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Ion-translocating ATPases in tendrils of *Bryonia dioica* Jacq.

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Abstract. Procedures have been developed which allow the preparation of highly pure endoplasmic reticulum and plasma membrane from tendrils of *Bryonia dioica*. These and further membrane fractions were used to study vanadate-sensitive ATPase activity as well as Mg^{2+}-ATP-driven transport of $^{45}Ca^{2+}$. Calcium-translocating ATPases were detected in the endoplasmic reticulum, the plasma membrane and the mitochondrial fraction and characterized kinetically and with respect to the effects of various inhibitors. The endoplasmic-reticulum Ca^{2+}-translocating ATPase was stimulated by KCl and was calmodulin-dependent. The plasma-membrane enzyme was not affected by these agents. These, as well as the inhibitor data, show that the Ca^{2+}-translocating ATPases of the endoplasmic reticulum and the plasma membrane are distinctly different enzymes. Upon mechanical stimulation, the activities of the vanadate-sensitive K^+, Mg^{2+}-ATPase and the Ca^{2+}-translocating ATPase(s) increased rapidly and transiently, indicating that increasing transmembrane proton and calcium fluxes are involved in the early stages of tendril coiling.

Key words: *Bryonia* – Ca^{2+}-ATPase – Endoplasmic reticulum – Plasmalemma – Tendril coiling

Introduction

Tendrils are among the most touch-sensitive organs of higher plants. When a tendril contacts a suitable support, coiling sets in. This reaction can be divided into a fast, reversible phase of local contact coiling followed – if the contact stimulus persists – by a slow, irreversible free-coiling process during which the whole tendril forms a tight, flexible spiral (see Jaffe and Galston 1968b, for review).

Recently, we have shown that methyljasmonate/jasmonic acid induces tendril free-coiling in *Bryonia dioica* Jacq. (Falkenstein et al. 1991; Weiler et al. 1993). Airborne methyljasmonate completely substitutes for the mechanical stimulus in inducing the response and in bringing it to completion. Other signalling molecules probably involved in this reaction are auxin (e.g. Reinhold 1967) and ethylene (e.g. Jaffe 1970). Very little is known about the molecular events underlying the perception of touch and the transduction of the stimulus into intracellular reactions. Available evidence, obtained for tendrils of *Pisum sativum* L., suggests that the contact-coiling response is driven by osmotic changes in the organ leading to contraction of the ventral side (Jaffe and Galston 1966a). Associated with this is a measurable leakage of electrolytes including protons and loss of water from the cells (Jaffe and Galston 1968a). Concomitantly, the level of ATP in the organ decreases drastically (Jaffe and Galston 1966b), and changes in "total tendril ATPase" activity were reported during contact-coiling (Jaffe and Galston 1967). From these early approaches, the field has not advanced towards understanding the molecular basis of touch perception and stimulus transduction from contact to coiling. Studies employing more recently developed, sensitive tools to analyze membrane transport processes are completely lacking.

As part of our effort to elucidate the structure and function of the tendril mechanoreceptor as well as its coupling to cellular effector systems, purification of membrane fractions from tendrils and characterization of their ion transporters was essential. Here, we report on the preparation and properties of highly pure plasma membranes (PMs) and endoplasmic reticulum (ER) from tendrils of *Bryonia dioica* Jacq. Specifically, we have focussed on Ca^{2+}-translocating ATPases, their properties, regulation and changes in activity during

Abbreviations: CAM = calmodulin; CCCP = carbonylcyanide *m*-chlorophenylhydrazone; IC_{50} = concentration giving 50% inhibition; PM = plasma membrane; rER = rough endoplasmic reticulum; sER = smooth endoplasmic reticulum; FC = fusicoccin; $U_3 + U_3$ = the two PM-rich upper phases obtained after phase partitioning of microsomal membranes

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contact coiling after mechanical stimulation. Calcium appears to play an important role in reactions of plants to mechanical forces (Braam and Davis 1990; Knight et al. 1991), and we have obtained evidence that the tendril mechanoreceptor, the tactile blep, contains large amounts of compartmentalized calcium (data not shown).

Materials and methods

Plant material. Bryonia dioica Jacq. (seeds originally obtained from N. Boyer, Clermont-Ferrand, France) was grown in 25-l pots from well-established rhizomes in phytotron chambers: 20° C day and 17° C night, 16 h photoperiod, 70–90 μmol photons $\cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation measured at 2 m distance from the light sources, relative humidity 70%.

Chemicals. Calcium chloride ($^{45}CaCl_2$, 15–60 TBq $\cdot mol^{-1}$) was purchased from Amersham (Braunschweig, Germany), ATP was from Boehringer (Mannheim, Germany), and A23187, enzyme inhibitors, other nucleotides and calmodulin (bovine brain) came from Sigma (Deisenhofen, Germany). Polyethyleneglycol (PEG 3350) was obtained from Union Carbide (Düsseldorf, Germany) and dextran PL 500 VC came from Pfeifer and Langen (Dormagen, Germany). Insoluble polyvinylpyrrolidone (PVP, Polyclar AT) was from Serva (Heidelberg, Germany).

Selection of tendrils and mechanical stimulation. Tendrils 12–18 cm long were used exclusively. These are most reactive towards touch. Tendrils were harvested and immediately immersed in liquid nitrogen. Mechanical stimulation was applied for 30 s to the ventral side of the organ by gently sliding a wooden stick along the whole organ in the apical direction. Specimens were used only if, following this treatment, visible contact-coiling occurred within 60 s after the stimulation (coiling by $\geq 90°$). Then, the tendril was brought into continuous contact with the wooden support adjacent to its distal third (for scheme, see Fig. 12). At the appropriate times, the whole tendril was harvested, avoiding the tendril suspensor (for discussion of tendril morphology, see Troll 1939) and immediately immersed in liquid nitrogen. It was noticed that even the time required from cutting the tendril until freezing brought about a detectable change in the physiology of the organ, specifically, in the activity of the ion pumps. Therefore, a second control was required: tendrils were harvested using a freeze-clamping technique (Benedetto and Slater 1987). In this case, the organ was quickly clamped, in situ, between two metal blocks cooled to the temperature of liquid nitrogen. The specimen, frozen between the metal blocks, was removed from the plant by withdrawal of the device and plunged into liquid nitrogen. These controls are designated "CO". For each datum point, 1 g fresh mass (usually 15 tendrils) was harvested.

Preparation of microsomes. The tendrils were ground under liquid nitrogen with a mortar and pestle and then further with 5 ml per g fresh mass of homogenization medium (50 mM Hepes-KOH, 3 mM dithiothreitol (DTT), 2 mM EDTA, 2 mM EGTA, 0.5 M sucrose, 0.6% (w/v) insoluble PVP, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5). The homogenate was passed through gauze and the tissue was re-extracted once with the above buffer. The combined extracts were centrifuged at 10000 $\cdot g$ and 4° C for 10 min. The supernatant was diluted with half its volume of 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$. Microsomal membranes were obtained by centrifugation of this preparation at 100000 $\cdot g$ and 4° C for 45 min.

Preparation of PMs. Microsomal sediments were resuspended in 5 mM potassium phosphate (pH 7.8), containing 0.33 M sucrose and subjected to aqueous two-phase partitioning according to Larsson (1985), but with several modifications. After phase separation, the phase systems (36 g size) consisted of 6.2% (w/v) dextran PL 500 VC and 6.2% (w/v) PEG 3350 and contained 0.33 M sucrose, 5 mM KCl and 5 mM potassium phosphate, pH 7.8. The upper phase (U_1) was re-extracted two times with fresh lower phase containing 8.2% (w/v) PEG. This lower phase (L_1) was re-extracted twice with the dextran-rich phase. The two final upper phases ($U_3 + U_3'$) were pooled, diluted with three volumes 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$, and the membranes were pelleted at 100000 $\cdot g$ and 4° C for 50 min.

The pellets were resuspended in 1.5 ml 5 mM Hepes-KOH (pH 7.1) with 6% (w/w) sucrose, 1 mM DTT, 2 mM EDTA, 0.5 mM PMSF and 50 μg $\cdot ml^{-1}$ chymostatin. This suspension was layered on top of a sucrose step gradient consisting of 6 ml 50%, 10 ml 40%, 11 ml 30%, 4 ml 20% (w/w) sucrose layers in 5 mM Hepes-KOH, 1 mM DTT, 2 mM EDTA, pH 7.1, and centrifuged at 100000 $\cdot g$ and 4° C for 3 h (rotor: TST 28.38; Kontron, Düsseldorf, Germany). Aliquots of 1 ml were then collected to determine the distribution of protein, Ca^{2+}-transporters and marker enzymes. To prepare PM free of contaminating ER and mitochondria, the fractions corresponding to a density between 1.140 and 1.175 g $\cdot cm^{-3}$ (33–40% sucrose) were collected, diluted with one volume of 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$, and the PM vesicles were pelleted for 30 min at 265000 $\cdot g$ and 4° C. The pellets were resuspended in the above buffer (0.5 mg of protein $\cdot ml^{-1}$) and shock-frozen by dropping the suspension in liquid nitrogen. Storage, without loss of calcium-transport activity, was at −80° C for a maximum of three months.

Preparation of ER. Microsomes for the preparation of ER were produced as above, but omitting EDTA from the homogenization buffer to retain the relative proportions of rough (rER) and smooth (sER) ER. After centrifugation (vide supra), microsomes were resuspended in 5 mM Hepes-KOH (pH 7.1) containing 6% (w/w) sucrose, 1 mM DTT, 3 mM $MgSO_4$, 0.5 mM PMSF and 50 μg $\cdot ml^{-1}$ chymostatin. This suspension (2–3 ml) was layered on top of a sucrose gradient prepared as described above, but without EDTA and containing 3 mM $MgSO_4$. In some cases, gradient composition was as follows: 4 ml 60%, 8 ml 50%, 10 ml 40% and 10 ml 30% (w/w) sucrose (these experiments are specified in *Results*). Centrifugation was done at 110000 $\cdot g$ and 4° C for 2.5 h (rotor: Kontron TST 28.38) followed by collection of 1-ml fractions. The material from the 21–30% sucrose layer (1.085 to 1.130 g $\cdot cm^{-3}$ density) was collected by centrifugation (265000 $\cdot g$, 4° C, 30 min) after dilution with 1 volume of 5 mM Hepes-KOH (pH 7.1) containing 1 mM DTT and 3 mM $MgSO_4$. Resuspension was in 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$ (0.8 mg of protein $\cdot ml^{-1}$) with aid of a glass homogenizer. This preparation contained sER (plus the tonoplast) and was designated the "sER-fraction".

The fractions from 32% to 40% sucrose (1.135–1.175 g $\cdot cm^{-3}$) contained the rER contaminated with PM and broken plastids. From this material, the rER was further purified by an EDTA-dependent density shift technique as follows: the pooled fractions from the 32% to 40% sucrose interface from two gradients were diluted with 1 volume of 5 mM Hepes-KOH (pH 7.1) containing 6% (w/w) sucrose, 1 mM DTT and 3 mM EDTA and centrifuged (vide supra). The sediments were resuspended in the same buffer containing 0.5 mM PMSF and 50 μg $\cdot ml^{-1}$ chymostatin and layered as a 2-ml aliquot on top of a sucrose gradient (vide supra) with $MgSO_4$ replaced by 3 mM EDTA. After centrifugation (110000 $\cdot g$, 4° C, 2.5 h, TST 28.38 rotor), the ER, now shifted to a density range of 1.085–1.130 g $\cdot cm^{-3}$ (21–30% sucrose) due to the loss of ribosomes and thus separated from contaminating membranes, was collected, diluted with 1 volume of 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$ and repelleted at 265000 $\cdot g$ (vide supra). The pellets, representing the original rER membranes, were resuspended at 0.5 mg protein $\cdot ml^{-1}$ in the above buffer, shock-frozen in liquid nitrogen and stored as described above. This material is hereafter referred to as "rER".

For some general experiments, the total density range represented by the 21–30% sucrose-fraction of EDTA-gradients which contained rER plus sER with contaminations of tonoplast and some envelope, was used. This fraction is later termed "total ER". However, for all crucial characterizations, the purified rER and/or sER was used.

Preparation of mitochondrial membranes. After differential centrifugation (vide supra), microsomal sediments were resuspended in 5 mM Hepes-KOH (pH 7.1) containing 6% (w/w) sucrose, 1 mM DTT, 3 mM EDTA, 0.5 mM PMSF and 50 $\mu g \cdot ml^{-1}$ chymostatin. This suspension (2–3 ml) was layered on top of a sucrose step gradient prepared as above and centrifuged at $100000 \cdot g$ and 4° C for 3 h (rotor: Kontron TST 28.38).

The pooled fractions from the 41–49% sucrose interface (density between 1.180 and 1.224 $g \cdot cm^{-3}$) were diluted with two volumes of 25 mM Hepes-KOH (pH 7.2) containing 0.25 M sucrose and 6 mM $MgSO_4$, and the membrane vesicles were collected by centrifugation for 30 min at $265000 \cdot g$ and 4° C. The sediments were resuspended in the above buffer and shock-frozen by dropping the suspension in liquid nitrogen.

Enzyme assays. The activity of the vanadate-sensitive, K^+-stimulated and Mg^{2+}-dependent ATPase (vanadate-sensitive K^+, Mg^{2+}-ATPase) was determined according to Hodges and Leonard (1974) with phosphate determinations as described by Lanzetta et al. (1979). The NO_3^--sensitive ATPase was determined as described in Gräf and Weiler (1989), catalase according to Lück (1962), succinate dehydrogenase as in Singer et al. (1973) and NADH-cytochrome *c* reductase (antimycin A-sensitive and insensitive) according to Moore and Proudlove (1983) and Lord (1983). The sidedness of the vesicles was determined from the latency of the vanadate-sensitive K^+, Mg^{2+}-ATPase in the presence or absence of 0.01% (w/v) Brij 58. Kinetic data (V_{max}, K_m) are apparent values.

Calcium-transport assays. The method of Gräf and Weiler (1989) was used. In brief, all assays were carried out at 25 °C and 0.1 ml final assay volume. Membrane fractions (PM: 5 µg; ER: 8 µg; microsomes: 9 µg and mitochondrial membranes: 10 µg of protein, respectively) were – if necessary – pre-incubated with the appropriate effectors (final volume 10 µl or, when individual fractions from sucrose gradients were analyzed, 30 µl) for 5 min. Then 90 µl (or 70 µl, respectively) of assay medium [25 mM Hepes-KOH, pH 7.2, containing 0.25 M sucrose, 6 mM $MgSO_4$, 3 mM ATP, 15 µM $^{45}CaCl_2$ (67–130 MBq, 15–60 $TBq \cdot mol^{-1}$)] were added, mixed and incubated for the times indicated (usually from 2 to 10 min). Controls were run in parallel without ATP. Reactions were terminated by adding 0.5 ml of stop-buffer (assay medium without ATP and $^{45}CaCl_2$ but with added 1 mM EGTA), mixing and vacuum filtration over nitrocellulose membranes (0.45 µm; Schleicher & Schuell, Dassel, Germany; Gräf and Weiler 1989). The filters were washed three times with 1 ml of stop-buffer, then dried and counted, after dissolution in 3 ml scintillation cocktail (Hydroluma; Baker Co., Deventer, The Netherlands) using a Phillips PW 4700 scintillation counter (window 0.4–1990 keV). The activity of the Ca^{2+}-ATPase was determined after correcting for ATP-independent $^{45}Ca^{2+}$ associated with the control (minus-ATP) vesicles. All modifications of this standard protocol are mentioned in the results section. Kinetic data (K_m, V_{max}) are apparent values.

To determine Ca^{2+}-efflux, the inside-out vesicles were loaded for 10–15 min with $^{45}Ca^{2+}$ as described above and reactions were terminated by adding 0.5 ml of stop-buffer (vide supra). Directly after termination, effectors (or buffer for controls) were added in final concentrations as indicated and the kinetics of release of $^{45}Ca^{2+}$ were determined at the indicated times. Filters were washed and prepared as described above.

Fusicoccin-binding assay. The fusicoccin-binding protein (FCBP) serves as the most specific PM marker available to date (Feyerabend and Weiler 1988; Oecking and Weiler 1991). Fusicoccin (FC)-binding activity was determined using [^{3}H]-9′-Nor-8′-hydroxy-fusicoccin (spec. act. $3.05 \cdot 10^{15}$ $Bq \cdot mol^{-1}$) as radioligand (Feyerabend and Weiler 1988). The assay was carried out as described by Schulz et al. (1990) using polyethyleneimine-coated glass-fiber filters to separate the free from the FCBP-bound radioligand.

Gel electrophoresis. Proteins were separated by one-dimensional polyacrylamide gel electrophoresis using a Laemmli system (Laemmli 1970) with specifications as in Oecking and Weiler (1991) and silver staining according to Blum et al. (1987).

Miscellaneous assays. Chlorophyll was determined according to Arnon (1949), and protein was assayed according to Bradford (1976). The density of the fractions was measured by refractometry.

Results

The goals for the work were twofold: (i) to develop a method for the preparation of highly pure PM as well as ER from tendril tissue of *B. dioica*, which was required for several ongoing studies on the early reactions of tendrils to touch and (ii) to characterize the Ca^{2+}-translocating ATPases of the tissue.

Microsomes prepared from tendrils accumulate freely (i.e. A23187-) exchangeable $^{45}Ca^{2+}$ in the presence but not in the absence of Mg^{2+}-ATP (Fig. 1). This process is significantly (1.7-fold) stimulated by the addition of calmodulin (CAM) with an apparent IC_{50} of about 0.25 µM ($n > 4$) (Fig. 1). The protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) affected ATP-driven accumulation of $^{45}Ca^{2+}$ into the microsomal vesicles only marginally. For up to 5 µM CCCP, calcium transport was inhibited by 10% while at 500 µM CCCP, only 18% inhibition was obtained. These data rule out the possibility that the microsomal $^{45}Ca^{2+}$ accumulation observed was due to a Ca^{2+}/H^+-exchange mechanism which would have been eliminated by 5 µM CCCP (Malatialy et al. 1988). Rather, our data show that primary active Ca^{2+}-translocating ATPases were responsible for most, if not all, of the microsomal Ca^{2+}-

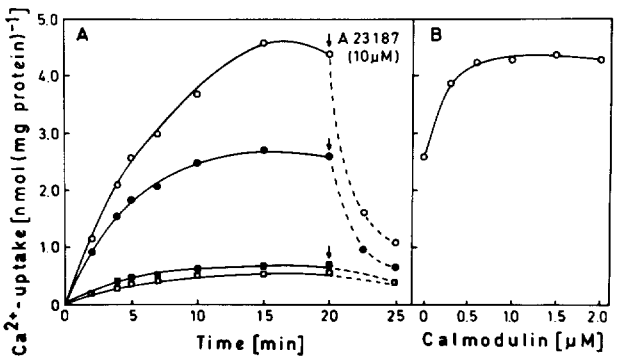

Fig. 1 A, B. Mg^{2+}-ATP-driven uptake of $^{45}Ca^{2+}$ into microsomal vesicles from straight tendrils of *Bryonia dioica* (**A**) in the presence (○, ●) or absence (□, ■) of 3 mM ATP using untreated (●, ■) or CAM (1 µM)-pretreated (○, □) membranes corresponding to 8 µg of microsomal protein (pH = 7). After 20 min, intravesicular $^{45}Ca^{2+}$ was discharged with A23187 (*arrows*). **B** Effect of different CAM concentrations on $^{45}Ca^{2+}$ uptake (loading for 15 min, otherwise standard conditions). Shown are typical sets of data from $n > 4$ independent experiments

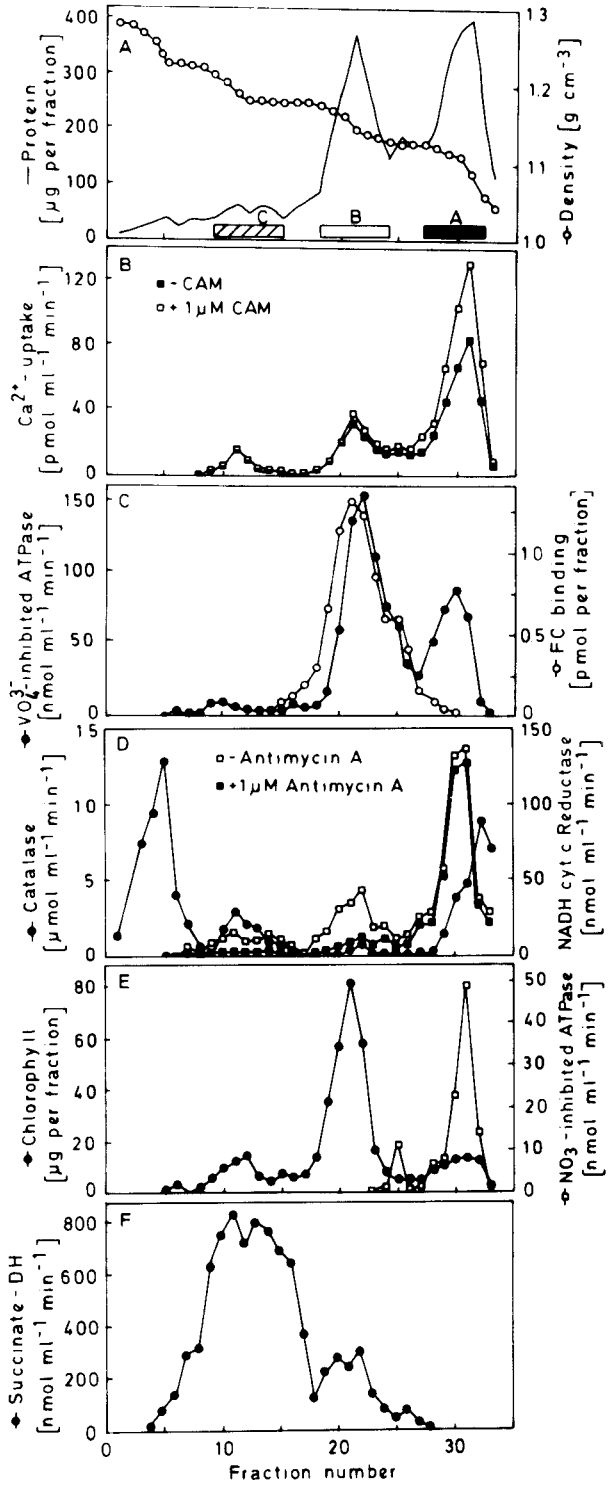

Fig. 2A–F. Distribution of protein and density (**A**), Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transport activities (**B**), FC-binding activity as well as vanadate-sensitive K^+, Mg^{2+}-ATPase (**C**) and marker enzymes (**D, E, F**) on a 30–60% sucrose density step gradient in the presence of 3 mM EDTA covering the density range from 1.28 to 1.04 g·cm^{-3}. Microsomes (5 mg of protein) obtained from straight tendrils of *Bryonia dioica* were spun at 4° C for 3 h and 100000·*g*; 1-ml fractions were collected and subjected to the various assays

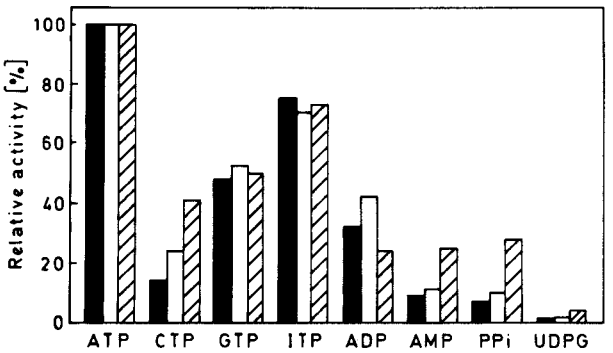

Fig. 3. Substrate specificity of the three different Ca^{2+}-transport systems in membranes corresponding to zone A (■), B (□) and C (▨) of the sucrose density gradient shown in Fig. 2 (panel A). Membrane vesicles from *Bryonia dioica* were collected by centrifugation and washed as described in *Materials and methods*. The assays were made under standard conditions at the pretermined pH-optima (listed in Table 2), using 3 mM substrate in each case. 100% = 6.6, 4.5 and 1.2 pkat per mg of protein for the enzymes from zones A, B and C, respectively

transport activity observed. This was further corroborated by the finding that $^{45}Ca^{2+}$ accumulation into the microsomal vesicles could be driven by ITP, which is a substrate of Ca^{2+}-ATPases, but not of the proton pump (vide infra). Regulation of the tendril calcium pumps through calcium and CAM was indicated by these data. A full understanding of the system, however, required a more detailed analysis of purified subcellular fractions.

Preparation and characterization of ER. When microsomal membranes were separated on 30–60% sucrose density step gradients in the presence of EDTA, three distinct vesicular fractions (zones A, B, C, marked in Fig. 2A) could be localized which exhibited Mg^{2+}-ATP-driven Ca^{2+}-transport activity (Fig. 2A, B). Peak densities as well as the co-localization of enzyme or other markers (Fig. 2C–F) allowed assignment of mitochondrial activity co-sedimenting with succinate dehydrogenase (zone C, density 1.18–1.22 g·cm^{-3}, 41–49% sucrose), followed by zone-B activity (density 1.130–1.175 g·cm^{-3}, 31–40% sucrose), which contained the PM, broken mitochondria and chloroplast membranes, as evident from the co-sedimentation of the major fraction of the vanadate-sensitive ATPase and FC-binding activity with antimycin A-sensitive NADH-cytochrome *c*-reductase and chlorophyll. The major Ca^{2+}-transport activity was associated with zone A (density 1.085–1.122 g·cm^{-3}, 21–29% sucrose) which contained ER and tonoplast and co-sedimented with antimycin-insensitive NADH-cytochrome *c*-reductase as well as with nitrate-sensitive ATPase. The microsomal preparations show that approx. 32% of the total microsomal uptake activity is associated with the PM (Fig. 2B), and a value of 38% is indicated in Table 1. The slight discrepancy (6%) is due to the fact that there is a small inhibition of the total uptake in whole microsomal preparations as compared to the activities after separation and removal of inhibitory material. Furthermore, microsomal as-

Table 1. Distribution of different markers and Mg^{2+}-ATP-dependent $^{45}Ca^{2+}$-transport activity in microsomal membranes (M) and PM-rich upper phases (U) after aqueous two-phase partitioning of microsomes from straight tendrils of *Bryonia dioica*. Shown are averaged data from $n \geq 3$ separate experiments

Parameter	Total activity		Recovery in U (% of M)	Specific activity[a]		Ratio U/M
	M	U		M	U	
Protein (mg)	8.70	0.73	8.4	–	–	–
Chlorophyll (mg)	0.78	0.00	0.0	–	–	–
$^{45}Ca^{2+}$ uptake (pkat)	52.20	19.70	38.0	6.00	27.00	4.50
Vanadate-sensitive K^+, Mg^{2+}-ATPase (nkat)	34.00	10.50	31.0	3.90	23.00	5.90
FC binding (pmol)	28.00	10.10	36.0	3.20	13.90	4.30
NADH-cytochrome *c* reductase						
antimycin insensitive (nkat)	13.50	1.20	9.0	1.55	1.64	1.06
antimycin sensitive (nkat)	2.80	0.17	6.0	0.32	0.23	0.72
NO_3^--sensitive ATPase (nkat)	9.60	0.10	1.0	1.10	0.13	0.12
Succinate dehydrogenase (nkat)	7.50	0.66	9.0	0.86	0.90	1.05
Catalase (μkat)	18.30	0.54	3.0	2.10	0.74	0.35

[a] Per mg of protein; second unit as under *Parameter*

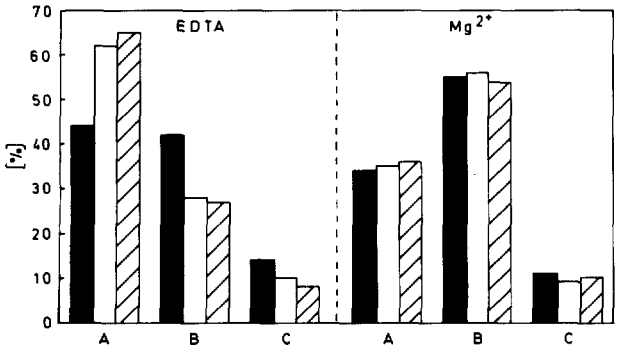

Fig. 4. Distribution of protein (■), Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transport activity (□) and NADH-cytochrome *c*-reductase activity (▨) in 30–50% sucrose density step gradients containing either EDTA (in the absence of Mg^{2+}) or Mg^{2+} (in the absence of EDTA) obtained from tendrils of *Bryonia dioica*. The density zones are those shown in Fig. 2 (A = 1.085–1.122 g·cm^{-3}, 21–29% sucrose; B = 1.130–1.175 g·cm^{-3}, 31–40% sucrose; C = 1.180–1.220 g·cm^{-3}, 41–49% sucrose). 100% levels (EDTA-gradient): protein, 4.5 mg; Ca^{2+}-transport, 530 pmol·min^{-1}; NADH-cytochrome *c*-reductase, 480 nmol·min^{-1}; (Mg^{2+}-gradient): protein, 4.7 mg; Ca^{2+}-transport, 650 pmol·min^{-1}; NADH-cytochrome *c*-reductase, 520 nmol·min^{-1}

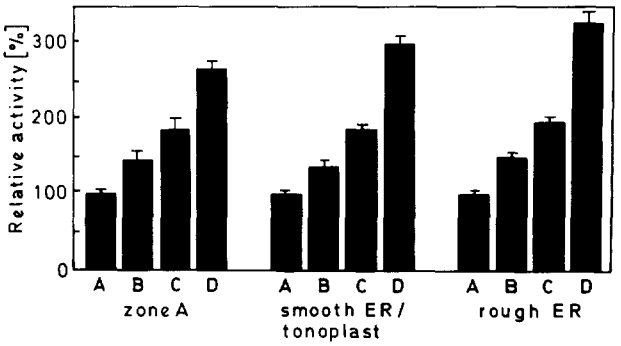

Fig. 5. Activities of Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transporters of zone A from sucrose density step gradients run in the presence of EDTA, (cf. Fig. 2, panel B), the sER/tonoplast fraction corresponding to the density zone A in identical sucrose density step gradients but containing Mg^{2+} in the absence of EDTA, and the rER fraction highly purified by the two-step Mg^{2+}-EDTA density-shift technique. The washed and re-pelleted fractions from *Bryonia dioica* were resuspended in assay buffer either without additions (*A*), or in the presence of 20 mM KCl (*B*), 1 μM CAM (*C*), or 1 μM CAM plus 20 mM KCl (*D*). 100% = 8.4, 7.2 and 5.6 pkat per mg of protein in the zone A, sER/tonoplast, or rER fraction, respectively

says have been conducted at the compromise pH (7.0) whereas the PM Ca^{2+}-ATPase has been assayed at its pH-optimum (7.2). Only the Ca^{2+}-ATPase in zone A was activated by CAM (Fig. 2B). The nucleotide specifities of the three transport systems (Fig. 3: ATP > ITP > GTP > CTP) were similar and are typical for other plant Ca^{2+}-ATPases (Gräf and Weiler 1989; Liß et al. 1991 and discussion therein). For all further experiments, the 60% sucrose cushion was omitted from the step gradients and, as detailed in *Materials and methods*, a 20% sucrose cushion was included. This allowed a better separation of zone A from the soluble enzymes.

That the zone-A activity contained the ER enzyme could also be shown by the Mg^{2+}-induced shift to higher densities (zone B) of both, NADH-cytochrome *c*-reductase and the Mg^{2+}-ATP-dependent $^{45}Ca^{2+}$-transport activity (Fig. 4). In the case of both enzymes, 29–30% of the total activity/amount was shifted (cf. Fig. 4). This fraction of Ca^{2+}-transport activity thus is associated with the rER. The remaining, non-shifting, Ca^{2+}-transport activity (approx. 32% of the total activity) presumably represents the enzyme from sER and the tonoplast.

Highly purified ER was prepared by the two-step process detailed in *Materials and methods*. In this process, microsomes were first separated on a sucrose gradient containing Mg^{2+} in the absence of EDTA. Collection of the density range 1.085–1.122 g·cm^{-3} (zone A) afforded the sER/tonoplast fraction. The fraction from 1.130–1.175 g·cm^{-3} (zone B), containing the rER as well as broken chloroplasts and PM, was collected separately, washed and re-run on a step gradient containing EDTA in the absence of Mg^{2+}. This shifted the rER,

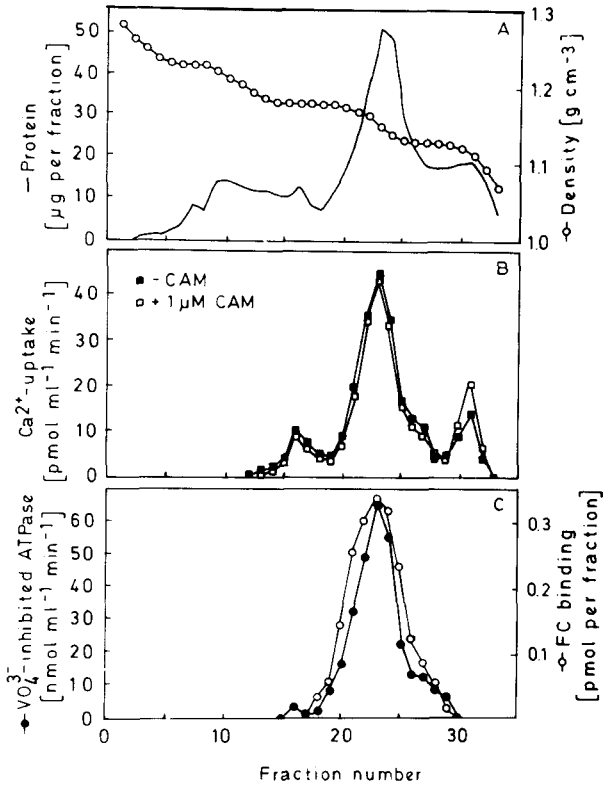

Fig. 6. Distribution of protein and density (**A**), Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transport (**B**) and vanadate-sensitive K^+, Mg^{2+}-ATPase as well as FC binding (**C**) in PM-rich upper phases (U_3+U_3') obtained from straight tendrils of *Bryonia dioica* by aqueous two-phase partitioning and then separated on a 20–60% sucrose density step gradient containing EDTA and no Mg^{2+}. Amount of protein loaded = 0.6 mg

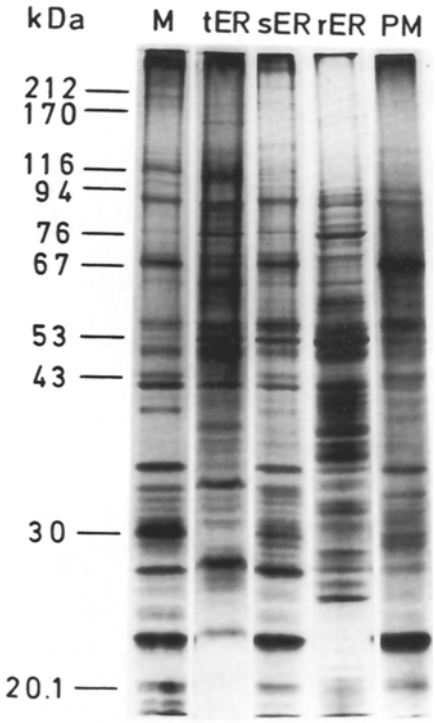

Fig. 7. Protein patterns of different membrane preparations from tendril tissue of *Bryonia dioica* obtained by SDS-PAGE on 10% acrylamide gels. *M*, microsomes; *tER*, zone A-fraction from sucrose-density step gradient run in the presence of EDTA; *sER*, smooth ER/tonoplast fraction (=zone A protein from density step gradients run in the presence of Mg^{2+}); *rER*, rough ER prepared by the two-step density-shift technique; *PM*, U_3+U_3'-phase protein obtained after aqueous two-phase partitioning and separated on a sucrose density step gradient in the presence of EDTA. Per lane, 25 µg of protein were loaded

by loss of ribosomes, to the 1.085–1.122 $g \cdot cm^{-3}$ density range from where it could be harvested in high purity, free of tonoplast, PM and chloroplast contaminations. Analysis of Mg^{2+}-ATP-driven $^{45}Ca^{2+}$ transport in the density zone A as well as in the sER/tonoplast fraction and in pure rER obtained as just described gave the same properties for the Ca^{2+}-transporter, being stimulated by CAM, KCl and largely so by CAM + KCl, when present simultaneously (Fig. 5). Thus, we conclude that the Mg^{2+}-ATP-driven Ca^{2+}-transport system present in zone A in sucrose-EDTA-gradients represents an ER-associated activity. We have not obtained any evidence for Ca^{2+}-transport by a tonoplast Ca^{2+}/H^+-antiport (Malatialy et al. 1988). Thus, because of the limited amount of tendril tissue available, the zone-A enzyme ("total ER" enzyme) was used for the general characterization of the ER Ca^{2+}-translocating ATPase.

Preparation and characterization of PMs. The purity of the PM fraction obtained from sucrose-density-gradient separation of microsomes was insufficient. Therefore, Larsson's technique of aqueous two-phase partitioning using dextran-polyethylene glycol phase-systems (Larsson 1985) was adapted for tendril tissue. Data for the optimized procedure are shown in Table 1. Phase partitioning afforded, in high and similar yields (31–38%), the two PM markers, vanadate-sensitive, K^+, Mg^{2+}-ATPase and FC-binding activity as well as a co-purifying Mg^{2+}-ATP-dependent $^{45}Ca^{2+}$-transporter representing the PM Ca^{2+}-ATPase. Chloroplast contaminations were completely removed, while the preparation, as evidenced by the other marker enzymes, still contained some ER and mitochondrial contamination (cf. Table 1). When the U_3+U_3'-membranes were run on linear sucrose-density gradients in the presence of Mg^{2+}, these contaminations could not be detected (not shown) due to insufficient separation. However, the optimized step gradient containing EDTA in the absence of Mg^{2+} clearly separated the residual ER, as well as the mitochondrial contaminations, from the PM and yielded an exceptionally pure PM preparation (Fig. 6). For most experiments and if not otherwise stated, however, due to the limited availability of tendril tissue, the combined U_3+U_3' phases obtained after phase-partitioning were used. The sidedness of these vesicles was determined from the latency of the vanadate-sensitive K^+, Mg^{2+}-ATPase. The preparation contained $40 \pm 6\%$ inside-out vesicles. The purity of the PM and ER fractions compared to microsomal preparations can also be assessed from the differential protein patterns revealed by SDS-PAGE (Fig. 7).

Vanadate-sensitive PM ATPase. The PM of tendril tissue contains a typical vanadate sensitive, Mg^{2+}-dependent

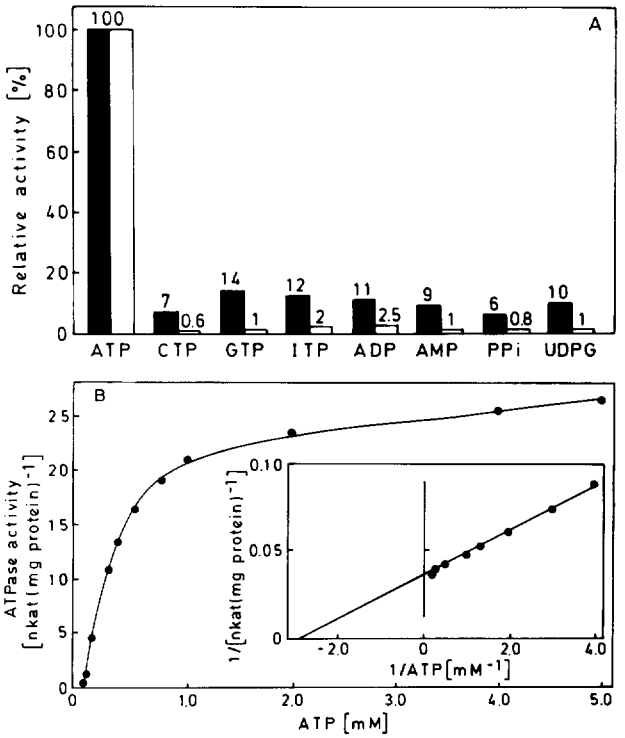

Fig. 8A, B. Substrate specificity (**A**) of the vanadate-sensitive K^+, Mg^{2+}-ATPase activity in microsomal (■) and PM-rich (U_3+U_3'-phase, □) vesicles obtained from straight tendrils of *Bryonia dioica*. All substrates (3 mM) assayed under standard conditions at pH 6.3. The numbers give the relative enzyme activities observed (100% = 3.4 (■) and 18.0 (□) nkat per mg of protein). **B** ATP-dependence of the enzyme from PMs (U_3+U_3'-phase) measured at pH = 6.3. K_m = 0.34 mM; V_{max} = 29 nkat per mg of protein

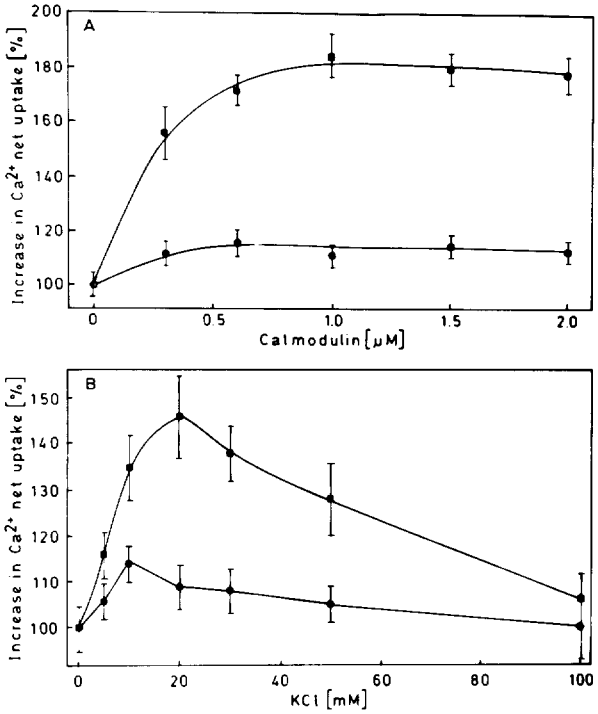

Fig. 9. Influence of bovine brain CAM (**A**) and KCl (**B**) on the Mg^{2+}-ATP-driven Ca^{2+}-uptake by the total ER fraction (zone A, Fig. 2) (■) and the PM (U_3+U_3'/fraction (●) from *Bryonia dioica*. Shown are means ± SD from $n > 4$ independent experiments. 100% = 6.2 (■), 11.0 (●) pkat per mg of protein

and K^+-stimulated ATPase presumably involved in proton pumping; although this was not determined within the framework of present study. The enzyme is highly nucleotide specific (Fig. 8A) and follows Michaelis-Menten kinetics (Fig. 8B) with respect to Mg^{2+}-ATP (V_{max} = 29 nkat per mg of protein, $K_{m,\ ATP}$ = 0.34 mM), has a pH-optimum of 6.3 and is inhibited by orthovanadate at pH 6.3 (IC_{50} = 0.23 mM).

Calcium-translocating ATPases. The properties of the three Ca^{2+}-translocating ATPases associated with the ER, the PM and the mitochondrial membranes were analyzed in detail. The major characteristics of these enzymes are compared in Table 2. Whereas the nucleotide preference of all enzymes is rather similar (see Fig. 3), they could clearly the distinguished based on their pH-optima, inhibitor sensitivities and stimulation by KCl and/or CAM. Notable differences were found between the PM and ER enzymes. While the enzyme from the ER was strongly stimulated by KCl and CAM, only a slight stimulation was observed for the Ca^{2+}-translocating ATPase from U_3+U_3' membranes (cf. Table 2, Fig. 9). This residual stimulation was found to be due to a trace contamination, by ER, of this fraction as is proven from the data in Fig. 6B. It is shown there that only the activity of the Ca^{2+}-ATPase associated with the minor ER band (at 1.085–1.122 g·cm^{-3} density) is CAM-stimulated while the PM activity is clearly

Table 2. Comparison of characteristics of the Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transport systems in the ER and PM from straight tendrils of *Bryonia dioica*. ER is the total ER fraction corresponding to zone A in Fig. 2, PM is the U_3+U_3' phase after phase partitioning of microsomal membranes. CAM = 1 μM final concentration. All data are means or means ± SD from $n > 3$ independent experiments. V_{max} in pkat per mg of protein, K_m in μM

Parameter		ER	PM
pH-Optimum		7.0	7.2
V_{max}, Ca^{2+} [a]	−CAM	13.0 ± 4	40.0 ± 6
	+CAM	18.0 ± 5	44.0 ± 5
K_m, Ca^{2+} [a]	−CAM	43.0 ± 5	10.0 ± 2
	+CAM	19.0 ± 2	10.0 ± 4
V_{max}, ATP [b]	−CAM	7.5 ± 1	28.0 ± 5
	+CAM	11.0 ± 1	31.0 ± 4
K_m, ATP [b]	−CAM	105.0 ± 5	14.0 ± 4
	+CAM	50.0 ± 2	13.0 ± 4
Activity in the presence of effectors (% of control)			
CAM (1 μM)		180 ± 9	114 ± 5
KCl (20 mM)		146 ± 8	108 ± 6
CAM (1 μM) + KCl (20 mM)		270 ± 20	145 ± 15
IC_{50}, vanadate (μM)		600.0 ± 100.0	230 ± 40
IC_{50}, erythrosine B (μM)		0.5 ± 0.4	0.0125 ± 0.0025
IC_{50}, thapsigargin (μM)		23.0 ± 10.0	≫100
IC_{50}, cyclopiazonic acid (μM)		≫100	≫100

[a] ATP concentration = 3 mM
[b] Ca^{2+} concentration = 15 μM

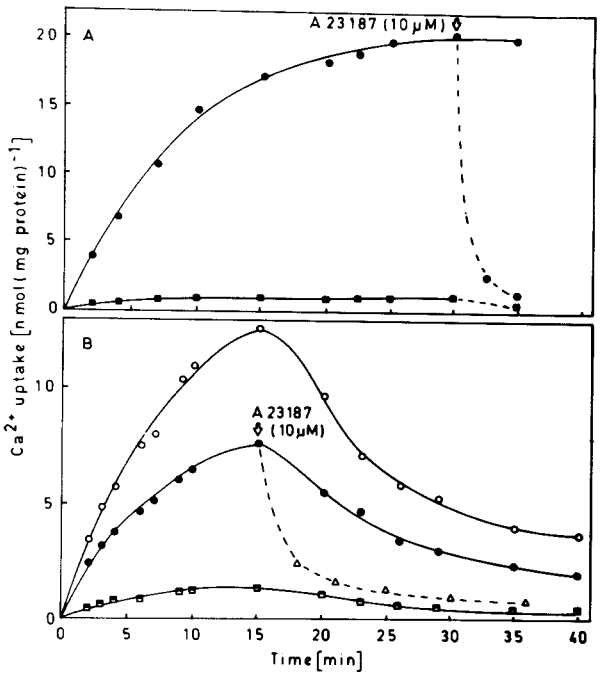

Fig. 10. $^{45}Ca^{2+}$-uptake into PM vesicles (U_3+U_3'-phase) (**A**) or ER vesicles (zone A, cf. Fig. 2, panel A) (**B**) in the absence (□, ■) or presence (○, ●) of ATP (3 mM) using untreated (■, ●) or CAM (1 μM) pretreated (□, ○) membranes from *Bryonia dioica*. At the times indicated by the *arrow*, A23187 (10 μM) was added and $^{45}Ca^{2+}$-efflux monitored for several minutes thereafter (*dashed lines*)

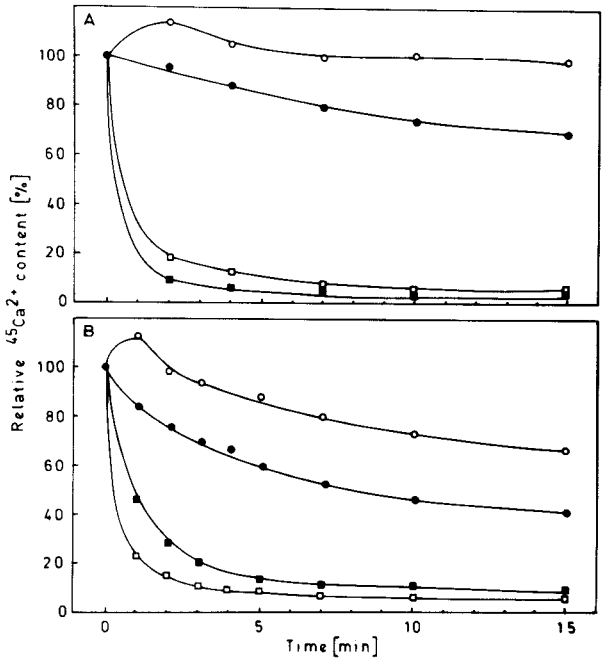

Fig. 11. Efflux of $^{45}Ca^{2+}$ from preloaded PM (U_3+U_3') vesicles (**A**) or ER vesicles (zone A. cf. Fig. 2, panel A) (**B**) from *Bryonia dioica*. After a total loading time of 12 min in the presence of Mg^{2+}-ATP (3 mM, see *Materials and methods* and Fig. 10), the process was stopped by the addition of EGTA buffer (t_o). The efflux of $^{45}Ca^{2+}$ was then monitored over 15 min in controls (●), in the presence of 0.1 mM $LaCl_3$ (○), 10 μM A23187 (■) or 100 μM trifluoperazine (□). 100% = 7.0 (**A**), 15 (**B**) nmol $^{45}Ca^{2+}$ per mg of protein

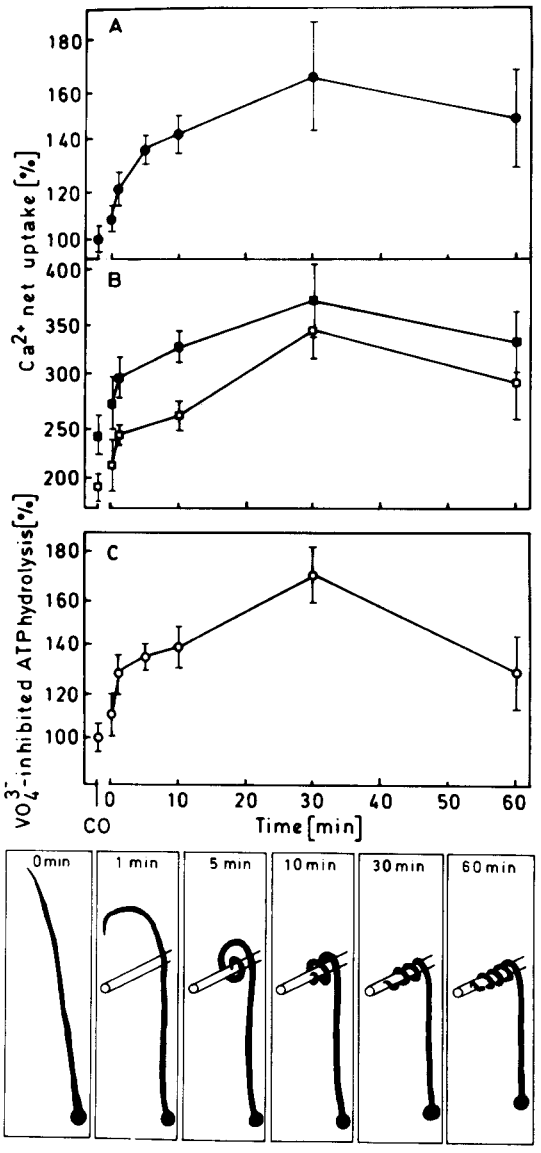

Fig. 12A, B. Mg^{2+}-ATP-driven $^{45}Ca^{2+}$-transport into microsomal vesicles prepared from touch-stimulated tendrils of *Bryonia dioica*. **A** Net $^{45}Ca^{2+}$-uptake into the vesicles, mean ± SD of $n=4$ experiments. 100% = 3.6 pkat per mg of protein; (**B**) effect of added CAM (□, 1 μM) or CAM (1 μM) plus KCl (20 mM) (■), $n=3$ experiments. **C** Kinetics of vanadate-sensitive K^+, Mg^{2+}-ATPase activity from microsomes of touch-stimulated tendrils (mean ± SD of $n=6$ experiments). 100% = 3.2 nkat per mg of protein. t_o, unstimulated tendrils harvested and frozen immediately; *CO*, unstimulated tendrils harvested by freeze-clamping. The lower panel gives the stage of the coiling process for the times indicated

not. The same holds true for the stimulation by KCl. Thus, it is clear that the ER Ca^{2+}-translocating ATPase is subject to regulation by the level of cellular calcium through CAM as well as, probably, potassium, while the enzyme in the PM is insensitive to these modulators. The mitochondrial activity is likewise not affected by CAM (cf. Fig. 2B), KCl or CAM+KCl (data not shown). The IC_{50} for CAM stimulation of the ER Ca^{2+}-translocating ATPase is approximately 0.2 μM (cf. Fig. 9). The stimulation is a result of increased binding affinity of the enzyme for both, Ca^{2+} as well as ATP,

Table 3. Stimulation of tendril ATPases during early coiling. Microsomes from freeze-clamped control tissue (CO) or from trendrils of *Bryonia dioica* after 30 min of continuous touch stimulation (cf. Fig. 12, lower panel) were separated by sucrose density step gradients in the presence of EDTA (minus Mg^{2+}), and fractions corresponding to zones A, B and C (see Fig. 2) were analyzed. Data are means or means ± SD of $n=3$ independent experiments

Sample	Assay pH	Enzyme activity		Increase [%]
		CO	30 min	
$^{45}Ca^{2+}$-transport [pkat·(mg protein)$^{-1}$]:				
Zone A	7.0	5.6	9.5	70±20
Zone B	7.2	2.4	3.5	45±15
Zone C	8.0	1.8	1.7	0±10
VO_4^{3-}-sensitive K^+, Mg^{2+}-ATPase [nkat·(mg protein)$^{-1}$]:				
Zone B	6.3	4.0	6.6	65±20
Zone A	6.3	3.5	3.9	15±12

as evident from the approximately 50% lower K_m values for both substrates (cf. Table 2). A concomitant, though slight, positive effect on V_{max} was also consistently observed.

Plasma-membrane vesicles loaded with $^{45}Ca^{2+}$ remained tightly sealed, as evident from the slow loss of $^{45}Ca^{2+}$ from the vesicles after stopping the loading reaction (Figs. 10A, 11A). However, treatment with A23187 instantaneously discharged the accumulated calcium showing that it was indeed contained within and not just bound to the vesicle (Fig. 10A). The release of $^{45}Ca^{2+}$ from PM vesicles was completely blocked by 100 µM $LaCl_3$, and, like A23187, trifluoperazine discharged the intravesicular pool of $^{45}Ca^{2+}$ (Fig. 11A). In contrast to the PM vesicles, ER vesicles accumulated $^{45}Ca^{2+}$ for a shorter period of time (15 min, cf. Fig. 10B), but then spontaneously released it again even in the continuous presence of Mg^{2+}-ATP and extravesicular $^{45}Ca^{2+}$ (see Fig. 10B). The A23187-induced discharge of $^{45}Ca^{2+}$ associated with the ER vesicles proved that the ion had been accumulated within the vesicles in a freely exchangeable form. Trifluoperazine was even more effective than A23187 in discharging this pool of $^{45}Ca^{2+}$ (see Fig. 11B) while $LaCl_3$ inhibited the efflux of Ca^{2+} from the vesicles only partially (cf. Fig. 11B).

Activities of ATPase in tendrils undergoing coiling. Due to the large numbers of tendrils required for the time-course analysis, ATPase determinations had to be carried out on microsomal membranes. It can be seen from Fig. 12, however, that during the course of mechanical stimulation, there is a rapid rise in the activities of both the vanadate-sensitive K^+, Mg^{2+}-ATPase (presumably the PM proton pump) and the Ca^{2+}-translocating ATPase activity which both reach a maximum after approximately 30 min of continuous touch stimulation. This increase in the activities of these enzymes appears to be transient. However, the period after 60 min of stimulation, i.e. when free-coiling sets in (usually after 2–3 h) has not been covered by the present study. The appearance of the tendril showing the stages of contact-coiling covered by the experiment can be derived from Fig. 12. Up to the stage reached after 5 min, the reaction is largely reversible, but later changes will be manifest. As panel B, compared to panel A, (Fig. 12) shows, the effects of mechanical stimulation, KCl and CAM on the microsomal $^{45}Ca^{2+}$ transporters remain additive at all stages analyzed.

In the final experiments, microsomes from control tendrils, harvested by freeze-clamping, and from tendrils stimulated for 30 min were separated on sucrose density step gradients containing EDTA, and the fractions cor-

Table 4. Kinetic parameters for the *Bryonia dioica* tendril ATPases from control (*CO*) or mechanically stimulated tendrils harvested after 30 min of continuous touch stimulation (30 min). Averages from $n=3$ independent determinations. For description of the sample composition, see text as well as Fig. 2 and Table 1). *n.a.* not applicable

Sample		Assay-pH	Enzyme activity			
			V_{max}, Ca^{2+}	K_m, Ca^{2+}	V_{max}, ATP	K_m, ATP
Ca^{2+}-transport:			[pkat·(mg protein)$^{-1}$]	[µM]	[pkat·(mg protein)$^{-1}$]	[µM]
Zone A	CO	7.0	18	67	7.0	120
	30 min	7.0	18	33	11.0	73
sER	CO	7.0	17	67	5.8	108
	30 min	7.0	17	40	7.8	65
rER	CO	7.0	13	63	3.5	125
	30 min	7.0	13	33	5.3	77
Zone B	CO	7.2	20	170	2.1	280
	30 min	7.2	20	110	2.7	150
VO_4^{3-}-sensitive K^+, Mg^{2+}-ATPase:					[nkat (mg·protein)$^{-1}$]	[µM]
Microsomes	CO	6.3		n.a.	2.5	870
	30 min	6.3		n.a.	4.6	645
Zone B	CO	6.3		n.a.	4.7	490
	30 min	6.3		n.a.	6.7	350
(U_3+U_3') phase	CO	6.3		n.a.	29.0	340
	30 min	6.3		n.a.	30.5	330

responding to zone A, B and C (see Fig. 2) were analyzed separately. The results (Table 3) show that the mitochondrial Ca^{2+} transport remains unaffected by mechanical stimulation, while the zone-B activity, shown above to contain the PM Ca^{2+}-translocating ATPase, is 45% more active. The ER-associated Ca^{2+} transport, however, is stimulated by 70%. The analysis also suggested that the increase in the total microsomal vanadate-sensitive ATPase observed after mechanical stimulation (cf. Fig. 12) involves activation of the PM-associated vanadate-sensitive K^+, Mg^{2+}-ATPase (Table 3, zone B enzyme). A detailed kinetic comparison of the respective enzymes from control versus stimulated tissue is provided in Table 4. It can be seen that the Ca^{2+}-translocating ATPase from the various ER fractions as well as zone B (containing PM) are affected in a similar way, in that K_m values for both, Ca^{2+} and ATP, were significantly lower as compared to controls and the V_{max} value for ATP was found also to be increased. A reduced K_m value for ATP and increased V_{max} were likewise observed for the vanadate-sensitive K^+, Mg^{2+}-ATPase (total microsomal or zone-B activity), but no change in these parameters was evident when PMs (U_3+U_3') prepared by phase partitioning were analyzed (cf. Table 4). Thus, activation of at least three ion pumps localized on at least two cellular membranes, the ER and the PM, occurs during early reactions of tendrils to tactile stimulation.

Discussion

The molecular basis of touch perception in tendrils of Cucurbitaceae, among the most touch-sensitive organs evolved in higher plants, is currently under study in our laboratory. During the course of this work, the need arose to develop techniques for the preparation of highly pure ER and plasmalemma from tendril tissue, which is available only in very limited quantity. Discontinuous or continuous sucrose density gradients did not afford suitably enriched PM. We therefore used dextran-polyethyleneglycol phase-partitioning (Larsson 1985) to remove contaminating membranes. Usually, repetitive partitioning steps (three to five) are carried out, keeping the composition of the phase systems constant (Larsson 1985; Gräf and Weiler 1989). Plasma membranes thus prepared from *Bryonia dioica* tendrils remained contaminated with chloroplast membranes (data not shown). This is also observed for other dicotyledonous tissues (e.g. Feyerabend and Weiler 1988). We noticed that a considerable improvement was obtained by washing the dextran-rich upper phase with a lower phase of increased polyethyleneglycol content as specified in *Materials and methods*. This treatment reduced chlorophyll from the U_3+U_3' upper phases to undetectable levels (Table 1). The enrichment factor for all PM markers was >4 compared to microsomes with yields >30%, i.e. as good as one finds for monocotyledonous tissues (e.g. Larsson 1985; Oecking and Weiler 1991). These usually lend themselves better to aqueous two-phase partitioning. Plasma membranes prepared by phase partitioning, although considerably purer than those obtained with other techniques, may contain remnants of ER and mitochondrial membranes. This was also noted in the case of *Bryonia* (see Table 1). We have been able to remove these effectively on a discontinuous sucrose density gradient containing EDTA in the absence of Mg^{2+} (cf. Fig. 6). Plasma membrane prepared by this two-step procedure is highly pure and consists of tightly sealed vesicles. Likewise, the two-step density-shift technique used to prepare ER yields a highly enriched fraction (from the original rough ER) virtually free of PM and other contaminants.

Access to different membrane fractions from tendril tissue has allowed the study and comparison of several membrane-associated ATPases involved in ion transport in this tissue. The early reactions of tendrils to an appropriate tactile stimulus are reversible and most probably involve turgor changes in the organ (e.g. Jaffe and Galston 1966a, 1968a). It will be important to understand the role of ion-translocating ATPases as well as other ion transporters in this context. Towards this end, Ca^{2+}-transporters were studied. Calcium transients as a reaction to mechanical stimulation were noted even in normal leaf tissue (Knight et al. 1991), suggesting that both the release of calcium from, as well as its relocation into, storage compartments, may be essential components of early reactions of plants to mechanical forces.

We analyzed Mg^{2+}-ATP-driven Ca^{2+} transport into vesicles prepared from different cellular membranes. Transporters accessible through this approach are (i) primary active Ca^{2+}-translocating ATPases and (ii) Ca^{2+}/H^+-exchangers energized by the proton motive force generated by H^+-translocating ATPases. Both systems have been shown to occur in plants in different membranes (e.g. Malatialy et al. 1988; Pfeiffer and Hager 1993). We have no evidence for a substantial contribution of a Ca^{2+}/H^+-exchange mechanism in our membrane preparations under the assay conditions. This conclusion rests on the fact that total microsomal ATP-driven Ca^{2+} transport was only weakly affected by the protonophore CCCP. Proton gradients are effectively dissipated by 5–10 μM CCCP (Zocchi and Hanson 1983; Malatialy et al. 1988); however, this treatment reduced Ca^{2+} transport by only 10%. Furthermore, the observation that for Ca^{2+} accumulation into vesicles of total ER (zone A, Fig. 2), ATP could be replaced by ITP, which is not a substrate of the H^+-ATPase (Fig. 8), but drives plant Ca^{2+}-ATPases (Gräf and Weiler 1989; De Michelis et al. 1993; for review, see Briskin 1990) excludes a Ca^{2+}/H^+-exchange mechanism for most, if not all, of the Ca^{2+}-transport capacity seen in microsomes from *Bryonia* tendrils.

At least three distinctly different Ca^{2+}-translocating ATPases were detected in tendril membranes based on enzymatic, kinetic and inhibitor data (Table 2, Fig. 2). These occur at the PM, the ER and a mitochondrial fraction. A fourth enzyme at the ER is suggested from some of the inhibitor data which, upon close inspection (data not shown), show that approx. 10–15% of the total ER Ca^{2+}-ATPase activity is highly sensitive to submicromolar concentrations of cyclopiazonic acid and thapsigargin, while the bulk activity has lower sensitivity to thapsigargin (Table 2) and is insensitive to cyclopiazonic acid up to 100 μM.

The picture thus emerging is the following: the PM of *Bryonia dioica* tendrils contains a Ca^{2+}-translocating ATPase which is strongly inhibited by nanomolar levels of erythrosine B, but is insensitive to thapsigargin and cyclopiazonic acid. This enzyme is not stimulated by potassium or CAM. The bulk of the ER Ca^{2+}-ATPase located at the rER is a distinctly different enzyme. It is activated by potassium and CAM, less sensitive to erythrosine B and inhibited by micromolar levels of thapsigargin, but not by cyclopiazonic acid. Overall, this enzyme is reminiscent in its properties of the animal PM Ca^{2+}-ATPase, best characterized from the erythrocyte membrane (for review, see Carafoli 1992). The tendril's PM Ca^{2+}-ATPase has no direct counterpart in animal cells. Altogether, our data are in agreement with other recent reports on plant PM and/or ER Ca^{2+}-ATPases (Gräf and Weiler 1989; Liß et al. 1991; Askerlund and Evans 1992; Chen et al. 1993; Thomson et al. 1993) with respect to substrate specificities, sensitivity towards a range of inhibitors and activation by CAM of these enzymes. However, in some species, a CAM-stimulated Ca^{2+}-ATPase associated with the PM was reported (see discussion in Briskin 1990; De Michelis et al. 1993). Whether this represents yet another type of enzyme or contamination of the PM by ER remains to be seen, and requires the use of rigorously purified PMs.

The sER/tonoplast fraction from tendrils also contains at least one Ca^{2+}-translocating ATPase with overall properties very similar to the enzyme we characterized from the rER membrane fraction. It is likely that these two enzymes are identical. However, at present we cannot wholly discount the tonoplast as a potential site of localization of the enzyme although this is highly unlikely. While earlier studies have led to the conclusion that the tonoplast membrane may contain a (low-affinity) Ca^{2+}/H^+-exchanger (e.g. Malatialy et al. 1988), but no primary active Ca^{2+}-pump, this view must be questioned based on recent findings from roots of *Zea mays* (Pfeiffer and Hager 1993). The authors have conclusively demonstrated the presence, in tonoplast membranes from this tissue, of a novel high-affinity Ca^{2+}-translocating ATPase with a high sensitivity to vanadate ($IC_{50} = 43$ μM) and diethylstilbestrol ($IC_{50} = 5.2$ μM) and a nucleotide acceptance similar to other Ca^{2+}-ATPases, including the enzymes from tendril tissue. If a similar enzyme is present in the sER/tonoplast fraction, it can constitute only a minor fraction of observed activity for several reasons: (i) We did not obtain evidence, within the limits of experimental error, for a highly vanadate-sensitive Ca^{2+}-ATPase associated with the zone-A activity. If the enzyme in the sER/tonoplast fraction, representing 50% of the total ER-associated activity, were of the novel tonoplast-type, its vanadate-sensitivity would have shown up in the inhibitor curves. (ii) The distribution of Ca^{2+}-ATPase activity between the "rER" and the "sER/tonoplast" fraction is identical to the distribution of the ER marker, NADH-cytochrome *c*-reductase (approximately 50:50) in the two fractions. (iii) In all properties the two enzymes were indistinguishable (cf. Fig. 5).

The operation of several Ca^{2+}-pumps located in different membranes and subject to separate modes of regulation allows the cell to adjust calcium levels in a flexible manner. The tendril's PM Ca^{2+}-ATPase has all the properties of a housekeeping enzyme, i.e. low K_m for its substrates, ATP and calcium, high capacity as evident from its V_{max} values, and the enzyme shows, as far as is known, little dependence on its ionic environment (K^+, Ca^{2+}). On the other hand, the ER enzyme is sensitive to K^+ and responds to Ca^{2+} via CAM. Calmodulin improves enzyme performance by lowering K_m for both ATP and Ca^{2+} and increases V_{max} moderately. Without CAM, the K_m values of this enzyme for both, Ca^{2+} and ATP, are considerably higher than those of the PM enzyme (see Table 2). Thus, at low cytoplasmic calcium levels, the ion would mainly be moved through the PM Ca^{2+}-ATPase, i.e. it would be exported from the cell into the apoplasm. This process may serve to continuously remove calcium entering the cell through passive diffusion, and the enzyme thus serves to maintain low cytoplasmic calcium levels. Any increase in cytoplasmic calcium, on the other hand, would activate, through CAM, the ER enzyme thus allowing effective retrieval of Ca^{2+} into the ER stores. The ER enzyme could consequently be part of a regulatory loop producing calcium transients in the tendril. Whether such transients occur in tendrils or are required as part of the process of mechanotransduction, remains to be seen. Evidence for mechanically induced calcium transients is, however, accumulating even for plant tissues not specifically evolved to sense mechanical force, such as the leaf (Braam and Davis 1990; Knight et al. 1991). Work in our laboratory has demonstrated that the reactions of tendrils to touch cannot be blocked by low (micromolar) levels of Gd^{3+}, but can be eliminated effectively by higher (>100 μM) levels of this ion (data not shown), suggesting, in accordance with the results obtained with tobacco (Knight et al. 1991), that calcium release from intracellular stores rather than calcium entering the cell through the PM is involved in tendril coiling. In addition, a Gd^{3+}-sensitive calcium channel has recently been identified at the ER of *B. dioica* trendrils. Taken together, our data suggest an important role for intracellular calcium in the process of mechanoperception and -transduction in tendrils, and possibly further for the reactions of tendrils to tactile stimulation. This view is supported by the observation that the activity of Ca^{2+}-translocating ATPases on both the ER and the PM, rapidly and transiently increase during mechanical stimulation of a tendril (cf. Fig. 12). In this context, it is interesting to note that Bourgeade et al. (1991) have observed that mechanical stress, applied to internodes of *B. dioica*, increases the activity of a microsomal ATP-dependent Ca^{2+} transport. The enzyme properties are similar to those of our ER activity. As Fig. 12 shows, the mechanism of activation of the ER enzyme is different from its stimulation by CAM or KCl, as the three factors act in an additive manner (cf. Fig. 12A with B). Also, the PM Ca^{2+}-translocating ATPase and the vanadate-sensitive ATPase, which is presumably the proton-pump and is stimulated by KCl, both do not respond to CAM and are both activated with the same kinetics. The molecular events underlying touch-induced activation of these enzymes are yet unknown and will be the subject of further stu-

dies. From the available data, it is, however, clear that several ion-translocating ATPases and different regulatory mechanisms operate in the early steps of tendril coiling.

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