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DIFFERENTIATION OF Ca^{2+} PUMPS LINKED TO PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM IN THE MICROSOMAL FRACTION FROM INTESTINAL SMOOTH MUSCLE

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ATP promotes ^{45}Ca uptake by the microsomal fraction from the longitudinal smooth muscle of guinea-pig ileum and this uptake is stimulated by oxalate. As the microsomal fraction is made up of various subcellular entities, we examined the localization of the Ca^{2+}-transport activity by density gradient centrifugation, taking advantage of the selective effect of digitonin (at low concentration) on the density of plasmalemmal elements. When the ^{45}Ca-uptake activity was measured in the absence of oxalate, its behavior in subfractionation experiments closely paralleled that of the plasmalemmal marker 5′-nucleotidase. In contrast, the additional Ca^{2+}-transport activity elicited by oxalate behaved like NADH-cytochrome *c* reductase, a putative endoplasmic reticulum marker. The endoplasmic reticulum vesicles constituted only a small part of the membranes in the microsomal fraction, which explains that their Ca^{2+}-storage capacity was not detectable in the absence of Ca^{2+}-trapping agent. Low digitonin concentrations selectively increased the Ca^{2+} permeability of the plasmalemmal vesicles. The two Ca^{2+}-transport activities were further differentiated by their distinct sensitivities to K^+, vanadate and calmodulin. In this respect, the oxalate-insensitive and oxalate-stimulated Ca^{2+}-transport systems resembled, respectively, the sarcolemmal and sarcoplasmic reticulum Ca^{2+} pumps in cardiac and skeletal muscle, in accordance with the subcellular locations established by density gradient centrifugation.

Introduction

Calcium plays a key role in excitation-contraction coupling in smooth muscle as in other types of muscle [1]. The level of ionized calcium (Ca^{2+}) in the cytosol of the smooth muscle cell is kept very low at rest despite a large electrochemical gradient favoring the entry of this cation from the extracellular milieu. Myofilament activation is mediated by a rise in the cytosolic Ca^{2+} concentration and the excess of Ca^{2+} must be removed from the myoplasm to allow relaxation. It is generally accepted that relaxation is brought about mainly through the operation of a Ca^{2+}-transport ATPase located in the endoplasmic reticulum of the smooth muscle cell, a membrane system that would thus fulfil a role analogous to that of the sarcoplasmic reticulum in cardiac or skeletal muscle [1,2]. However, another Ca^{2+}-pumping mechanism is needed at the level of the plasma membrane to maintain calcium homeostasis in smooth muscle. Indeed, many stimulatory agents act, at least partly, by inducing an influx of extracellular Ca^{2+}. To prevent accumulation of calcium, an energy-requiring Ca^{2+}-extrusion system must thus operate at the plasma membrane. It is likely that Ca^{2+} is pumped out of the smooth muscle cell mainly by a Ca^{2+}-ATPase, rather than via a Na^+-Ca^{2+} exchange driven by the Na^+ pump [2].

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

Ideally, adequate characterization of these Ca^{2+}-transport systems would require isolation of the host membranes in a highly purified state. In view of the difficulties of this undertaking, several groups have studied smooth muscle Ca^{2+} pumps in relatively crude microsomal fractions, relying on analytical density gradient centrifugation to establish the subcellular location of the Ca^{2+}-transport activity [3–7]. In this approach, the microsomal fraction is equilibrated in a density gradient and the distribution of the Ca^{2+}-transport activity is compared to that of putative marker enzymes for various types of membranes. This methodology may be refined by taking advantage of the differential effects of digitonin on cholesterol-rich and cholesterol-poor membranes. The plasma membranes (cholesterol-rich) are shifted by digitonin towards higher equilibrium densities, whereas the membranes from mitochondria or endoplasmic reticulum (cholesterol-poor) are not shifted [8,9]. This technique enabled us to demonstrate that in the microsomal fraction isolated from rat aorta a calmodulin-activated Ca^{2+} pump is associated with plasma membrane elements [7]. In the present work, we have extended this approach to the ATP-driven Ca^{2+}-transport by the microsomal fraction from the longitudinal smooth muscle of guinea-pig ileum. We found that two distinct Ca^{2+} pumps, located respectively in the plasma membrane and in the endoplasmic reticulum, coexist in this preparation, as originally suggested by Godfraind et al. [10]. The plasma membrane Ca^{2+}-transport activity was preferentially measured in the absence of oxalate. Indeed, the bulk of the ^{45}Ca was then sequestered within vesicles that behaved like plasma membrane elements in density gradient experiments. This result might be related to the composition of the microsomal fraction from intestinal smooth muscle, where plasma membrane elements formed the major membrane component. Oxalate, a 'Ca^{2+}-trapping' agent that has long been used to enhance Ca^{2+} storage by sarcoplasmic reticulum vesicles from striated muscle [11], selectively stimulated Ca^{2+} transport into endoplasmic reticulum vesicles in this preparation. The two Ca^{2+}-transport systems were further differentiated on the basis of their sensitivity to monovalent cations [10], vanadate, digitonin and calmodulin. This protein stimulated the plasmalemmal Ca^{2+} transport but had apparently no effect on Ca^{2+} transport into endoplasmic reticulum vesicles. The results presented here have been previously reported in condensed form [12].

Methods

Preparation and subfractionation of the microsomal fraction. Ileum segments were taken from albino guinea-pigs weighing 300–400 g and immersed in Krebs medium (37°C) equilibrated with 95% O_2/5% CO_2. The longitudinal muscle layer was separated as previously described [10] and transferred into ice-cold 0.25 M sucrose buffered at pH 7.4 with 5 mM Tris-HCl (sucrose-Tris). The tissue (1.5–2 g) was minced with scissors and homogenized with 15–20 ml of buffered sucrose in an all-glass Potter-Elvehjem-type grinder (Braun, Melsungen, F.R.G.) kept at 2°C, by four strokes of the pestle rotating at 1500 rev./min. The homogenate was spun at $1000 \times g$ for 5 min in a table-top centrifuge. The pellet was rehomogenized as above with 15 ml of sucrose-Tris and the resulting suspension centrifuged at $1000 \times g$ for 5 min. The supernatants from the low-speed runs were combined and centrifuged at $20\,000 \times g$ for 30 min in a W40/128 rotor of an Omega II Ultracentrifuge (Heraeus-Christ, Osterode am Harz, F.R.G.). The supernatant (including the gelatinous material covering the pellet) was spun at $100\,000 \times g$ for 60 min. The microsomal pellet was usually suspended in 5–10 ml of sucrose-Tris.

For density gradient centrifugation experiments, microsomal pellets were suspended in 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole-HCl. Samples supplemented with a small amount of digitonin and control samples were subfractionated by isopycnic centrifugation as previously described [7,9]. Usually, the digitonin/protein weight ratio was 0.2. (As contamination by contractile protein varied widely among preparations, the amount of digitonin added with respect to membrane material present is best expressed in terms of mg digitonin/mg phospholipid, as it is in Results).

Measurement of ^{45}Ca uptake. Microsomal fractions were kept at 0°C until incubated with ^{45}Ca and were used within 24 h after preparation. Fractions (50–100 μg protein/ml) were incubated at 37°C with 20 mM maleate, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM $^{45}CaCl_2$ (1.6 μCi/ml), 0.317 mM EGTA,

3 mM ATP and 5 mM NaN_3, in the presence or absence of 5 mM oxalate. Maleate, ATP, EGTA and oxalate solutions were adjusted to pH 6.6 (37°C) with Tris. The free Ca^{2+} concentration was 10 μM, as determined previously [10].

^{45}Ca uptake was started by adding ATP with or without oxalate. At the end of incubation, duplicate or triplicate samples (0.6 ml) were filtered on Sartorius filters (SM 11306, 0.45 μM) prewashed with 1 M KCl [10]. The radioactivity retained after washing with sucrose-Tris (0°C, 20 ml) was measured by liquid scintillation [7]. The ATP-dependent ^{45}Ca uptake was obtained by subtracting the radioactivity retained on the filters when the subcellular fraction was incubated without ATP.

Biochemical determinations. Enzymes, protein and phospholipid were measured as previously reported [7,9].

Reagents. Sodium monovanadate was obtained from E. Merck AG. (Darmstadt, F.R.G.) and dissolved by boiling in 5 mM NaOH. The origin of the other reagents has been given previously [7,9].

Results

As shown previously [10], 5 mM oxalate markedly stimulates the ATP-dependent ^{45}Ca uptake by microsomal fractions from the longitudinal muscle of guinea-pig ileum. In the absence of oxalate, ^{45}Ca accumulation slows down after a few minutes and reaches a plateau (20–30 nmol/mg protein) after 20 min. In the presence of oxalate, the rate of Ca^{2+} transport remains high for at least 30 min (4–8 nmol/min per mg protein). Subsequently we shall refer to the oxalate-stimulated Ca^{2+} uptake, which is obtained by subtracting the uptake in the absence of oxalate from the uptake in the presence of oxalate.

Subcellular localization of Ca^{2+}-transport activities

Godfraind et al. [10] reported that the Ca^{2+} transport measured in the absence of oxalate and the oxalate-stimulated Ca^{2+} transport are differently influenced by parameters such as the pH or the type of monovalent cation added to the medium. Moreover, these two Ca^{2+}-uptake activities (which were measured in the presence of azide to inhibit mitochondrial Ca^{2+} transport [13]) are differently distributed between the nuclear, mitochondrial and microsomal fractions, which suggests that they are associated with distinct subcellular entities.

To test this hypothesis, we analyzed microsomal fractions by density equilibration in sucrose-H_2O gradients. As shown in Fig. 1, when referred to the amount of phospholipid as index of membrane material, the capacity to take up ^{45}Ca in the absence of oxalate did not vary much along the density gradient. In contrast, the oxalate-stimulated uptake increased markedly with the density of subfraction. In the lightest subfraction ^{45}Ca uptake was not stimulated by oxalate, whereas in the two subfractions with the greatest densities, the oxalate-stimulated uptake was about 3-fold higher than in the original microsomal fraction.

To determine the subcellular origin of the vesicles accumulating ^{45}Ca, we compared their density distributions to those of 'marker' enzymes: 5'-nucleotidase, located in the plasma membrane [3–10] and the rotenone-insensitive NADH-cytochrome *c* reductase, presumed to be largely associated, as in microsomal fractions from other tissues, with vesicles derived from endoplasmic reticulum [3–9]. In these experiments, part of the microsomal preparation was

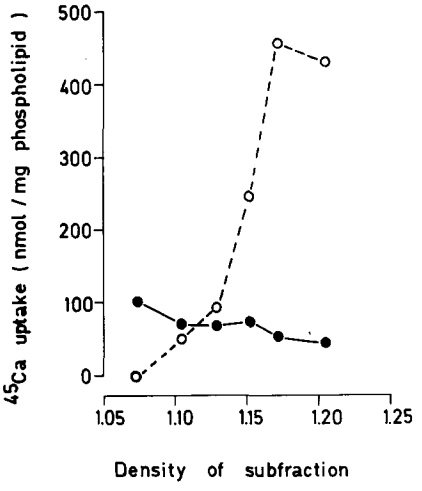

Fig. 1. ATP-dependent ^{45}Ca uptake by microsomal subfractions obtained by density equilibration in a sucrose-H_2O gradient (see Methods). ^{45}Ca uptake (10 min) in the absence of oxalate is indicated by the solid line and oxalate-stimulated uptake by the broken line. ^{45}Ca uptake by the original microsomal fraction amounted to, respectively, 72 nmol/mg phospholipid (without oxalate) and 157 nmol/mg phospholipid (oxalate-stimulated uptake).

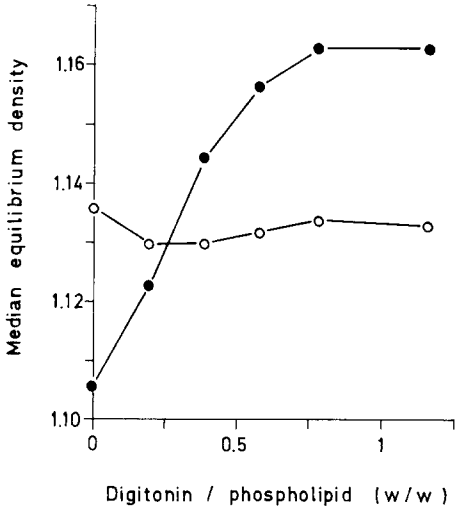

Fig. 2. Influence of digitonin on the median equilibrium density of 5′-nucleotidase (●———●) and NADH-cytochrome *c* reductase (○———○). Increasing amounts of digitonin (0.05 to 0.3 mg/mg protein) were added to microsomal samples (0.21 mg phospholipid/mg protein), which were then subfractionated by isopycnic centrifugation. Each gradient was cut into seven subfractions, from which the density distributions of the two enzymes were determined. Median densities were computed as reported previously [8].

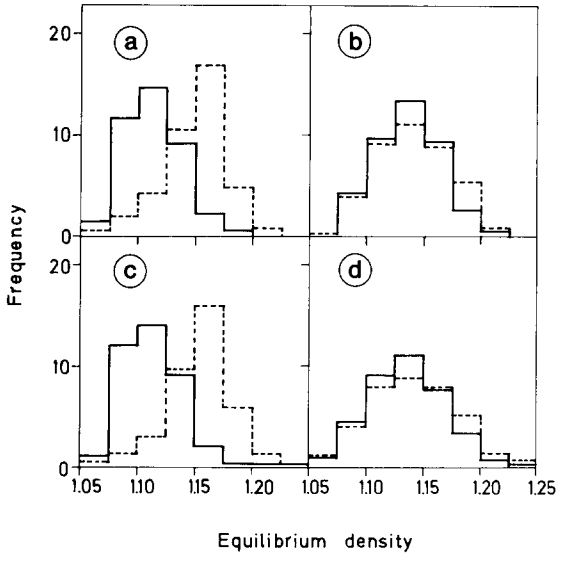

Fig. 3. Density distribution patterns of enzymes and ^{45}Ca-uptake activities in untreated (solid line) and digitonin-treated (broken line) microsomal samples. The amount of digitonin added was 0.2 mg/mg protein, or 0.52 mg/mg phospholipid. After isopycnic centrifugation, eight subfractions were recovered from each gradient and assayed for (a) ^{45}Ca uptake (10 min) in the absence of oxalate, (b) oxalate-stimulated ^{45}Ca uptake, (c) 5′-nucleotidase and (d) NADH-cytochrome *c* reductase. Recoveries of ^{45}Ca uptake or enzyme activities (sum of activities in subfractions expressed as percent of activity in sample layered on gradient) were (%): (a) 89.6 (untreated microsomes) and 85.9 (digitonin-treated microsomes); (b) 76.3 and 85.7; (c) 106.5 and 98.9; (d) 98.3 and 104.8. Density distributions were normalized as described [8]. The frequency is the fraction of activity recovered in a given subfraction, divided by the density increment across this subfraction [8]. Similar results were obtained in two other experiments.

treated with digitonin before density gradient centrifugation. As illustrated in Fig. 2, this treatment sharply distinguished the plasmalemmal elements (5′-nucleotidase), whose density was increased by digitonin in a dose-dependent manner, from the membrane entities bearing NADH-cytochrome *c* reductase, which retained the same equilibrium density after addition of digitonin. The density shift of 5′-nucleotidase reached a plateau (approx. 0.05 density units) at 0.7–0.8 mg digitonin per mg phospholipid, probably because the accessible cholesterol of the plasma membrane elements had then completely reacted with digitonin.

Fig. 3 compares the density distributions of the two enzymes and of the ^{45}Ca-uptake activities in a control microsomal preparation and in a preparation supplemented with 0.5 mg digitonin per mg phospholipid. In the untreated sample (solid line), the distribution profile of the oxalate-stimulated Ca^{2+}-transport activity peaked at higher densities than the profile of the transport activity measured in the absence of oxalate, but the two distribution profiles largely overlapped each other. The difference in the response to digitonin (broken line) was much more clear-cut, the Ca^{2+}-transport activity without oxalate being shifted towards markedly higher densities, while the oxalate-stimulated transport activity did not move. With both microsomal preparations, the ^{45}Ca-uptake activity measured in the absence of oxalate was distributed in the gradient almost exactly like 5′-nucleotidase, and thus showed a typical plasmalemmal behavior, whereas the oxalate-stimulated ^{45}Ca-uptake activity closely followed NADH-cytochrome *c* reductase, and thus behaved in a manner

that was clearly suggestive of an endoplasmic reticulum origin.

We found also that the density distribution of phospholipid was very similar to that of 5′-nucleotidase, in both untreated (Fig. 4a) and digitonin-treated (Fig. 4b) preparations. Obviously, plasma membrane elements represented the major membrane component in our microsomal fractions. Therefore, it is not surprising that the ^{45}Ca uptake in the absence of oxalate did not vary appreciably among microsomal subfractions, when referred to their phospholipid content (see Fig. 1).

Treatment with digitonin not only influenced the equilibrium density of the vesicles that took up ^{45}Ca in the absence of oxalate, but also depressed the level of ^{45}Ca accumulation by these vesicles. As shown in Fig. 5, the inhibition increased as a function of the digitonin/phospholipid ratio. In contrast, the oxalate-stimulated ^{45}Ca-uptake was remarkably insensitive to digitonin over the range of concentrations tested.

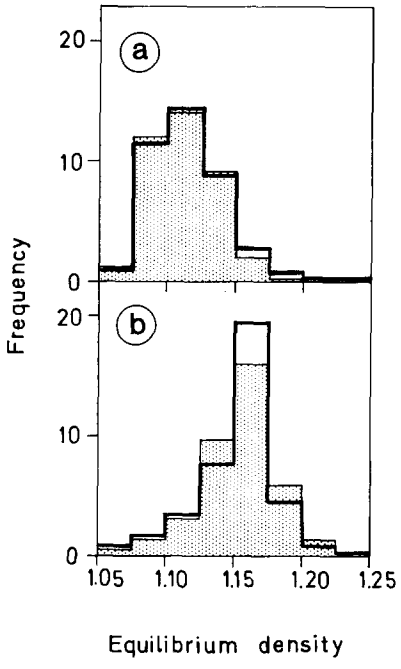

Fig. 4. Density distribution profiles of phospholipid in untreated (a) and digitonin-treated (b) microsomal samples. Same experimental conditions as in Fig. 3. The recoveries of phospholipid were 94.2% (a) and 108.2% (b). The shaded histograms give the distribution profiles of 5′-nucleotidase.

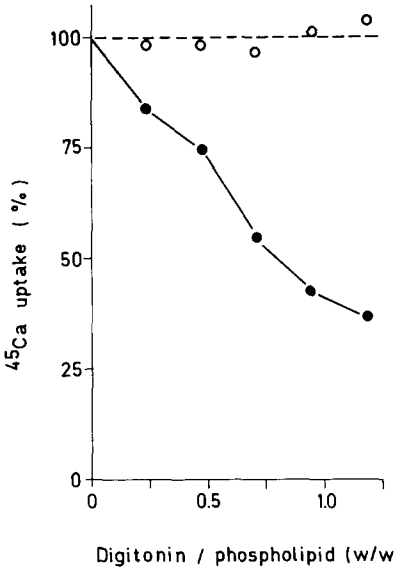

Fig. 5. Effect of digitonin on ATP-dependent ^{45}Ca uptake. Microsomal suspensions were diluted twice with digitonin solutions (in 0.25 M sucrose–3 mM imidazole) to yield the indicated digitonin/phospholipid ratios. Digitonin concentrations in the ^{45}Ca incubation medium ranged from 0 to 7.5 μg/ml. The solid line refers to ^{45}Ca uptake (10 min) in the absence of oxalate and the broken line to the oxalate-stimulated ^{45}Ca uptake. Results are expressed as percent of uptake in samples incubated without digitonin.

Differential sensitivity of the endoplasmic reticulum and plasma membrane Ca^{2+} pumps to various agents

As pointed out recently by Caroni and Carafoli [14], in heart tissue the plasmalemmal and sarcoplasmic reticulum Ca^{2+} pumps can be differentiated according to a variety of criteria, in particular the sensitivity to K^+, vanadate and calmodulin. We have used a similar approach to further distinguish the two Ca^{2+}-transport systems present in microsomal fractions from intestinal smooth muscle.

The oxalate-stimulated Ca^{2+}-transport is considerably reduced when K^+ is replaced by choline$^+$ in the incubation medium, whereas this substitution does not affect the Ca^{2+} accumulation determined in the absence of oxalate [10]. In the experiment shown in Fig. 6, the K^+ concentration was varied while the ionic strength was kept constant with choline$^+$. Half-maximal oxalate-stimulated uptake was observed at about 35 mM K^+, as calculated from Lineweaver-Burk plots (not shown). Substitution of 100 mM Na^+

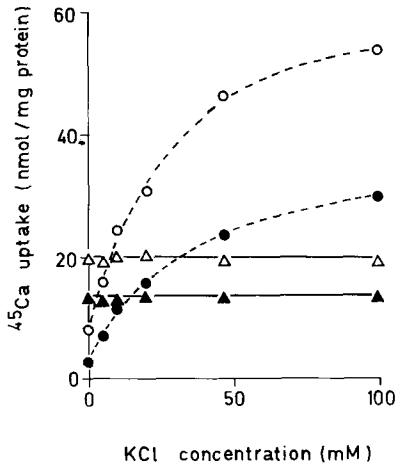

Fig. 6. Influence of K^+ concentration on ATP-dependent ^{45}Ca uptake. Solid lines indicate ^{45}Ca uptake in the absence of oxalate and broken lines oxalate-stimulated ^{45}Ca uptake, after incubation for 10 min (closed symbols) or 20 min (open symbols). When K^+ concentration was less than 100 mM, ionic strength was kept constant by replacing K^+ by choline$^+$. NaN_3 was omitted from the incubation medium in this experiment. Similar results were obtained with another microsomal preparation.

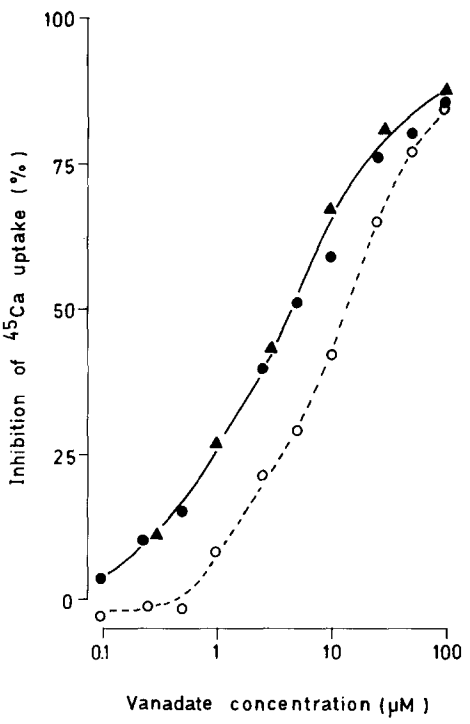

Fig. 7. Effect of vanadate on ATP-dependent ^{45}Ca uptake. Microsomal fractions were preincubated for 15 min at 37°C in the medium described in Methods supplemented with vanadate at various concentrations, and further incubated for 10 min after addition of ATP. The solid line refers to ^{45}Ca uptake in the absence of oxalate and the broken line to oxalate-stimulated ^{45}Ca uptake. Inhibition by vanadate is expressed as percent of the uptake measured in the absence of this compound. Circles and triangles refer to different microsomal preparations.

(instead of choline$^+$) for 100 mM K^+ had little effect [10]. These properties are compatible with the presumed subcellular locations of the two Ca^{2+}-transport systems. The Ca^{2+} pump in heart [15] and skeletal muscle [16,17] sarcoplasmic reticulum is also highly dependent on monovalent cations such as K^+ or Na^+, whereas plasmalemmal Ca^{2+} pumps seem less influenced by these cations [14].

Vanadate, a good inhibitor of Ca^{2+}-transport ATPases [14,18,19], proved to be more potent, in our preparation, on the ^{45}Ca-uptake activity measured in the absence of oxalate (Fig. 7). Half-maximal inhibition (I_{50}) of this activity was observed at 4 μM vanadate, as compared with 12 μM for the oxalate-stimulated transport. This order of potency is compatible with the proposed locations of the two Ca^{2+}-transport systems, since, as reported by others, under conditions optimal for inhibition, the I_{50} values are 0.6–1.5 μM and 5 μM for, respectively, plasmalemmal Ca^{2+} pumps [14,18] and sarcoplasmic reticulum Ca^{2+}-transport ATPase [19]. It seems unlikely that in our system the presence or absence of oxalate could by itself account for the difference in sensitivity to vanadate. Indeed, in sarcoplasmic reticulum preparations the inhibitory effect of vanadate is independent of the presence of oxalate when determined under conditions similar to those we have used (i.e. after preincubation of the membranes with vanadate and 100 mM K^+) [19].

We have previously shown that in rat aorta the plasmalemmal Ca^{2+} pump may be activated 2.5-fold by calmodulin after treatment of the microsomal fraction with EGTA [7]. This treatment presumably removes membrane-bound endogenous calmodulin. In the microsomal fraction from intestinal smooth muscle (Table I), the plasmalemmal (oxalate-insensitive) Ca^{2+} uptake was reduced by about 20% after

TABLE I

EFFECT OF EGTA-TREATMENT AND OF CALMODULIN ON ATP-DEPENDENT ^{45}Ca UPTAKE

The microsomal pellet (see Methods) was suspended in 30 ml of sucrose-Tris buffer containing 0.2 mM dithioerythritol and 1 mM EGTA, or in the same solution without EGTA. After centrifugation at 100 000 × *g* for 60 min, the pellets were resuspended in sucrose-Tris. EGTA-treated and control samples were incubated for 10 min at 37°C in the ^{45}Ca-containing medium (without ATP), in the absence or presence of calmodulin (6 μg/ml). ^{45}Ca uptake was started by addition of ATP with or without oxalate and was stopped by filtration after 10 min. Results are means ± S.E. from three determinations.

EGTA treatment	Calmodulin	^{45}Ca uptake (nmol/mg protein)		
		Without oxalate	With oxalate	Oxalate-stimulated
–	–	17.7 ± 0.36	49.9 ± 0.31	32.2 ± 0.40
–	+	21.8 ± 0.12 [a]	52.5 ± 0.75	30.7 ± 0.76
+	–	14.6 ± 0.16 [b]	25.6 ± 0.32	11.0 ± 0.36
+	+	19.4 ± 0.40 [a]	31.1 ± 0.51	11.7 ± 0.65

[a] Effects of calmodulin significant at $P < 0.01$ (Student's *t*-test).
[b] Effect of EGTA-treatment significant at $P < 0.01$).

EGTA-treatment. Calmodulin enhanced by 30–35% Ca^{2+} accumulation in the absence of oxalate after such a treatment. Stimulation by calmodulin was almost maximal at 3 μg/ml and was little influenced by incubation time or free Ca^{2+} concentration (data not shown). The rather weak effect of calmodulin on the plasmalemmal Ca^{2+} transport in this preparation might be due to the persistence of tightly bound endogenous calmodulin despite the EGTA treatment. It has been shown recently that a similar treatment does not effectively remove endogenous calmodulin from heart sarcolemmal membranes [14].

The endoplasmic reticulum (oxalate-stimulated) Ca^{2+} uptake was much more sensitive to the EGTA-treatment, but the addition of calmodulin did not evoke any stimulation of Ca^{2+} transport. The effect of EGTA is consistent with earlier reports indicating that the sarcoplasmic reticulum Ca^{2+}-transport ATPase is inactivated irreversibly and time-dependently upon removal of bound Ca^{2+} [20,21]. A slight stimulation of Ca^{2+} transport by calmodulin in heart sarcoplasmic reticulum preparations has been reported [22,23]. This effect is probably mediated by a calmodulin-dependent phospholamban kinase [22].

Differential release of ^{45}Ca from endoplasmic reticulum and plasma membrane vesicles after ATP-dependent loading

In the experiment shown in Fig. 8, microsomal samples loaded with ^{45}Ca were further incubated after addition of the Ca^{2+} ionophore A23187 (5 μM), or of 5 mM EGTA. The ionophore induced a very rapid (less than 2 min) release of ^{45}Ca from microsomes loaded in the absence of oxalate and a slower ($t_{1/2} \simeq 5$ min) release of the oxalate-stimulated ^{45}Ca

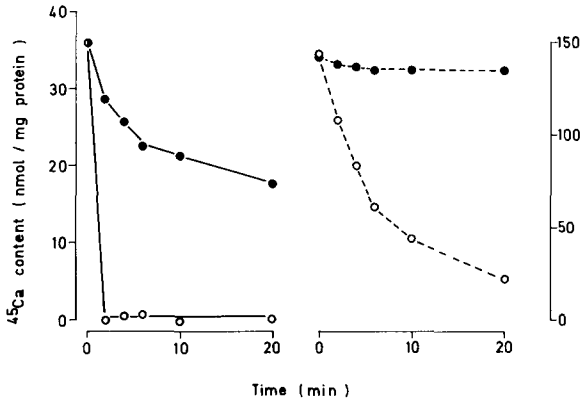

Fig. 8. Time-course of ^{45}Ca release induced by EGTA (•) or A23187 (○). Microsomal fractions were loaded with ^{45}Ca in the absence or presence of oxalate in a final volume of 8 ml. After filtration of duplicate 0.6 ml samples, 0.7 ml of 50 mM EGTA or 50 μM A23187 were immediately added (zero time). Duplicate samples were then filtered at various time intervals. The ^{45}Ca content after loading in the absence of oxalate is indicated by solid lines, and the oxalate-stimulated ^{45}Ca content by broken lines. Similar results were obtained in a second experiment.

content. The slower discharge of the extra ^{45}Ca accumulated in the presence of oxalate might reflect the time needed for the dissolution of calcium oxalate crystals, which have indeed been observed within microsomal vesicles from stomach smooth muscle [24]. Anyhow the effect of A23187 supports the view that ATP promotes the transport of Ca^{2+} into the internal space of the vesicles against a concentration gradient.

Addition of 5 mM EGTA lowered the free Ca^{2+} concentration to a level at which further ^{45}Ca uptake was almost abolished [10]. In this situation (Fig. 8), no ^{45}Ca was discharged from the vesicles into which Ca^{2+} loading had been promoted by oxalate. These vesicles appear to be impermeable to Ca^{2+} when incubated in a medium containing a very low Ca^{2+} concentration. This behavior is reminiscent of that of sarcoplasmic reticulum vesicles from striated muscle [25,26]. In contrast, the ^{45}Ca taken up in the absence of oxalate fell to half of its initial value 20 min after addition of EGTA.

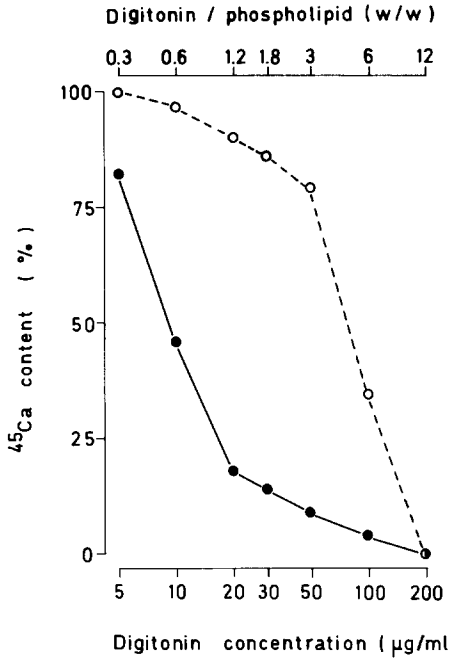

Fig. 9. Effect of digitonin on ^{45}Ca release. Microsomal fractions loaded with ^{45}Ca for 15 min in the absence or presence of oxalate were further incubated for 15 min after addition of 5 mM EGTA and of digitonin at the indicated final concentrations. Results are expressed as percent of ^{45}Ca content after addition of 5 mM EGTA without digitonin. The ATP-dependent ^{45}Ca content after loading in the absence of oxalate is indicated by the solid line and the oxalate-stimulated ^{45}Ca content by the broken line.

As shown above (Fig. 5), in the absence of oxalate, digitonin-treated microsomal fractions accumulated less ^{45}Ca than control samples. As this lower level of uptake could be caused by a higher Ca^{2+} permeability of digitonin-treated plasmalemmal vesicles, we investigated the effect of digitonin on the Ca^{2+} efflux induced by the addition of 5 mM EGTA. Fig. 9 shows that, indeed, low concentrations of digitonin specifically enhanced ^{45}Ca efflux from the vesicles loaded in the absence of oxalate. When digitonin was omitted, EGTA induced the release of 35% of this ^{45}Ca after 15 min, in fair agreement with the data shown in Fig. 8. In the presence of 20 μg digitonin/ml the release of this ^{45}Ca amounted to 90%. Under the same conditions, 90% of the additional ^{45}Ca taken up in the presence of oxalate remained associated with the microsomal fraction. Appreciable release of this pool of ^{45}Ca occurred only at digitonin concentrations higher than 50 μg/ml. The selective effects of digitonin on the Ca^{2+} permeability and on the equilibrium density of plasmalemmal vesicles were observed at similar digitonin/phospholipid ratios and are probably both attributable to the binding of digitonin to the plasmalemmal cholesterol.

Discussion

In the absence of oxalate, most of the ^{45}Ca accumulated in an ATP-dependent manner by the microsomal fraction from intestinal smooth muscle was associated with vesicles originating from the plasma membrane. This situation reflects the composition of our microsomal fraction, in which plasma membranes were the predominant membrane component. In the absence of oxalate, ^{45}Ca sequestration by the outnumbered endoplasmic reticulum vesicles was not detectable. Oxalate specifically enhanced the storage capacity of endoplasmic reticulum vesicles, which then exceeded that of the plasmalemmal elements.

Our results clearly indicate that the presence or absence of a Ca^{2+}-trapping agent is a key factor in studies devoted to Ca^{2+}-transport systems in subcellular fractions from smooth muscle (see also Refs. 5 and 27), and probably from some other tissues as

well. Failure to take this factor into consideration may account for the conflicting conclusions reached previously as regards the type of vesicle that accumulates ^{45}Ca in microsomal fractions isolated from the longitudinal muscle of guinea-pig ileum. Hurwitz et al. [3] reported that the Ca^{2+}-transport activity, measured in the presence of oxalate, was located in plasma membrane elements, which seems at variance with our own results. However, the incubation conditions of these authors (short incubation time, pH 7.4) were not suitable to obtain an appreciable stimulatory effect of oxalate (see also Ref. 10). More recently, Chaturvedi et al. [6] examined the submicrosomal distribution of the ^{45}Ca-uptake activity under conditions of strong oxalate-stimulation and they concluded that this activity was associated with endoplasmic reticulum elements, in fair agreement with our data. However, these authors failed to detect the plasmalemmal Ca^{2+} transport, probably because they did not investigate ^{45}Ca-uptake activity in the absence of oxalate.

The apparent lack of oxalate effect on Ca^{2+} accumulation by our plasmalemmal vesicles is in accordance with earlier observations on plasma-membrane-rich fractions from smooth muscle [28], as well as from cardiac [14] or skeletal muscle [29]. Membrane vesicles from red cells seem to be an exception in this respect [30]. The absence of oxalate effect on smooth-muscle plasma membrane vesicles might be the consequence of their low permeability to oxalate [31]. On the other hand, it is well known that oxalate diffuses into sarcoplasmic reticulum vesicles from skeletal or cardiac muscle and increases up to a 100-fold the calcium-storage capacity of these vesicles [11]. Stimulation by oxalate of Ca^{2+} accumulation by our endoplasmic reticulum vesicles seems of the same order of magnitude. Indeed, if 5% of the ^{45}Ca taken up in the absence of oxalate is located in these vesicles, their oxalate-independent ^{45}Ca content would amount to 1–2 nmol/mg microsomal protein, as compared with 80–150 nmol for the oxalate-stimulated content (after 30 min incubation). In view of this high degree of stimulation by oxalate of the Ca^{2+} transport associated with endoplasmic reticulum vesicles, even a tiny contamination of purified plasma membrane fractions by such vesicles may result in a significant enhancement of ^{45}Ca uptake by those fractions in the presence of oxalate. For this reason, the recent contention that oxalate activates Ca^{2+}-transport by myometrium plasma membranes appears questionable [32].

Under our experimental conditions, the ^{45}Ca uptake activity associated with plasmalemmal vesicles from intestinal smooth muscle does not reflect, even partly, the combined activities of a $(Na^+ + K^+)$-ATPase and of a Na^+-Ca^{2+} exchange system. Indeed, the ATP-dependent ^{45}Ca uptake was not influenced when sodium ions were completely omitted from the incubation medium, or when the Na^+ pump was blocked with 50 μM digitoxigenin (data not shown). A $(Ca^{2+} + Mg^{2+})$-ATPase activity is demonstrable in the microsomal fraction from intestinal smooth muscle [10], but this activity is low with respect to the basal Mg^{2+}-ATPase activity, which considerably complicates the study of this facet of Ca^{2+}-transporting systems in our preparations. Anyhow, the properties of the ATP-driven Ca^{2+}-transport activity in plasma membranes from intestinal or vascular [7] smooth muscle are clearly suggestive of a close kinship with more extensively characterized plasmalemmal Ca^{2+}-transport ATPases in other tissues. It remains to be demonstrated, however, that this Ca^{2+}-transport system is linked to inside-out plasmalemmal elements and is thus the genuine equivalent of the plasma membrane Ca^{2+}-extrusion ATPase postulated to exist in smooth muscle cells.

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