The effect of calmodulin on the active calcium-ion transport and $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase in microsomal fractions of smooth muscle compared with that in erythrocytes and cardiac muscle

Frank WUYTACK, Greet DE SCHUTTER and Rik CASTEELS
Laboratorium voor Fysiologie, Universiteit Leuven, B-3000 Leuven, Belgium

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The Ca^{2+} uptake and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of the porcine coronary-artery smooth-muscle microsomal fraction ('microsomes') are only slightly stimulated by calmodulin. The Ca^{2+} uptake after 2 min in the absence of oxalate, corrected for the ATP-independent binding, increased by a factor of 1.44, whereas the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase is stimulated 1.39 times. These findings contrast with the effect observed in human erythrocyte 'inside-out' microsomes. In these vesicles calmodulin increases the Ca^{2+} uptake after 20 min in an oxalate-free medium and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase respectively by a factor of 3.82 and 6.18. The magnitude of the calmodulin stimulation of the Ca^{2+} transport in coronary-artery microsomes is similar to that observed in heart microsomes.

The stimulation by calmodulin of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase (Bond & Clough, 1973; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1978; Lynch & Cheung, 1979) and of the Ca^{2+} transport (Macintyre & Green, 1978; Larsen & Vincenzi, 1979) in microsomal preparations obtained from human erythrocytes is well documented. More recently also a stimulation of the Ca^{2+} transport in cardiac microsomal fractions has been reported (Katz & Remtulla, 1978). Because smooth muscle is a rich source of calmodulin (Grand *et al.*, 1979), we have investigated whether calmodulin also stimulates the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase and the Ca^{2+} transport in smooth-muscle microsomal fraction ('microsomes') and compared this action with that obtained in the microsomes of human erythrocytes and canine heart.

Materials and methods

Smooth-muscle microsomes were prepared from the intima-media layers of the large extramural branches of the porcine coronary artery (Wuytack *et al.*, 1978). In this tissue the only cell types present are smooth-muscle cells and a small number of endothelial cells. Microsomes from 'ghosts' of human erythrocytes were prepared by the method of Steck & Kant (1974). The centrifugation on the dextran barrier was omitted and the 'sideness' of the vesicles was checked by measuring the

acetylcholinesterase latency as described by the same authors. Of the vesicles 30% were found to be 'inside-out'. Microsomes from dog ventricular myocardium were prepared by the method of Katz & Remtulla (1978).

The Ca^{2+} uptake and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase activity of the microsomes were measured in similar solutions in parallel experiments as described previously (Wuytack & Casteels, 1980). The ionized Ca^{2+} concentration in these solutions was kept at $10\mu\text{M}$ by addition of 1mM -EGTA and the free $[\text{Mg}^{2+}]$ was 1mM . These experimental procedures were chosen because it was shown previously that under these conditions the Ca^{2+} transport reached its V_{max} value (Wuytack & Casteels, 1980). The Ca^{2+} uptake by the microsomes was measured with the Millipore-filtration method and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase activity was measured spectrophotometrically by using a coupled enzyme system in which ATP hydrolysis is coupled to NADH oxidation (Barnett, 1970).

The calmodulin used in these experiments was either obtained from bovine brain by the organic solvent method of Grand *et al.* (1979) and was kindly supplied by Dr. R. J. A. Grand, or from canine or bovine brain by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography (Sharma & Wang, 1979). Each of these three calmodulin preparations increased the activity of the calmodulin-dependent phosphodiesterase prepared from bovine heart and

of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of the erythrocyte microsomes.

Results and discussion

The components of the ATPase activity measured in the microsomes obtained from coronary artery and from human erythrocytes are given in Table 1. This Table also gives the effect of a supramaximal concentration of calmodulin on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase. The concentration of calmodulin used in all the experiments ($10\mu\text{g/ml}$) is supramaximal, because maximal stimulation of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of erythrocyte microsomes was already reached at $1\mu\text{g}$ of calmodulin/ml. Also in the control test of calmodulin on the modulator-dependent phosphodiesterase prepared from bovine heart (Sharma & Wang, 1979) full Ca^{2+} -dependent activation was reached at $1\mu\text{g/ml}$. This high concentration of calmodulin was deliberately chosen to saturate modulator-binding proteins that might be present in our microsomal fraction and that could neutralize calmodulin if added at lower concentration (Grand & Perry, 1979). It is clear from Table 1 that the stimulating effect of calmodulin on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of erythrocyte microsomes is much larger than on that of the coronary-artery microsomes.

The effect of calmodulin on the Ca^{2+} uptake by coronary-artery microsomes in the presence of 5 mM-oxalate and in the absence of oxalate and on the ATP-independent 'binding' is represented in Fig. 1. The results depicted in Fig. 1 were obtained in 15 paired experiments. The stimulatory effect of calmodulin on the Ca^{2+} uptake is small. A paired t test shows, however, that the stimulation by calmodulin is significant ($P < 0.01$, single-sided) for the 2 min and 10 min points of the Ca^{2+} uptake measured in the absence of oxalate. Although in the presence of calmodulin the plateau for Ca^{2+} uptake reached after 20–30 min tends to be higher compared with the controls, it is not significantly higher. In the presence of oxalate the Ca^{2+} uptake is significantly higher after 2 min ($P < 0.05$, single-sided), but not after longer times. It is important to point out the effect of calmodulin on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase was always studied within 3–6 min after addition of Ca^{2+} or calmodulin and in the absence of oxalate. Oxalate interferes with the pyruvate kinase in the coupled enzyme system used for the ATPase assay. The effect of calmodulin on the Ca^{2+} uptake by the erythrocyte microsomes is quite different (Fig. 2). It increases the plateau of Ca^{2+} uptake in an oxalate-free medium and the rate of Ca^{2+} uptake in an oxalate-containing medium. Also the ATP-independent 'binding' of the Ca^{2+} to the erythrocyte microsomes is augmented by calmodulin.

We have considered the possibility that the

Fig. 1. $^{45}\text{Ca}^{2+}$ uptake by the microsome fraction of the porcine coronary-artery smooth muscle

The $^{45}\text{Ca}^{2+}$ uptake was measured at 37°C by means of the Millipore-filtration method as described previously (Wuytack & Casteels, 1980). The uptake medium was, except for the omission of pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH, the same as the medium used for measuring the ATPase activity. The uptake was started by the addition of $0.87\text{ mM-}^{45}\text{CaCl}_2$ resulting in $[\text{Ca}^{2+}] = 10\mu\text{M}$. Calmodulin was added at a concentration of $10\mu\text{g/ml}$ approx. 1 min before addition of $^{45}\text{CaCl}_2$. When ATP was omitted, the free $[\text{Mg}^{2+}]$ was kept at 1.0 mM by adding 1.0 mM-MgCl_2 instead of 5.7 mM . The values are means for 15 paired experiments and the bars indicate $\pm 1\text{ S.E.M.}$ Filled symbols represent the controls and the open symbols show the values obtained in the presence of $10\mu\text{g}$ of bovine brain calmodulin/ml. Triangles represent the uptake in the presence of 5 mM-oxalate, circles the uptake in the absence of oxalate, squares the uptake in the absence of ATP. The calmodulin stimulation of the Ca^{2+} uptake was only statistically significant for the 2 min and the 10 min values of the uptake measured in the absence of oxalate ($P < 0.01$, single-sided paired t test). After 2 min the control uptake amounted to 27.48 ± 2.60 , whereas in the presence of calmodulin a value of $34.75 \pm 3.19\text{ nmol of Ca}^{2+}/\text{mg of protein}$ was reached; after 10 min the control was 35.18 ± 3.06 and in the presence of calmodulin $41.76 \pm 3.65\text{ nmol of Ca}^{2+}/\text{mg of protein}$. The Ca^{2+} uptake measured in the presence of oxalate was only significantly different for the 2 min period ($P < 0.05$ single-sided paired t test). The control was $34.30 \pm 2.92\text{ nmol of Ca}^{2+}/\text{mg}$ and in the presence of calmodulin increased to $43.05 \pm 5.35\text{ nmol of Ca}^{2+}/\text{mg}$.

Table 1. *ATPase components in the microsomal fractions*

The values represent means $\pm$ 1 S.E.M. and the numbers of observations are given in parentheses. The ATPase was measured at 37°C by following the decrease in A_{340} of a 1 ml solution containing (mM): KCl, 100; imidazole/HCl (pH 6.8), 30; MgCl_2 , 5.7; disodium ATP, 5.0; NaN_3 , 5.0; EGTA, 1.0; phosphoenolpyruvate, 1.5; NADH, 0.26; pyruvate kinase, 40 units/ml; lactate dehydrogenase, 30 units/ml; microsomal protein, 50–100 $\mu\text{g}/\text{ml}$. ($\text{Na}^+ + \text{K}^+$)-dependent ATPase was defined as the decrease in ATPase activity measured in this medium after addition of 0.1 mM-ouabain. The remaining activity corresponds to the basal Mg^{2+} -dependent ATPase. ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase is determined from the increase in ATPase activity obtained by adding 0.87 mM- CaCl_2 to the medium (resulting in a $[\text{Ca}^{2+}] = 10 \mu\text{M}$). The effect of addition of bovine brain calmodulin (10 $\mu\text{g}/\text{ml}$) is expressed by the ratio of (d) to (c).

	ATPase (nmol/mg of protein per min)	
	Porcine coronary-artery microsomes	Human erythrocyte microsomes
(a) Basal Mg^{2+} -dependent ATPase	38.41 ± 1.33 (20)	6.12 ± 0.73 (6)
(b) ($\text{Na}^+ + \text{K}^+$)-dependent ATPase	10.16 ± 0.97 (20)	5.31 ± 0.70 (6)
(c) ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase	18.90 ± 1.14 (20)	6.67 ± 0.81 (6)
(d) ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase + calmodulin (10 $\mu\text{g}/\text{ml}$)	22.66 ± 1.75 (20)	41.96 ± 0.33 (6)
($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase in the presence of calmodulin	1.39 ± 0.04 (20)	6.18 ± 0.55 (6)
($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase in the absence of calmodulin

Fig. 2. The $^{45}\text{Ca}^{2+}$ uptake by the microsomal fraction prepared from human erythrocytes by the method of Steck & Kant (1974)

The experimental conditions and the meaning of the symbols are described in the legend to Fig. 1. (a) Control without calmodulin. (b) Data obtained in the presence of 10 μg of calmodulin/ml.

smaller stimulation of the Ca^{2+} transport and of the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase by calmodulin in coronary-artery microsomes compared with erythrocyte microsomes might be due to some factor masking the calmodulin effect in coronary-artery microsomes. We have therefore tried several experi-

mental modifications that might help to demonstrate a larger stimulation by calmodulin of the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase and Ca^{2+} transport in coronary-artery microsomes. However, in none of the following modifications did calmodulin increase the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent

ATPase activity or the Ca^{2+} transport by a factor greater than 2.

The small effect of calmodulin on the Ca^{2+} transport and on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of coronary-artery microsomes could be due to a slow rate of binding of calmodulin or of the Ca^{2+} -calmodulin complex to its binding sites. We have therefore initiated, in one series of experiments, the Ca^{2+} accumulation by adding ATP to the medium after a 30 min preincubation of the microsomes in $10\mu\text{M-Ca}^{2+}$ and $10\mu\text{g}$ of calmodulin/ml. No larger calmodulin effect was obtained. We also have envisaged the possibility that addition of calmodulin has such a small effect on the Ca^{2+} uptake and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase by these microsomes because they already contained an almost saturating amount of endogenous calmodulin. To remove this putative calmodulin from the microsomes, they were exposed for 30 min at 0°C to a solution containing 0.25M -sucrose, 2mM -sodium EDTA (pH 7.4), 1mM -dithiothreitol, centrifuged ($220000g$ for 30 min) and the sediment was resuspended in 0.25mM -sucrose. In a second procedure the microsomes were extracted at 0°C overnight in a large volume (100ml) of a solution of 0.5mM -sodium phosphate buffer, pH 8.5, followed by resuspension in a small volume of the same buffer (Steck & Kant, 1974). These treatments did not increase the stimulation of Ca^{2+} transport or $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase significantly by subsequent addition of calmodulin. Identical negative results have furthermore been obtained with microsomes prepared from tissues that were homogenized in the standard medium but supplied with 1mM -sodium EDTA at pH 6.8.

Because of this large discrepancy between the calmodulin action on coronary-artery microsomes and that on erythrocyte microsomes, it seemed worthwhile to compare these effects with those obtained in canine cardiac microsomes, in which a stimulation of the Ca^{2+} transport by calmodulin has been reported by Katz & Remtulla (1978). This was even more justified because the calmodulin stimulation of the Ca^{2+} transport in cardiac microsomes reported by these authors was also small (<2 times). These microsomes were prepared as described by Katz & Remtulla (1978) and the Ca^{2+} uptakes were measured under the same experimental condition as described by these authors, i.e. in solutions containing $1\mu\text{M-Ca}^{2+}$. The microsomes accumulated Ca^{2+} as represented in Fig. 3 and this uptake was appreciably augmented by adding $1\mu\text{M}$ -cyclic AMP and $50\mu\text{g}$ of protein kinase/ml.

Calmodulin ($10\mu\text{g/ml}$) induced a small increase of the Ca^{2+} uptake in the absence of cyclic AMP + protein kinase, but this stimulation could not be observed in the presence of cyclic AMP + protein kinase. These findings confirm the observation by

Fig. 3. The $^{45}\text{Ca}^{2+}$ uptake by the microsomal fraction prepared from canine cardiac microsomes was performed as described by Katz & Remtulla (1978) at 37°C in a medium containing $1\mu\text{M}$ ionized Ca^{2+} and 5mM -oxalate

Filled symbols represent controls and open symbols the values obtained in the presence of $10\mu\text{g}$ of calmodulin/ml. The uptake in the absence of cyclic AMP and protein kinase is represented by circles, and the uptake in the presence of $1\mu\text{M}$ -cyclic AMP + $50\mu\text{g}$ of bovine heart cyclic AMP-dependent protein kinase/ml (Sigma) is represented by the squares. Results are means $\pm$ S.E.M. for six observations.

Katz & Remtulla (1978) that the effect of calmodulin on the cardiac microsomal Ca^{2+} accumulation is small and at the same time they suggest that there might be a fundamental difference between the action of calmodulin on the Ca^{2+} -transport system of erythrocyte microsomes and that of cardiac- and smooth-muscle microsomes. It is also possible that in the case of coronary-artery microsomes and heart microsomes calmodulin acts only on a small subpopulation of the microsomes, whereas the

remaining fractions of microsomes have either become insensitive to calmodulin during the preparation process or contain a Ca^{2+} transport system that is different from the one in human erythrocytes, may be more resembling the Ca^{2+} -transport system in fragmented sarcoplasmic reticulum of skeletal muscle.

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