Biochimica et Biophysica Acta 819 (1985) 148–152
Elsevier

BBA 70242

BBA Report

Subcellular origin of the oxalate- or inorganic phosphate-stimulated Ca^{2+} transport by smooth muscle microsomes: revisitation of the old problem by a new approach using saponin

Chiu-Yin Kwan

Smooth Muscle Research Program, Department of Neurosciences, McMaster University Health Sciences Centre, 1200 Main Street, West Hamilton, Ontario L8N 3Z5 (Canada)

(Received May 20th, 1985)

Key words: Smooth muscle; Saponin; Ca^{2+}-transport; Plasma membrane; Endoplasmic reticulum

Saponin, a cell-skinning reagent which perforates the cell membrane via its specific interaction with plasmalemmal cholesterol, was used to identify the subcellular origin of ATP-dependent Ca^{2+} accumulation in the presence and absence of inorganic phosphate and oxalate by microsomal fractions isolated from rat vas deferens and dog aorta. The purified plasma membranes from rat gastric fundus muscle, which elicit the stimulation of ATP-dependent Ca^{2+} accumulation by inorganic phosphate but not by oxalate, were used as a control reference. Saponin at concentrations effective for skinning smooth muscle fibres (10–50 μg/ml) inhibited Ca^{2+} binding in the absence of ATP to a similar extent in all fractions, but the inhibition of ATP-dependent Ca^{2+} accumulation was more pronounced in dog aorta microsomes and rat gastric fundus muscle plasma membranes than in rat vas deferens microsomes. The resistance of phosphate- and oxalate-stimulated ATP-dependent Ca^{2+} accumulation to inhibition by saponin was much greater in rat vas deferens than in dog aorta microsomes. Our results suggest that phosphate- and oxalate-stimulated ATP-dependent Ca^{2+} accumulation also occurs in plasma membrane vesicles isolated from smooth muscle and is by no means an unique property of endoplasmic reticulum.

Smooth muscle microsomal membrane vesicles prepared by conventional differential centrifugation have been widely used to study the ATP-driven Ca^{2+}-transport properties [1–4]. It is well recognized that such microsomal membrane fractions are highly heterogeneous, containing plasma membranes, endoplasmic reticulum vesicles and small amounts of mitochondrial fragments, all of which are capable of accumulating Ca^{2+} in the presence of ATP. Although the contribution by mitochondrial membranes to the Ca^{2+} transport can be eliminated by inclusion of azide or Ruthenium red in the assay medium, the subcellular origin of the azide-insensitive ATP-dependent Ca^{2+} transport has long been an issue of debate [2,3,5,6] because of the lack of specific inhibitor to differentiate the plasma membrane Ca^{2+}-pump from the endoplasmic reticulum Ca^{2+}-pump. Since ATP-dependent Ca^{2+}-transport by the sarcoplasmic reticulum vesicles isolated from the striated muscle is greatly stimulated in the presence of oxalate and phosphate ions, it has become habitual to extrapolate this property to the isolated smooth muscle microsomal membranes and attribute this anion-stimulated ATP-dependent Ca^{2+} transport to endoplasmic reticulum. Such an extrapolation requires special caution because: (a) lack of an oxalate stimulation has been reported in some crude smooth muscle microsome preparations [7,8]; (b) no oxalate effect was found in microsomal subfractions enriched in endoplasmic reticulum isolated from rat mesenteric artery, whereas the

plasma-membrane-enriched fraction showed the highest phosphate stimulation of the active Ca^{2+} transport [9]; (c) ATP-dependent Ca^{2+} accumulation in the presence of oxalate and phosphate by some smooth muscle membranes showed different subcellular distributions [11,10]; (d) subcellular distribution of ATP-dependent Ca^{2+} accumulation in the presence of oxalate by human myometrium [12] and dog aortic muscle membranes [13] paralleled that of the plasma membrane marker enzyme activities; (e) pretreatment with digitonin of the smooth muscle microsomes isolated from rat vas deferens and dog aorta prior to sucrose density gradient centrifugation resulted in a shift toward higher density of oxalate-stimulated ATP-dependent Ca^{2+} accumulation (together with that of plasma marker enzyme activities) in dog aortic [13] but not in rat vas deferens preparation [14]. Such a shift toward higher density of plasma-membrane-associated properties is due to the specific interaction of digitonin with the plasmalemmal cholesterol [15]. Recently, we have shown that the ATP-dependent Ca^{2+} accumulation by rat vas deferens microsomes was much less sensitive in the presence than in the absence of oxalate to the inhibition by saponin [16], which perforates the plasma membranes via its interaction with plasmalemmal cholesterol and is widely used as a skinning reagent to destroy selectively the smooth muscle plasma membrane [17–19]. In this report, we describe the effect of this plasma-membrane-specific reagent, saponin, on the Ca^{2+}-binding ATP-dependent CA^{2+} transport in the presence and absence of oxalate and inorganic phosphate anions in three different smooth muscle membrane preparations.

The detailed procedures and characterization of microsomal membranes isolated from rat vas deferens and dog aortic smooth muscle by differential centrifugation have previously been reported [13,20]. Rat gastric fundus smooth muscle plasma membrane was used as an internal reference membrane which is, primarily, purified plasma membrane [21] and does not elicit oxalate stimulated ATP-dependent Ca^{2+} accumulation [22]. Azide-insensitive Ca^{2+} accumulation over a period of 10 min, in the presence and absence of 5 mM ATP, with and without 2 mM oxalate or 20 mM inorganic phosphate, was investigated using the Millipore filtration method, as is routinely used in this laboratory [9,13,16,20,21].

Table I shows the levels of Ca^{2+} accumulation under various conditions similarly employed for three different smooth muscle membrane preparations. In vas deferens microsomes, Ca^{2+} binding in the absence of ATP was the lowest, whereas the stimulation of ATP-dependent Ca^{2+} accumulation by either oxalate or phosphate was the highest. Dog aortic microsomes also showed oxalate and phosphate-stimulated ATP-dependent Ca^{2+} accumulation but with less magnitude compared to vas deferens microsomes. Gastric fundus plasma membranes showed no oxalate-stimulated Ca^{2+} transport as previously observed [22], but were capable of actively accumulating Ca^{2+} in the presence of 20 mM inorganic phosphate, being similar to the plasma membranes isolated from rat mesenteric arteries [9]. Also, the effect of oxalate in the case of vas deferens microsomes was greater than that of phosphate, whereas in dog aortic microsomes, the reverse relationship was observed. It was also reported that in rat myometrial plasma membranes, the effect of phosphate at 25 mM was greater than the effect of oxalate at 5 mM [10]. Therefore, it is clear that different degrees of the stimulation of ATP-dependent Ca^{2+} accumultion by oxalate and phosphate anions exist in different smooth muscle preparations. The oxalate and phosphate anions may act either separately on different submicrosomal membranes or commonly on similar submicrosomal membranes with different efficacies.

Fig. 1 shows that 30–100 μg/ml of saponin, the concentrations effective for skinning of smooth muscle fibre without affecting the endoplasmic reticulum [17–19], substantially inhibited the binding of Ca^{2+} in the absence of ATP. The patterns of inhibition were quite similar for all three smooth muscle membranes, suggesting that a large portion of Ca^{2+} binding in smooth muscle microsomes was of plasma membrane origin. This is consistent with our previous studies using microsomal subfractions isolated from a number of smooth muscles on the sucrose density gradient [9,13,20,21]. A potent inhibition by saponin of ATP-dependent Ca^{2+} accumulation was also observed for these smooth muscle membrane preparations. The pattern of saponin inhibition was similar for both rat fundus plasma membranes and dog aortic micro-

TABLE I

ACCUMULATION OF Ca^{2+} BY MEMBRANE FRACTIONS ISOLATED FROM RAT VAS DEFERENS, DOG AORTA AND RAT GASTRIC FUNDUS MUSCLES

Ca^{2+} accumulation was determined in a final volume of 250 μl imidazole (50 mM) buffered (pH 6.87, 37°C) sucrose (250 mM) solution containing 5 mM ATP, 20 μM free Ca^{2+}, trace amount of $^{45}Ca^{2+}$, 5 mM sodium azide and Mg^{2+} (0.5 mM and 5.0 mM in the absence and presence of ATP, respectively). The reaction was started by the addition of 50 μl membranes containing 10–50 μg protein) and stopped after 10 min by rapid filtration using Millipore HAWP filter (0.45 μm pore size). Ca^{2+} accumulation is expressed in μmol/g per 10 min. Data are expressed as mean ± S.D. from the number of experiments indicated in parentheses.

	Vas deferens microsomes (6)	Dog aorta microsomes (5)	Gastric fundus plasma membranes (4)
ATP-independent Ca^{2+} binding	0.83 ± 0.32	1.48 ± 0.21	1.57 ± 0.32
ATP-dependent Ca^{2+} accumulation			
Basal	20.84 ± 5.52	16.52 ± 2.18	19.70 ± 2.02
2 mM oxalate	163.75 ± 57.81	29.60 ± 3.49	19.24 ± 2.64
20 mM phosphate	76.12 ± 19.57	41.07 ± 10.16	30.40 ± 4.84

somes with an IC_{50} of approx. 5 μg/ml saponin. This finding is very similar to that obtained using inside-out erythrocyte cell membranes [23,24]. For rat vas deferens microsomes, similar inhibition by saponin was observed except for a small portion of saponin-resistant ATP-dependent Ca^{2+} accumulation, which was reflected as a shoulder near 30 μg/ml saponin. This suggests that such a saponin-resistant Ca^{2+} transport is not derived from the cholesterol-enriched plasma membranes of this smooth muscle, and represents only a small portion of the functional ATP-dependent Ca^{2+} transport in the microsomes.

Fig. 2 shows that the effects of saponin on the ATP-dependent Ca^{2+} transport in the presence of 20 mM inorganic phosphate and 2 mM oxalate among these smooth muscle membrane were quite contrasting. In the case of rat fundus plasma membranes, the pattern of inhibition by saponin (10–50 μg/ml) of phosphate-stimulated ATP-dependent Ca^{2+} accumulation, whether corrected for the basal ATP-dependent Ca^{2+} accumulation or not, is very similar to that seen in Fig. 1, with an IC_{50} of 5 μg/ml saponin. The portion of saponin-resistant Ca^{2+} transport increases in dog aortic microsomes and is greatest in rat vas deferens microsomes. Similar patterns of inhibition by saponin of oxalate-stimulated ATP-dependent Ca^{2+} accumulation by rat vas deferens and dog aortic microsomes were also obtained, except that no less than 90% of the saponin-resistant Ca^{2+} transport was observed in rat vas deferens at 10–50 μg/ml saponin and, in contrast, only a very small portion of such a saponin-resistant Ca^{2+} transport was observed in dog aortic microsomes at 30 μg/ml saponin. The IC_{50} of saponin inhibition of Ca^{2+} transport in the presence of oxalate in rat vas deferens was approx. 80 μg/ml, which is comparable to the corresponding value of about 100 μg/ml reported using isolated cardiac sarcoplasmic reticulum. Although it is tempting to suggest that most of the saponin-resistant, oxalate-stimulated Ca^{2+} transport is derived from the smooth muscle endoplasmic reticulum, one should be cautious in that our present study does not rule out the possible contribution from the cholesterol-poor plasma membrane subfractions. The fact that a substantial fraction of the oxalate-stimulated ATP-dependent Ca^{2+} transport by dog aortic microsomes was saponin-sensitive, in contrast to that found using rat vas deferens microsomes, is consistent with our previous findings that digitonin pretreatment of the dog aortic microsomes shifted the modal density of oxalate-stimulated Ca^{2+} transport activities to a higher density in a continuous sucrose density gradient [13], but this did not occur with rat vas deferens microsomes [14]. It appears that the plasma-membrane-related oxalate effect is greater

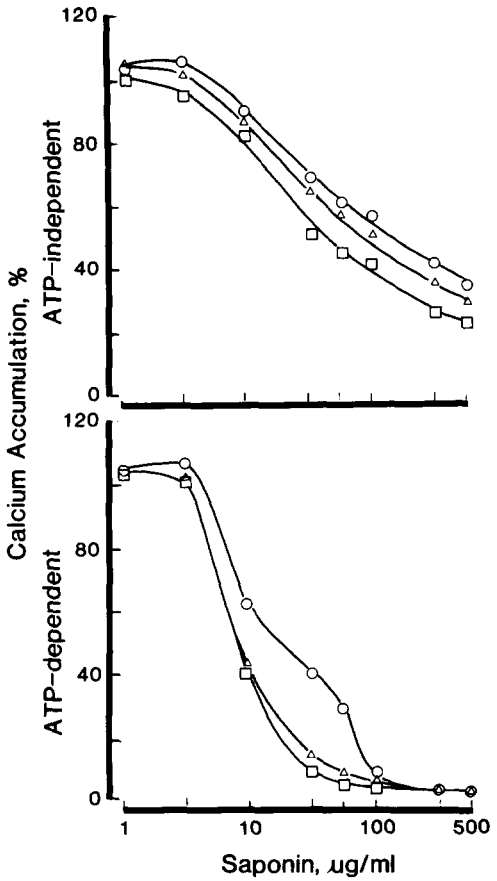

Fig. 1. Effect of saponin on the Ca^{2+}-accumulation by rat vas deferens microsomes (○), dog aortic muscle microsomes (△) and rat gastric fundus muscle plasma membranes (□) in the absence (top) and presence (bottom) of ATP. Conditions are the same as described in Table I. Each point represents the mean value from three separate experiments using three replicate samples in each experiment. Standard deviations are less than 10% of the mean and are excluded for clarity. All data points were normalized using the values obtained in the absence of saponin as 100%.

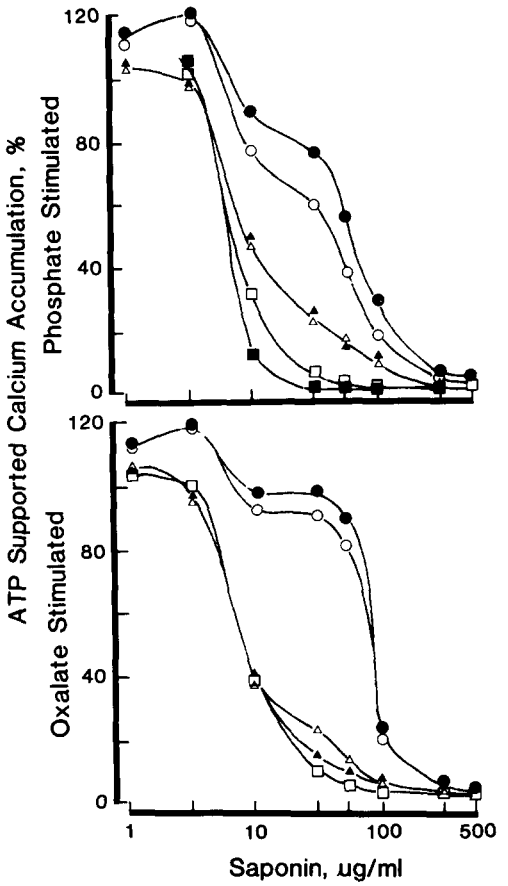

Fig. 2. Effect of saponin on the ATP-driven Ca^{2+} accumulation by rat vas deferens microsomes (●, ○), dog aortic muscle microsomes (▲, △) and rat gastric fundus muscle plasma membranes (■, □) in the presence of 20 mM inorganic phosphate (top) and 2 mM oxalate (bottom). Open symbols represent the total ATP-dependent Ca^{2+} accumulation in the presence of oxalate or phosphate. Closed symbols represent the anion-stimulated ATP-dependent Ca^{2+} accumulation after subtraction of the basal Ca^{2+}-accumulation from the total Ca^{2+} accumulation. Presentation and normalization of the data remain the same as described in Fig. 1.

in dog aortic microsomes than in rat vas deferens microsomes, whereas the plasma-membrane-associated phosphate effect is greatest in rat fundus and least in rat vas deferens. Thus, in conclusion, our present and previous studies [9,12,13] do not support the view that oxalate- or phosphate-stimulated ATP-dependent Ca^{2+} accumulation is a unique property of endoplasmic reticulum in smooth muscle. But rather, we suggest that oxalate- or phosphate-stimulated ATP-dependent Ca^{2+}-transport is a property of plasma membranes as well as of endoplasmic reticulum. The relative magnitude of stimulation by these anions at subcellular membrane sites depends upon the source of smooth muscle used. Therefore, such an anion-stimulated ATP-dependent Ca^{2+} transport should not be used as a membrane marker for smooth muscle endoplasmic reticulum.

This work was supported by the Heart and Stroke Foundation of Ontario. The author very much appreciated the technical assistance of Ms. S. Sipos and Mrs. V. Gaspar and the helpful discussion with Dr. E.E. Daniel.

References

1 Carsten, M.E. (1969) J. Gen. Physiol. 53, 414–426
2 Daniel, E.E., Crankshaw, D.J. and Kwan, C.Y. (1979) in Trends in Autonomic Pharmacology Vol. 1 (Kalsner, S., ed.), pp. 443–486, Urban & Schwarzenberg Inc., Baltimore
3 Sloane, B.F. (1980) in Handbook of Pharmacology, Vol. 2 (Bohr, D.F., Somlyo, A.P. and Sparks H.V. eds.), pp. 121–132, Am. Physiol. Soc., Bethesda, MD
4 Ford, G. (1976). Fed. Proc. 35, 1298–1301
5 Ford, G.D. and Hess, M.L. (1982) Am. J. Physiol. 242, 242–249
6 Kwan, C.Y. (1983) Am. J. Physiol. 244, 115–118
7 Baudouin-Legros, M. and Meyer, P. (1973) Br. J. Pharmacol. 47, 377–385
8 Allen, J.C. (1977) Blood Vessels 14, 91–104
9 Kwan, C.Y., Lee, R.M.K.W. and Daniel, E.E. (1981) Blood Vessels 18, 171–186
10 Grover, A.K. and Kwan, C.Y. (1983) Life Sci. 32, 2655–2660
11 Raeymaekers, L., Wuytack, F., Eggermont, J., DeSchutter, D. and Casteels, R. (1983) Biochem. J. 210, 315–322
12 Crankshaw, D.J., Challis, J.R.G., Patrick, J.E. and Daniel, E.E. (1979). Gyn. Ob. Invest. 10, 298–305
13 Kwan, C.Y., Triggle, C.R., Grover, A.K., Lee, R.M.K.W. and Daniel, E.E. (1984) J. Mol. Cell. Cardiol. 16, 747–764
14 Grover, A.K. and Kwan, C.Y. (1984) Arch. Int. Pharmacodyn. Ther. 267, 4–12
15 Wibo, M., Duong, A.T. and Godfraind, T. (1980) Eur. J. Biochem. 112, 87–94
16 Kwan, C.Y. and Lee, R.M.K.W. (1984) Mol. Physiol. 5, 105–114
17 Endo, M., Yagi, S. and Iino, M. (1982) Fed. Proc. 41, 2245–2250
18 Stout, M.A. and Diecke, F.P.J. (1983) J. Pharmacol. Exp. Ther. 225, 102–111
19 Saida, K. and Nonomura, Y. (1978). J. Gen. Physiol. 72, 1–14.
20 Kwan, C.Y., Lee, R.M.K.W. and Grover, A.K. (1983) Mol. Physiol. 3, 53–69
21 Kwan, C.Y., Sakai, Y., Grover, A.K. and Lee, R.M.K.W. (1982) Mol. Physiol. 2, 107–120
22 Grover, A.K., Kwan, C.Y. and Daniel, E.E. (1982). Mol. Physiol. 2, 301–313
23 Suematsu, E., Hirata, M. and Kuriyama, H. (1984) Biochim. Biophys. Acta 773, 83–90
24 Inamitsu, T. and Ohtsuki, I. (1984) Eur. J. Biochem. 145, 115–121