated Ca^{2+} pump associated with the microsomal fraction is located in plasmalemmal elements.

Introduction

Several ATP-driven Ca^{2+} pumps might be involved in the reduction of the cytoplasmic Ca^{2+} concentration during the relaxation of vascular smooth muscle cells and in the maintenance of a steep Ca^{2+} concentration gradient with respect to the extracellular milieu [1]. Ca^{2+}-transport systems have been described in the mitochondria [2], and in microsomal [3–6] or related subcellular fractions [7,8] which contain, in variable proportion, vesicular elements derived from intracellular membranes, in particular the endoplasmic reticulum, and from the plasma membrane. However, the relative contribution of these subcellular entities to the regulation of the Ca^{2+} level in arteries is still controversial (for a review, see Ref. 9). A prominent feature of plasmalemmal Ca^{2+} pumps, as demonstrated in studies on red blood cells [10–13], brain [14] and adipocytes [15], is their sensitivity to calmodulin, a ubiquitous Ca^{2+}-binding protein that modulates many Ca^{2+}-dependent cellular activities [16]. These studies have led to better characterization of the properties of these pumps and to strengthening of the evidence for their role in physiological regulation. Therefore, we decided to examine the effect of calmodulin on the ATP-dependent ^{45}Ca uptake by rat aorta microsomes and to investigate the submicrosomal localization of the Ca^{2+}-transport activities. In contrast with a recent report on pig coronary artery microsomes [17], we found that calmodulin markedly enhanced ^{45}Ca uptake by EGTA-treated microsomes from rat aorta. The ^{45}Ca was accumulated inside vesicles which behaved like plasmalemmal elements in density gradient centrifugation.

Methods

Preparation of microsomes

30–50 female Wistar rats weighing 200–250 g were killed by decapitation. Thoracic aortae were immersed in Krebs medium (37°C) equilibrated with 95% O_2/5% CO_2. After removal of the adherent connective and fatty tissue, the aortae were transferred

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

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into ice-cold 0.25 M sucrose, buffered at pH 7.4 with 5 mM Tris-HCl (sucrose-Tris), and finely minced with scissors. The tissue pieces were homogenized with 25 ml of sucrose-Tris in a Potter-Elvehjem-type all-glass tissue grinder (Braun, Melsungen, F.R.G.) kept at 2°C, by three strokes of the pestle rotating at 1500 rev./min.

The homogenate was processed as described by Fitzpatrick et al. [3]. It was centrifuged at $1500 \times g$ for 10 min in a refrigerated Christ Zeta 17 Centrifuge (rotor 8750, Heraeus-Christ, Osterode am Harz, F.R.G.). The supernatant was centrifuged at $27\,000 \times g$ for 20 min in a W 40/128 rotor of a Christ Omega II Ultracentrifuge, and the resulting supernatant at $100\,000 \times g$ for 60 min in the same rotor. The microsomal pellet was either suspended in a small volume of sucrose-Tris by means of a Potter-Elvehjem-type homogenizer fitted with a Teflon pestle (untreated microsomes), or further processed as follows. The microsomes were suspended in 30 ml of a chilled solution comprising 10 mM Tris-HCl, pH 7.4/0.2 mM dithiothreitol/1 mM EGTA and then centrifuged at $100\,000 \times g$ for 60 min. The pellet was suspended in 2–3 ml sucrose-Tris or, for density gradient experiments, 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole hydrochloride. This suspension was designated EGTA-treated microsomes.

Treatment with digitonin and isopycnic centrifugation

Linear density gradients (3.8 ml) extending from 18.8 to 53.9% sucrose (g sucrose/100 g solution), i.e., from 1.08 to 1.26 in density (0°C), were delivered to tubes containing 0.2 ml of 66% sucrose (density 1.33) by a Beckman gradient former (Beckman, Palo Alto, CA, U.S.A.). All sucrose solutions were buffered at pH 7.4 with 3 mM imidazole hydrochloride. Before centrifugation, part of the microsomal preparation was treated with digitonin as described previously [18,19]. The digitonin, dissolved in 0.5 vol. 0.25 M sucrose/3 mM imidazole was added dropwise to the preparation kept in ice. The amount of digitonin (0.25 mg/mg microsomal protein) was approximately equimolar to the microsomal cholesterol [19]. After 15 min at 0°C, the digitonin-treated and untreated samples were layered on density gradients and then centrifuged for 12 h at $100\,000 \times g$ in an S 52/61 rotor. Subfractions were collected by puncturing the bottom of the centrifuge tube and their sucrose concentration was determined by refractometry. Normalized density frequency histograms were constructed as described by Beaufay and Amar-Costesec [20].

Measurement of ^{45}Ca uptake

Microsomes (10–20 μg protein/ml) were incubated at 37°C with 20 mM maleate, adjusted to pH 6.6 with Tris, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, ^{45}Ca (1.6 μCi/ml), EGTA (as needed to achieve the required free Ca^{2+} concentration [21]), 3 mM Tris-ATP, 10 mM creatine phosphate and creatine kinase (3 U/ml), in the presence or absence of calmodulin (usually 6 μg/ml). The ATP-regenerating system was needed to avoid a rapid fall in ATP concentration caused by the very active Mg^{2+}-ATPase of rat aorta microsomes [19]. Incubation was first carried out for 15 min in the absence of ATP, to allow for the interaction of the calcium-calmodulin complex with the microsomes, and thereafter for 15 or 20 min in the presence of ATP. Samples were also incubated in the absence of ATP to determine the ATP-independent ^{45}Ca uptake. At the end of incubation, 0.6 or 0.9 ml portions were filtered under suction on Sartorius filters (0.45 μm, SM 01386) disposed on a Millipore Sampling Manifold. The filters were rapidly washed with 20 ml sucrose-Tris and then dissolved in a scintillation mixture containing Pico-Fluor 30 (Packard), toluene and ethylene glycol monomethylether (2 : 7 : 1, v/v). The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Results were corrected for ^{45}Ca binding to the filters in the absence of microsomes. In order to reduce this binding, the filters were washed with 2 ml 1 M KCl, followed by 5 ml of sucrose-Tris, 15 min before use.

In most experiments, the free Ca^{2+} concentration was adjusted to 10 μM (see legends of figures). When Ca^{2+}-uptake activities were measured in microsomal subfractions obtained by density equilibration (Fig. 5), the free Ca^{2+} concentration was increased 5-fold, to enhance the level of ^{45}Ca accumulation by these comparatively diluted samples.

Enzyme determinations

Sulfatase C was assayed by the procedure of Canonico et al. [22]. The other enzyme activities were measured as described previously [19].

Protein was estimated by the method of Lowry et

al. [23], using human serum albumin as standard, or, when required, by the more sensitive fluorescamine assay [24].

Reagents

Calmodulin was a generous gift of Dr. H. Van Belle (Janssen Pharmaceutica, Beerse, Belgium). It was isolated from rat brain according to conventional procedures [25], and further purified by isoelectric focusing. A23187 was a gift of Dr. R.L. Hamill (E. Lilly Co., Indianapolis, IN, U.S.A.). Calcium stock solutions were prepared from $CaCO_3$ by titration with HCl. $^{45}CaCl_2$ was supplied by the Radiochemical Centre (Amersham, U.K.). ATP, creatine phosphate and creatine kinase were obtained from Boehringer (Mannheim, F.R.G.). Tris-ATP was prepared by neutralisation of the free acid eluted from a Dowex-50 column (H^+ form). Fluorescamine was from Hoffmann-La Roche (Basle, Switzerland). Most other chemicals were obtained from E. Merck AG. (Darmstadt, F.R.G.).

Results

Effect of calmodulin on the ATP-dependent ^{45}Ca uptake

As shown in Fig. 1, the ATP-dependent ^{45}Ca uptake by EGTA-treated microsomes increased linearly with time for a few minutes after addition of ATP and levelled off after 15 min. When the preparation had been preincubated with calmodulin (in the presence of Ca^{2+}), the ^{45}Ca uptake was faster and plateaued at a higher level. With 10 μM free Ca^{2+}, the ATP-dependent uptake at 15 min amounted to 4.5 ± 0.2 nmol/mg protein (±S.E., eight experiments) in the absence of calmodulin, and to 11.5 ± 1.1 nmol/mg protein (five experiments) in the presence of calmodulin (6 μg/ml); thus the average stimulation by calmodulin was 2.5-fold. The effect of calmodulin was almost maximal at a concentration of 3 μg/ml (Fig. 2), which is about twice the optimal concentration for the Ca^{2+} pump of red blood cells [10]. The same amount of serum albumin did not enhance Ca^{2+} uptake. ATP-independent ^{45}Ca binding (about 1 nmol/mg) was not affected by calmodulin. The ATP-dependent ^{45}Ca uptake was insensitive to 5 mM azide.

Microsomes which had been washed with 10 mM Tris/0.2 mM dithiothreitol (without EGTA) accumulated about twice as much ^{45}Ca as EGTA-treated microsomes (at 10 μM free Ca^{2+}), but they were only slightly responsive to the addition of calmodulin (data not shown). Thus, the EGTA treatment might reduce Ca^{2+} transport by stripping endogenous calmodulin from the membranes, as in erythrocyte vesicles [26–28]. The time-course of ^{45}Ca uptake was not affected by the EGTA treatment.

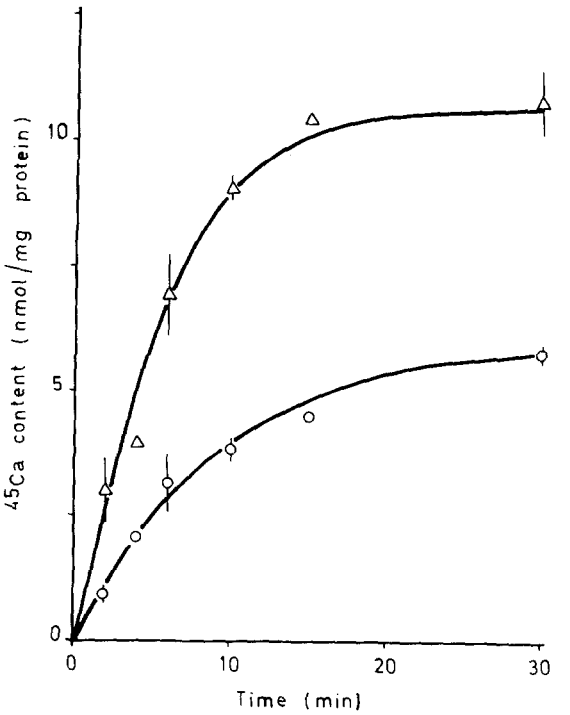

Fig. 1. Time-course of ATP-dependent ^{45}Ca uptake by EGTA-treated microsomes. Microsomes were incubated with (△) or without (○) calmodulin (6 μg/ml). The medium was supplemented with 0.32 mM EGTA, to yield 10 μM Ca^{2+}. Vertical bars indicate range of values obtained from two microsomal preparations.

As shown in Fig. 3a, calmodulin enhanced the activity of the Ca^{2+}-transport system of EGTA-treated microsomes by increasing its apparent affinity for Ca^{2+}. The Ca^{2+} concentration giving half-maximal Ca^{2+} accumulation (K_{Ca}) was lowered from 50 μM to 10 μM. ^{45}Ca uptake by untreated microsomes was also half-maximal at about 10 μM free Ca^{2+} (Fig. 3b). This finding is consistent with the view that untreated microsomes contain bound calmodulin. Our K_{Ca} values are in the range of those reported for the Ca^{2+}

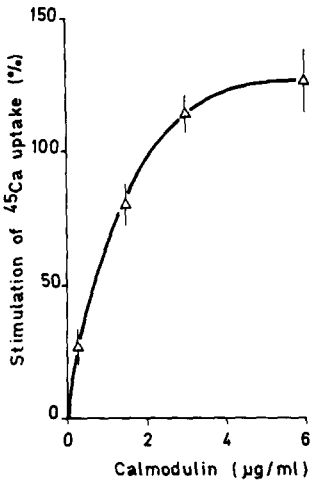

Fig. 2. Effect of calmodulin concentration on ATP-dependent ^{45}Ca uptake by EGTA-treated microsomes. Microsomes were incubated with calmodulin at various concentrations in the presence of 10 μM free Ca^{2+} (0.32 mM EGTA). The ^{45}Ca uptake was measured 20 min after addition of ATP. The additional uptake induced by calmodulin is expressed as a percentage of the uptake in the absence of calmodulin. Vertical bars represent S.E. from six determinations on a single microsomal preparation.

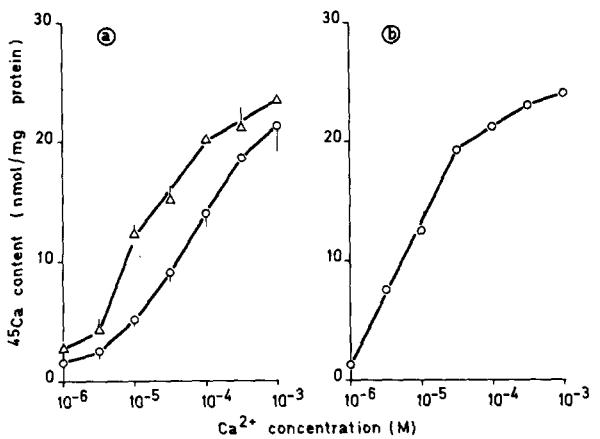

Fig. 3. Effect of free Ca^{2+} concentration on ATP-dependent ^{45}Ca uptake by various microsomal preparations. Microsomes were incubated for 20 min after the addition of ATP, at various Ca^{2+} concentrations. Up to 10^{-4} M Ca^{2+}, the total Ca was $2 \cdot 10^{-4}$ M and the EGTA concentration was varied as indicated previously [21]. a. EGTA-treated microsomes, with (△) or without (○) calmodulin (6 μg/ml). S.E. from 3–9 determinations on two microsomal preparations are indicated by vertical bars, when these are larger than the symbols. b. Untreated microsomes without added calmodulin. Each value is the mean from two determinations on a single preparation.

pump in red blood cells, where K_{Ca} is 30–45 μM in calmodulin-depleted inside-out vesicles, and is lowered to 1–15 μM after addition of calmodulin [10,27,29]. In EDTA-treated plasma membranes from adipocytes, calmodulin also stimulates Ca^{2+}-transport by increasing the apparent affinity for Ca^{2+} [15].

Release of ^{45}Ca from preloaded microsomes

In order to obtain further insight into the nature of the Ca-uptake process, microsomes preloaded with ^{45}Ca in the presence or absence of calmodulin were further incubated after addition of 2 mM EGTA, to lower the extravesicular free Ca^{2+} below 0.1 μM, or of 8.5 μM A23187. As shown in Fig. 4, the ionophore induced a rapid release of ^{45}Ca, which was nearly complete after 2 min. This suggests that the ^{45}Ca had been accumulated inside the vesicles against a concentration gradient. The release induced by EGTA was slower, but indicated a higher degree of calcium leakage from these vesicles than from inside-

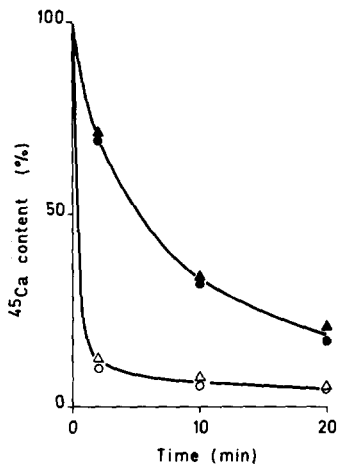

Fig. 4. Time-course of ^{45}Ca release from preloaded microsomes. EGTA-treated microsomes were preloaded with ^{45}Ca for 20 min in the standard medium except that the $CaCl_2$ concentration was 20 μM and no EGTA was added. Samples were incubated with (triangles) or without (circles) calmodulin (6 μg/ml). The release of ^{45}Ca was induced by the addition of EGTA (2 mM final concentration, closed symbols) or A23187 (8.5 μM final concentration, open symbols). Results were corrected for dilution and expressed as percent of the ^{45}Ca content at time 0. The amount of ethanol added as solvent of A23187 had no effect on the ^{45}Ca content (not shown). Each value is a mean from two microsomal preparations.

out erythrocyte vesicles [13,29] or from microsomes prepared from porcine coronary arteries [6]. The rate of ^{45}Ca release from untreated microsomes (not shown) did not differ from that observed with EGTA-treated microsomes.

Subcellular origin of the vesicles accumulating ^{45}Ca

EGTA-treated microsomes were subfractionated by isopycnic centrifugation and the density distributions of the ^{45}Ca-uptake activities were compared to those of enzymes of known subcellular location. Parallel runs were performed with microsomal samples that had been treated with digitonin in such a way as to induce a marked density perturbation of plasmalemmal elements without clear-cut effects on the density of mitochondria and endoplasmic reticulum vesicles [19,30,31]. These quantities of digitonin reduced by 10–20% ^{45}Ca accumulation by the microsomes.

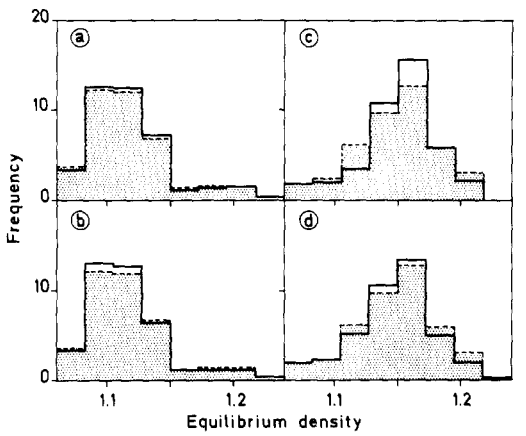

Fig. 5. Density distributions of ^{45}Ca-uptake activities in EGTA-treated microsomes. Influence of digitonin on equilibrium densities. A microsomal sample treated with digitonin (right-hand panels) and a control sample (left-hand panels) were subfractionated by isopycnic centrifugation. The subfractions were incubated with 50 μM free Ca^{2+} (0.15 mM EGTA) in the absence (a, c) or presence (b, d) of calmodulin (6 μg/ml) and the ATP-dependent ^{45}Ca uptake was determined from samples filtered in triplicate 15 min after addition of ATP (solid line). Calmodulin enhanced ^{45}Ca uptake in the control and digitonin-treated microsomal preparations by 55%. The recoveries (sum of Ca uptakes in subfractions expressed as percent of Ca uptake in the sample layered on the gradient) were: (a) 81%, (b) 102%, (c) 64%; (d) 79%. The shaded histogram in each panel gives the density distribution of 5′-nucleotidase. The frequency is the fraction of the ^{45}Ca uptake or enzyme activity recovered in a given subfraction, divided by the density increment across this subfraction [20]. Similar results were obtained in a second experiment.

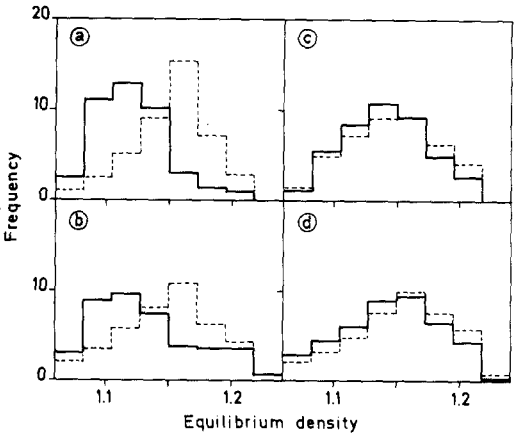

Fig. 6. Density distributions of enzymes and protein in EGTA-treated microsomes. Influence of digitonin on equilibrium densities. The distribution profiles obtained from digitonin-treated samples (broken line) and from control samples (solid line) are superimposed in each panel. The density distributions from several preparations were normalized and averaged. (a) Mg^{2+}-ATPase (n = 3); (b) protein (n = 3); (c) NADH-cytochrome c reductase (n = 2); (d) sulfatase C (n = 1). Recoveries ranged from 87 to 117%.

As shown in Fig. 5, the Ca-uptake activity, measured in the absence or in the presence of calmodulin was distributed almost exactly like the plasma-membrane marker 5′-nucleotidase between the subfractions from both the untreated (Fig. 5a and b) and the digitonin-treated (Fig. 5c and d) samples. When digitonin had not been added to the microsomes, the Ca^{2+}-transport activity and 5′-nucleotidase were concentrated in the low-density part (1.08–1.15) of the gradient, whereas, after digitonin treatment, they were markedly shifted towards higher densities (1.11–1.19).

Fig. 6 shows that the distribution patterns of Mg^{2+}-ATPase and of protein were also profoundly affected after addition of digitonin, whereas those of NADH-cytochrome c reductase and of sulfatase C were hardly modified. In the absence of digitonin, the two latter enzymes were characterized by higher equilibrium densities (peak at 1.14–1.15) than those of the other enzymes and of Ca^{2+}-uptake activities (peak at 1.11–1.12).

Discussion

In density-gradient centrifugation experiments, the behavior of the vesicles that took up ^{45}Ca and

responded to calmodulin mimicked that of the plasmalemmal enzymes 5′-nucleotidase and Mg^{2+}-ATPase [6,8,19,30], but differed markedly from that of NADH-cytochrome *c* reductase and sulfatase C. The latter enzyme is located in the endoplasmic reticulum in rat liver and in mouse peritoneal macrophages [22]. As in other tissues, the rotenone-insensitive NADH-cytochrome *c* reductase could be associated with both the endoplasmic reticulum and the outer mitochondrial membrane in arteries [6,8,19,30]. Participation of residual mitochondria in ^{45}Ca uptake in our microsomal fractions may be ruled out because (i) ^{45}Ca uptake was insensitive to azide, an inhibitor of Ca^{2+} transport by mitochondria [32], and (ii) mitochondria equilibrate at rather high densities in sucrose gradient and are not shifted by digitonin [19]. Thus, our results indicate clearly that the calmodulin-stimulated Ca^{2+} pump is located in plasmalemmal elements. Interestingly, plasma membrane elements seem to be a major component of our microsomal fraction, as suggested by the similarity between the distribution patterns of protein and those of 5′-nucleotidase and Mg^{2+}-ATPase.

In membrane fractions from rat mesenteric arteries the ATP-dependent Ca^{2+} accumulation has also been reported to be closely associated with plasma membrane marker enzymes [8]. In contrast, in their study on rat aorta microsomes, Moore et al. [5] found that the ^{45}Ca-uptake activity was distributed in density gradients like NADH oxidase, rather than 5′-nucleotidase, and they concluded that the ^{45}Ca was taken up by endoplasmic reticulum elements. The discrepancy between our findings and those of Moore et al. [5] could be linked to the use of oxalate by these authors to enhance Ca^{2+} accumulation. Indeed, Wuytack et al. [6] have shown in microsomes from pig coronary arteries that the density distribution of ^{45}Ca-uptake activity, measured in the absence of oxalate, resembles that of 5′-nucleotidase and adenylate cyclase, but differs from that of NADH-cytochrome *c* reductase, whereas the reverse occurs when the ^{45}Ca uptake is measured in the presence of oxalate. A plausible interpretation of these results is that stimulation of Ca^{2+} transport by oxalate is much more pronounced at the level of the endoplasmic reticulum elements [6]. In accordance with this view, it has been reported that oxalate does not affect ^{45}Ca accumulation by plasma membrane-rich fractions from rat aorta or mesenteric arteries [7,8]. The differences in the sensitivity to pH and to monovalent cations between the oxalate-stimulated and basal Ca^{2+} transports in smooth muscle microsomes from guinea-pig ileum also suggested that these two processes might be associated with distinct subcellular entities [21].

Our finding of a sizeable stimulation of ^{45}Ca uptake into rat aorta microsomes by calmodulin is at variance with the rather weak effect reported recently by Wuytack et al. [17] in pig coronary microsomes. This discrepancy might suggest that the two microsomal fractions are largely different with respect to their origin in the cell, since up to now the sensitivity to calmodulin seems to be restricted to plasmalemmal Ca^{2+} pumps [10,14,15]. Alternatively, it could be that in some smooth muscles the plasmalemmal Ca^{2+} pump is less sensitive to calmodulin, or contains more tightly bound endogenous calmodulin. We have recently found (N. Morel, M. Wibo and T. Godfraind, unpublished observations) that EGTA-treated microsomes from the longitudinal muscle of guinea-pig ileum possess a plasmalemmal Ca^{2+} pump that is distinctly less stimulated by calmodulin than its counterpart in rat aorta microsomes.

In intact rat aorta, the rate of Ca^{2+} extrusion from the cells has been estimated to vary from about 10 nmol/min per g wet wt. in resting tissue to 50 nmol/min per g in the presence of 10^{-5} M noradrenalin [33]. When EGTA-treated microsomes were incubated with 10 μM free Ca^{2+} in the presence of calmodulin, the initial rate of ^{45}Ca uptake was of the order of 1–1.5 nmol/min per mg protein (Fig. 1), i.e., 3–5 nmol/min per g wet wt. As the recovery of plasma membranes in the microsomal fraction is about 30% [19], the activity of the plasmalemmal Ca^{2+} pump in the whole homogenate may be estimated to be 10–15 nmol/min per g tissue. As the ATP-dependent Ca^{2+} accumulation is presumably associated with inside-out vesicles, the proportion of which is unknown, but might plausibly be around 30%, the activity of the calmodulin-stimulated Ca^{2+} pump described here may easily account for the rates of Ca^{2+} extrusion from the cytoplasm which have been observed in intact tissue.

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