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Calcium Fluxes across the Plasma Membrane of *Commelina communis* L. Assayed in a Cell-Free System[1]

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ABSTRACT

The inside-out fraction of plasma membrane-rich vesicles prepared from leaves of *Commelina communis* L. by aqueous two-phase partitioning was loaded with $^{45}Ca^{2+}$ through the action of the plasma membrane Ca^{2+}-ATPase. While the Ca^{2+}-loaded vesicles were tightly sealed, trifluoperazine (TFP) (effective concentration giving 50% of maximum effect [EC_{50}] = 70 micromolar) and W-7 (EC_{50} = 100 micromolar), but to a much lesser extent, W-5 (EC_{50} = 500 micromolar) led to a rapid efflux of $^{45}Ca^{2+}$ from the vesicles. This efflux could be blocked efficiently with low (<1 millimolar) concentrations of La^{3+}, but it remained unaffected by the addition of calmodulin (CM). Further experiments with vesicles incubated in $^{45}Ca^{2+}$ in the absence of ATP, as well as experiments performed with control liposomes and nonloaded as well as Ca^{2+}-loaded plasma membrane vesicles using the indicator dye arsenazo III showed, that TFP and W-7 and, again to a lesser extent, W-5 mobilized a pool of membrane-bound Ca^{2+} from the vesicles. No indications for a detergent effect of TFP and W-7 were obtained. The EC_{50}-values of these compounds for mobilizing membrane-associated Ca^{2+} (TFP = 100 micromolar, W-7 = 100 micromolar, W-5 = 500 micromolar) or for the triggering of Ca^{2+} release from Ca^{2+}-loaded vesicles (see above) were very similar, suggesting a common basis of antagonist action on both processes. Our results suggest the presence of a Ca^{2+} channel in the plasma membrane of *C. communis*. The channel is obtained in a Ca^{2+}-inactivated state after preparation and Ca^{2+}-loading of the vesicles. The inactivation is removed by TFP or W-7, presumably due to the Ca^{2+}-mobilizing effect of these compounds. The activated Ca^{2+} channel is La^{3+} sensitive and, in the cell, would allow for passage of Ca^{2+} into the cell. The possibility that TFP or W-7 act independent of CM, or through CM tightly associated with the plasma membrane, is discussed. The system described allows a cell free analysis of Ca^{2+} influx, displaying channel properties, in a higher plant.

Calcium is intimately involved in the maintenance of cell structure and function. This becomes evident from the extremely severe effects of Ca^{2+} deficiency on plant development. Intracellular Ca^{2+} plays an important role in the regulation of a multitude of cellular processes (22) and some of the molecular mechanisms are now becoming clearer. The interaction of the Ca^{2+} ion with cellular components might directly or indirectly alter their function. For example, it has been shown recently by Schroeder and Hagiwara (38) that a rise in the level of $[Ca^{2+}]_c$[2] inactivates inward-rectifying K^+ channels and triggers an anion efflux leading to depolarization and opening of K^+ efflux channels in the plasma membrane of guard cells of *Vicia faba*. Thus fluctuations of $[Ca^{2+}]_c$ are key to an understanding of the osmoregulation of guard cells and, most likely, of plant cells in general. The presence of CM (8, 26 and refs. therein) and other Ca^{2+}-binding proteins (28) in plants and the multitude of processes modulated by or dependent upon calmodulin provides further support for a central role of Ca^{2+} in the regulation of plant cell function. Calcium enters the cell from the apoplastic compartment and is sequestered into several organelles such as the vacuole, the ER or the mitochondria, while $[Ca^{2+}]_c$ is maintained at a relatively constant resting level of approximately 150 to 400 nM (14–16). These steep Ca^{2+} gradients established across plant cell membranes, most notably across the plasma membrane and the tonoplast might provide the basis for the triggering of certain cell functions through flucations of $[Ca^{2+}]_c$. It has been shown that upon irradiation of *Mougeotia* cells with red, but not with far red light, $^{45}Ca^{2+}$ rapidly associates with and presumably is taken up by the algal cells (12). Calcium influx into guard cells of *Commelina communis* L. was recently demonstrated by $^{45}Ca^{2+}$ flux measurements using isolated guard cells in epidermal strips (31) and there is indirect evidence from several studies (1, 19, 21, 23, 29, 34) that Ca^{2+} channels might provide a pathway for Ca^{2+} entry into cells of higher plants.

On the other hand, a Ca^{2+}-translocating ATPase with very high affinity for Ca^{2+} is located at the plasma membrane (3, 17), and this enzyme would efficiently remove intruding Ca^{2+} or Ca^{2+} released from intracellular stores and thus maintain or restore low $[Ca^{2+}]_c$ to terminate a Ca^{2+} message.

While the Ca^{2+}-ATPase of *C. communis* has now been solubilized and reconstituted into liposomes in functional form and its purification is in progress (18), Ca^{2+} influx systems have not been characterized biochemically in plants to date. We describe here a cell-free system from a higher plant which allowed us to demonstrate the presence of a Ca^{2+}

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[2] Abbreviations: $[Ca^{2+}]_c$, cytoplasmic free calcium; CM, calmodulin; EC_{50}, effective concentration giving 50% of maximum effect; TFP, trifluoperazine; W-5, N-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

influx pathway with properties of a Ca^{2+} channel in the plasma membrane of higher plants.

MATERIALS AND METHODS

Plant Material

Commelina communis L. was grown exactly as described (17), at a RH of 80% and 16 h of light (from fluorescent tubes, Osram cool-white NT6, photosynthetically active radiation: 200 $\mu E\ m^{-2}\ s^{-1}$). The temperature was maintained at 25°C (day) and 20°C (night).

Aqueous Two-Phase Partitioning

A plasma membrane-rich vesicle fraction was obtained from leaf microsomes of 3 week old plants using the polyethylene glycol-3350 dextran T 500 two-phase partitioning procedure (30). The details as well as the characterization of the plasma membrane fraction (U_3 + $U_{3'}$) have been reported (17). The plasma membrane vesicles, consisting of 64 to 69% right-side-out and 31 to 36% inside-out vesicles using K^+,Mg^{2+}-ATPase latency, determined in the presence or absence of 0.02% (w/v) Triton X-100, as criterion (17) were finally collected by centrifugation (100,000*g*, 45 min), resuspended in 25 mM Hepes-KOH containing 250 mM sucrose (pH 7.2) and 1 mg/mL of protein, as determined by the Bradford (2) assay and BSA as standard. If not used immediately, membranes were kept frozen at −20°C. Storage under these conditions for up to 14 d did not lead to detectable losses in Ca^{2+} transport activity of the membrane vesicles.

$^{45}Ca^{2+}$ Loading and Ca^{2+} Release

ATP-dependent loading of inside-out, sealed plasma membrane vesicles was performed as described (17). Briefly, 100 μg of plasma membrane vesicles were incubated in 25 mM Hepes-KOH containing 250 mM sucrose, 2 mM $MgSO_4$, 1 mM ATP, and 1 μCi (37 KBq) of $^{45}CaCl_2$ (15–60 TBq mol^{-1}) in a total assay volume of 1 mL (pH 7.2) (assay buffer). If desired, the loading assay was scaled up proportionally. Loading was performed at 23°C for 1 h and then stopped by depleting the ATP by the addition of glucose (1 mM final concentration) plus hexokinase (3 units/ml) (20). Aliquots of $^{45}Ca^{2+}$-loaded membrane vesicles were transferred to 1.5 mL Eppendorf tubes containing 0.12 to 0.6 ml, per 12 μg of protein, buffer (1 mM EGTA, 250 mM sucrose, 25 mM Hepes-KOH [pH 7.2]) and additions as indicated for each experiment. If not indicated otherwise, 100 μM TFP was added at the start of an experiment, and the vesicles were collected by filtration after 10 min at 23°C. If La^{3+} (as $LaCl_3$) or CM was used, these were preincubated with the vesicles for 10 min prior to the addition of TFP. Vesicles were collected by vacuum filtration on Schleicher and Schuell BA 85 nitrocellulose membranes (pore size 0.45 μm). Vesicles (corresponding to 10 μg of protein in a volume of 0.1 to 0.5 mL) were added to the filters presoaked in buffer and washed with 3 mL of assay buffer containing 1 mM EGTA. The filters were then immersed in 3 mL of scintillation fluid (Hydroluma, Baker) and the activity of the filters determined in a Philips PW 4700 liquid scintillation counter. Each experiment was carried out at least three times with different plasma membrane vesicle preparations. The results for one typical experiment are shown.

Spectrophotometric Assay of Ca^{2+}

Ca^{2+}-induced changes in absorption of the indicator dye, arsenazo III (sodium salt, Sigma) were determined in a single beam, dual wavelength spectrophotometer (Sigma ZWS 11) at 650 nm and 730 nm (reference), bandwidth 3 nm. Membrane vesicles (100 μg of protein) were loaded with unlabeled Ca^{2+} (2.5 mM, as $CaCl_2$) as described above and the reaction was stopped by the addition of glucose (1 mM), hexokinase (3 units/mL) and EGTA (2 mM). The vesicles were then washed twice by centrifugation (30 min, 100,000*g*) and resuspended in assay buffer with $MgSO_4$. To determine Ca^{2+} release in the presence of TFP and A23187, the loaded, washed vesicles (100 μg of protein in 0.1 mL assay buffer) were added to 1.8 mL assay buffer without $MgSO_4$ containing arsenazo III (50 μM, final concentration). TFP (100 μM final concentration) and A23187 (5 μM final concentration) were added in the sequences indicated. The absorption changes (ΔA_{650}–A_{730}) were calibrated by the addition of $CaCl_2$ or EGTA (final concentration each: 1 μM). In control experiments, nonloaded plasma membrane vesicles were treated with TFP and A23187 as above and the absorption changes of arsenazo III were recorded. A further control involved the use of liposomes prepared from purified asolectin (soybean phosphatidyl-choline type IV-S) by the detergent dialysis technique in the presence of 2 mM $CaCl_2$ (9).

RESULTS

$^{45}Ca^{2+}$ Loading and Stability of the Vesicles

The preparation of U_3 + $U_{3'}$ phases obtained from leaf microsomes of *Commelina communis* consisted of 31 to 36% inside-out and 64 to 69% right-side-out, sealed vesicles enriched for the plasma membrane markers, vanadate-sensitive K^+,Mg^{2+}-ATPase, glucan synthase II and erythrosin B-sensitive Ca^{2+}-ATPase, by a factor of about 5 as compared to the microsomal fraction. A detailed characterization of the preparation has been published (17). The Ca^{2+}-ATPase of these vesicles has a high affinity for Ca^{2+} (K_m = 4.4 μM) and ATP (K_m = 0.3 mM). When $^{45}Ca^{2+}$ plus Mg^{2+}-ATP (1 mM) are added to the plasma membrane vesicles in the absence of Triton X-100, $^{45}Ca^{2+}$-transport exclusively reflects import into the inside-out fraction of the vesicles (17). Calcium loading was complete in 60 min (Fig. 1). The addition of low levels of the ionophore A23187 in the presence of EGTA (1 mM), to the loaded vesicles resulted in very rapid and nearly completely release of $^{45}Ca^{2+}$ from the vesicles (Fig. 1). The data show that only little (about 2–5%) of $^{45}Ca^{2+}$ remained associated with the vesicles and thus 95 to 98% of total $^{45}Ca^{2+}$ reflects soluble, intravesicular Ca^{2+}. Control vesicles, in the presence of EGTA but in the absence of the ionophore slowly loosed about 20% of their $^{45}Ca^{2+}$ over a period of 1 h (Fig. 1), probably reflecting Ca^{2+} diffusion out of the vesicles. Thus, the plasma membrane vesicles used in our study appear tightly

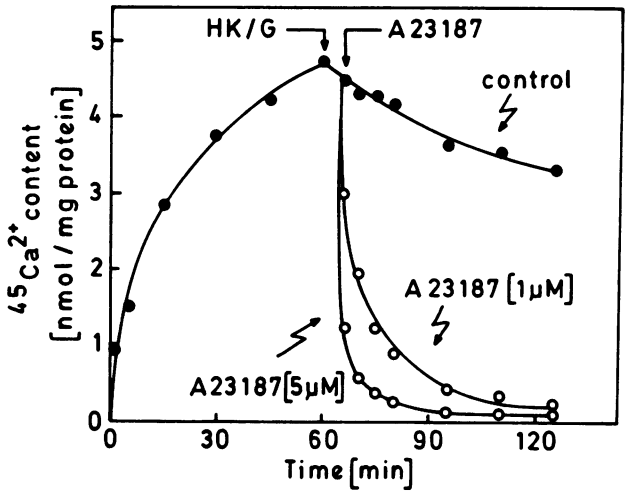

Figure 1. Kinetics of ATP-driven $^{45}Ca^{2+}$ uptake into, and release from, plasma membrane (U_3 + $U_{3'}$) vesicles (10 μg protein per data point) prepared from leaves of *C. communis* L. Ca^{2+} loading was terminated after 1 h by the addition of hexokinase and glucose (HK/G) as well as 1 mM EGTA. While control vesicles (●) were incubated without further additions, intravesicular Ca^{2+} was discharged by the addition of A23187 (○).

sealed for $^{45}Ca^{2+}$. The stability of the $^{45}Ca^{2+}$-loaded vesicles was optimal at 20 to 25°C and nearly independent of assay pH in the range of 5 to 9 (Fig. 2).

TFP-Induced Ca^{2+} Release

The present study was based on the earlier observation that TFP, in micromolar concentrations, appeared to inhibit the plasma membrane Ca^{2+}-ATPase present in the same membrane preparations. This inhibition was shown to be actually due to a loss of Ca^{2+} from the vesicles rather than resulting from an inhibition of the Ca^{2+}-ATPase activity (17). When $^{45}Ca^{2+}$-preloaded vesicles were treated for 10 min at 23°C with TFP, and residual $^{45}Ca^{2+}$ was determined, a progressive loss of the ion at TFP concentrations >20 μM was observed (Fig. 3). Complete loss of $^{45}Ca^{2+}$ occurred at 400 to 500 μM TFP and 50% loss was obtained with about 70 μM TFP. W-7 was also effective although at slightly higher levels ($EC_{50} \approx 100$ μM in different experiments) while the W-7 analog, W-5 was drastically less effective ($EC_{50} \approx 500$ μM).

Low levels of La^{3+}, a potent blocker of Ca^{2+} channels of animal cells, strongly inhibited the TFP-induced efflux of $^{45}Ca^{2+}$ from ATP-loaded vesicles (Fig. 4). Since TFP and W-7 are regarded as effective CM antagonists, whereas W-5 is usually much less potent (24), the influence of CM on $^{45}Ca^{2+}$ efflux from control as well as TFP-treated vesicles was investigated. When $^{45}Ca^{2+}$-loaded vesicles were pretreated with 1 μM CM (from bovine brain) in the presence of Ca^{2+} (0.1 μM–1 mM), no effect on basal as well as TFP-induced $^{45}Ca^{2+}$ efflux was observed. The data suggest that the action of TFP in this system is independent of soluble CM (Fig. 5). It is known that TFP affects membranes in several animal cells through exchange of membrane associated Ca^{2+} (for review, see ref. 7).

To test this hypothesis, plasma membrane vesicles and control liposomes were challenged with either A23187 or TFP in the presence of the Ca^{2+} indicator dye, arsenazo III (Fig. 6, A, B, C). Control liposomes prepared from purified asolectin by the detergent dialysis technique in the presence of 2 mM Ca^{2+} did release only minute amounts of Ca^{2+}, detected as an absorbance change of the indicator dye, when challenged with 100 μM TFP (Fig. 6A, trace b), but released large amounts of (presumably intravesicular) Ca^{2+} after addition of the ionophore A23187 (5 μM, Fig. 6A, trace a). A23187 or TFP, at the concentrations used, neither produced these absorbance changes in the absence of liposomes, nor did these compounds affect the sensitivity of the dye toward detecting changes in Ca^{2+} level (Fig. 6A, trace d, e). From these findings, it appears unlikely that TFP exerts an unspecific, detergent-like effect on plant lipid bilayers at the concentrations used in this study. This conclusion is also supported by the finding that the effect of TFP on $^{45}Ca^{2+}$ release was readily reversible. Plasma membrane vesicles were loaded with $^{45}Ca^{2+}$ through the action of the Ca^{2+}-ATPase as usual and pretreated with 100 μM TFP to deplete them of $^{45}Ca^{2+}$. These vesicles were then collected by centrifugation and washed to remove the TFP. After these pretreatments, the vesicles could be reloaded with $^{45}Ca^{2+}$ in the presence of ATP. This reloading was as effective as the loading of the control vesicles (Fig. 7).

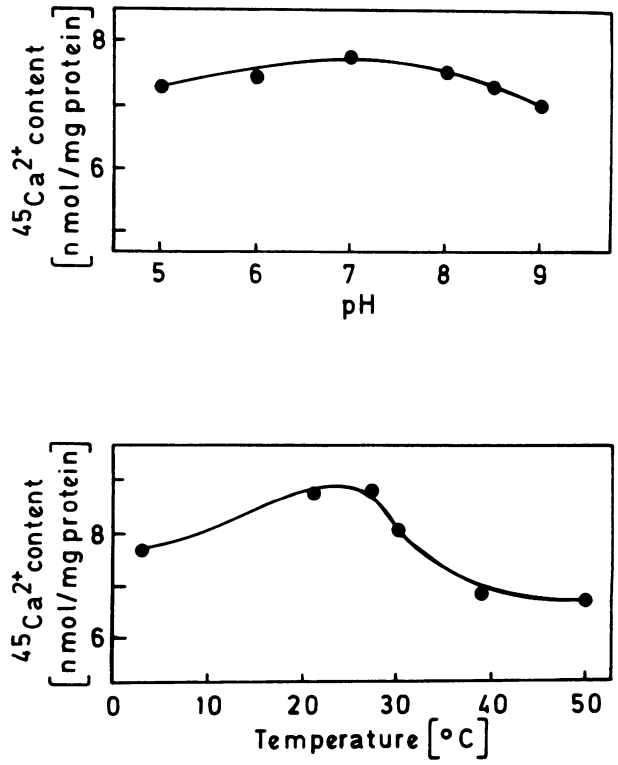

Figure 2. Stability of $^{45}Ca^{2+}$-loaded plasma membrane vesicles of *C. communis*. $^{45}Ca^{2+}$-loaded vesicles (10 μg protein per data point) were incubated for 30 min in buffer containing 1 mM EGTA, pH as shown, at 23°C or at pH 7.2 and temperature as shown and the residual $^{45}Ca^{2+}$ was determined thereafter.

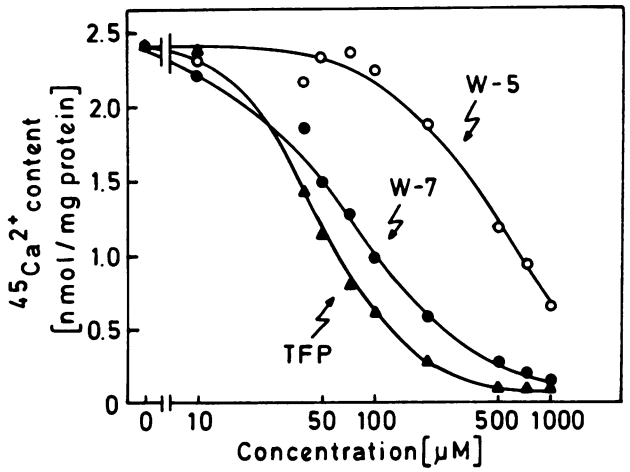

Figure 3. Influence of calmodulin antagonists on the $^{45}Ca^{2+}$ content of vesicles loaded with $^{45}Ca^{2+}$ under standard conditions. After termination of $^{45}Ca^{2+}$ loading by the addition of hexokinase and glucose, the antagonists were added (final concentrations as indicated). Residual $^{45}Ca^{2+}$ was determined after 10 min.

When, instead of liposomes, plasma membrane vesicles (these were not loaded, and used as obtained from two-phase partitioning) were subjected to treatments with A23187 and/or TFP in the presence of arsenzo III, it could be shown that A23187 released only little Ca^{2+}, indicating that only small amounts of Ca^{2+} were trapped inside the vesicles during their preparation and processing. However, if well after the application of A23187, TFP was added, an additional large amount of Ca^{2+} was released (Fig. 6B, trace a), which thus cannot reflect free, intravesicular Ca^{2+}, but rather must represent membrane associated Ca^{2+} (Fig. 6B, trace a). In these experiments, TFP, when given before A23187 (Fig. 6B, trace b) appeared to release not only membrane bound Ca^{2+}, but also the A23187-accessible Ca^{2+} pool to some extent. This became clearer in another series of experiments, in which Ca^{2+}-loaded plasma membrane vesicles were used in the presence of arsenazo III (Fig. 6C). While after A23187 pretreatment, TFP released significant additional amounts of Ca^{2+} from vesicles (Fig. 6C, trace a), A23187 released little Ca^{2+} when given shortly after TFP (Fig. 6C, trace c) and virtually none, when given a few minutes after TFP (Fig. 6C, trace b). Because arsenazo III is not absolutely specific for Ca^{2+} (6), the above results may not reflect exclusively movements of Ca^{2+}. Therefore, additional data were sought from experiments with $^{45}Ca^{2+}$. Plasma membrane vesicles were washed with 1 mM EGTA to remove all freely exchangeable Ca^{2+}. They were then equilibrated with $^{45}Ca^{2+}$ in the absence of ATP, collected by centrifugation and resuspended in $^{45}Ca^{2+}$-free buffer to which A23187 (5 μM) to deplete all intravesicular Ca^{2+} and $MgCl_2$ (2 mM) to reduce nonspecific association of $^{45}Ca^{2+}$ with the membranes were added. TFP and W-7, but to a lesser extent W-5, depleted these membranes from associated $^{45}Ca^{2+}$ (Fig. 8). The relative activities and EC_{50} values of the antagonists were similar to those found to trigger the release of $^{45}Ca^{2+}$ from ATP-loaded vesicles (*cf.* Fig. 3). The radiotracer as well as the dye experiment thus lead to the conclusion that the effect of TFP is dual and consist of (a) the mobilization of a pool of membrane associated Ca^{2+} and (b) triggering of the release of the free intravesicular pool of Ca^{2+}.

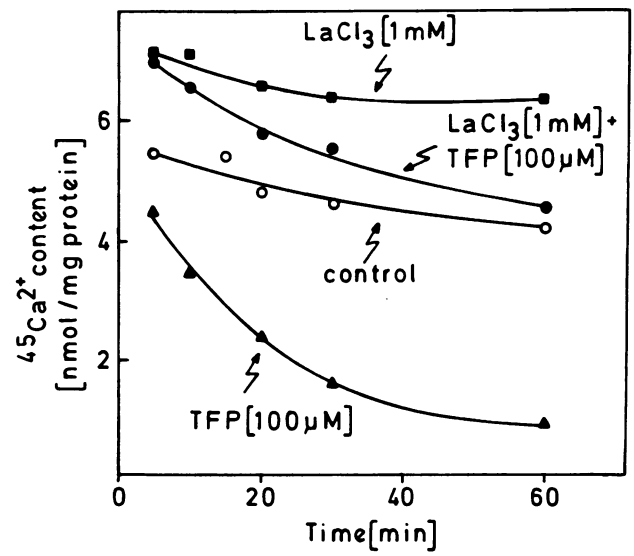

Figure 4. Effect of $LaCl_3$ on TFP-induced release of $^{45}Ca^{2+}$ from loaded plasma membrane vesicles. Following $^{45}Ca^{2+}$ loading under standard conditions, $LaCl_3$ (1 mM final concentration) or buffer as added. TFP (or buffer) was added 10 min later (t = 0 min) to the appropriate vesicles and the kinetics of release of $^{45}Ca^{2+}$ were followed.

DISCUSSION

While it is clear that all Ca^{2+} ultimately enters the plant cell from the extracellular space and although considerable evi-

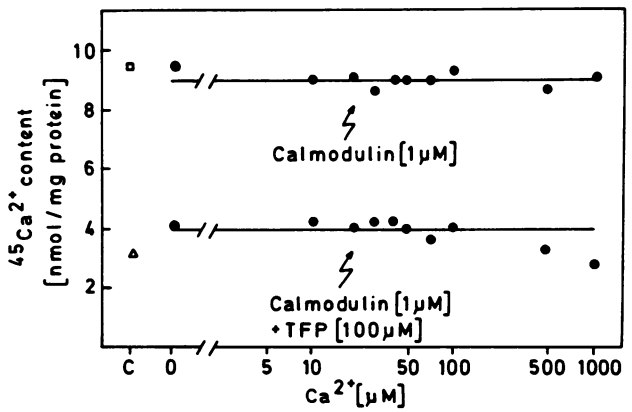

Figure 5. Effect of bovine brain CM on the $^{45}Ca^{2+}$ content of control as well as TFP-treated vesicles. CM (1 μM) together with increasing amounts of Ca^{2+}, final concentrations as indicated, was preincubated with $^{45}Ca^{2+}$-loaded vesicles followed by the addition of TFP (or buffer for controls) in the presence of 1 mM EGTA. Ten min after this addition, residual $^{45}Ca^{2+}$ was determined. C = controls; (□), vesicles loaded in the absence of TFP and CM; (△), vesicles loaded in the presence of TFP, but in the absence of CM).

dence suggests that Ca^{2+} uptake into the cell accompanies many physiological processes, Ca^{2+} influx systems have not been identified or isolated from plants to date. Due to the steep gradients in Ca^{2+} activity across the plasma membrane, a continuous diffusion of the ion into the cytoplasm will take place. However, there is indirect evidence for the presence of additional Ca^{2+} influx systems in the plasma membrane. Studies with protoplasts or membrane vesicles have revealed the occurrence of high-affinity binding sites for organic Ca^{2+} channel blockers, especially of the phenylalkylamine type (1, 19, 21) but also of the dihydropyridine type (11, 23). The inorganic Ca^{2+} channel blocker, $LaCl_3$, strongly inhibits depolarization-induced Ca^{2+} influx into corn root segments (34) as well as other processes seemingly dependent on extracellular Ca^{2+} (36). This would suggest the presence of Ca^{2+} channels in plasma membranes of higher plants. However, due to the difficulties in measuring unidirectional Ca^{2+} fluxes in intact cells or even protoplasts (39), the uncertainties in measuring transients in free cytoplasmic Ca^{2+}, and the problem of detecting putative Ca^{2+} channels against the high background of other conductivities by electrophysiological techniques, evidence remains circumstantial. As a step toward the identification and ultimately, isolation of Ca^{2+} channels from higher plants, a cell free system for the analysis of Ca^{2+} fluxes is required. While a number of earlier studies have conclusively shown an ATP-dependent Ca^{2+} uptake into microsomes or enriched plasma membrane vesicles from several species (4, 5, 10, 13, 20, 32, 33, 35, 40), such Ca^{2+}-loaded vesicles usually loose little Ca^{2+} upon removal of ATP, indicating the absence of active Ca^{2+} channels. In a recent study (17), highly pure plasma membrane vesicles of known orientation, prepared by aqueous two-phase partitioning from *Commelina communis* leaves, were shown to contain a Ca^{2+}-translocating ATPase of high affinity for Ca^{2+}, which transports Ca^{2+} from the cytoplasmic to the apoplastic side of the membrane. This process is conveniently monitored as ATP-driven $^{45}Ca^{2+}$ loading of inside-out vesicles (Fig. 1) and establishes a Ca^{2+} gradient in physiological orientiation across the vesicle membrane. This system was used in the present study to analyze potential Ca^{2+} influx system in the *Commelina* plasma membrane (measured as efflux from $^{45}Ca^{2+}$-loaded vesicles).

While Ca^{2+}-loaded vesicles appeared tightly sealed for Ca^{2+} (Fig. 1) and relatively stable over wide pH and temperature ranges (Fig. 2), they released their $^{45}Ca^{2+}$ rapidly (Fig. 3) upon addition of micromolar concentrations of TFP or W-7. Unspecific or detergent-like effects of TFP have been reported (7, 15, 37), but these can be excluded here for several reasons: (a) Ca^{2+}-loaded asolectin liposomes did release their Ca^{2+} upon additon of the ionophore A23187, but not when TFP was added at levels which were highly effective on $^{45}Ca^{2+}$-loaded plasma membrane vesicles (Fig. 6A, trace b); (b) TFP-induced $^{45}Ca^{2+}$ was strongly inhibited by the Ca^{2+} channel blocker, $LaCl_3$ (Fig. 4); (c) the effect of TFP was readily

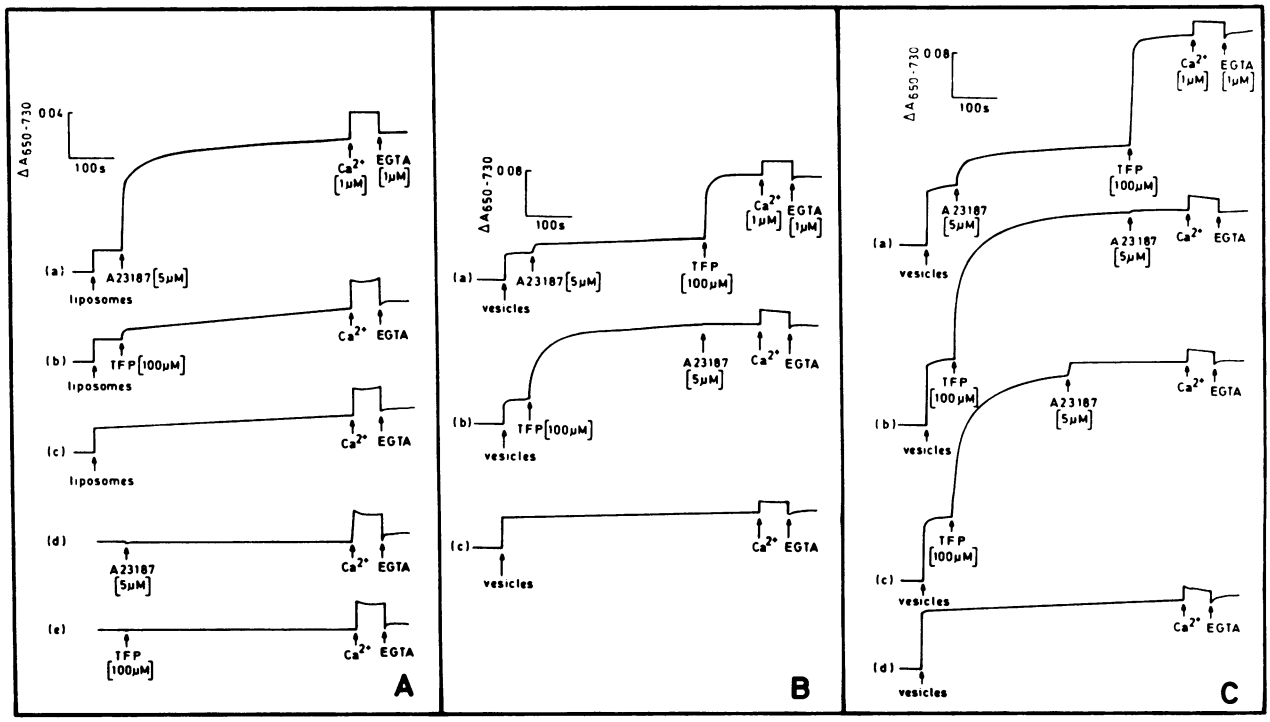

Figure 6. TFP and A23187 induced absorption changes of arsenazo III using asolectin liposomes (A), plasma membrane vesicles (B), or plasma membrane vesicles loaded with Ca^{2+} in the presence of ATP (C). Assays were performed with 50 μM arsenazo III (final concentration of the dye in assay buffer without $MgSO_4$, pH 7.2). Additions were as indicated on each trace: 100 μg protein was used in each experiment (B, C), final assay volume: 2 mL. Controls: the effect of TFP or A23187 on the absorption of arsenazo III in the absence of liposomes or vesicles is shown in A, trace (d) and (e), the absorption changes produced by vesicles or Ca^{2+}-loaded vesicles alone are shown in B, trace (c) and C, trace (d).

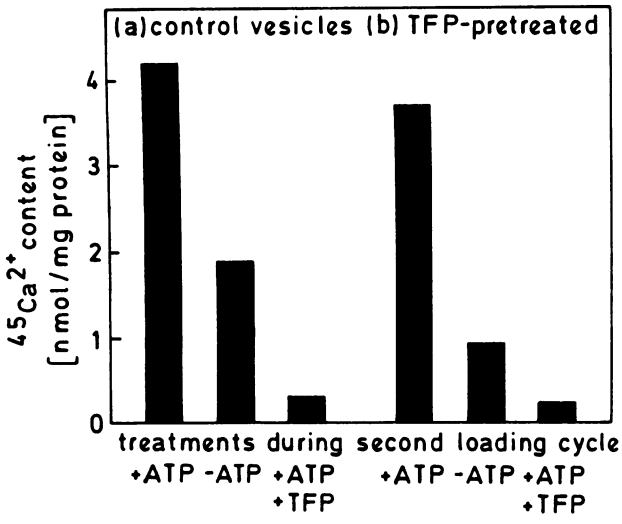

Figure 7. Reversibility of TFP action on Ca^{2+} uptake and release by membrane vesicles of *C. communis*. Vesicles loaded with $^{45}Ca^{2+}$ for 30 min under otherwise standard conditions were incubated in the absence (a) or presence (b) of 100 μM TFP as usual. They were then washed in assay buffer and collected by centrifugation. The two populations of vesicles were then subjected to a second loading cycle in the absence of ATP (for control), in the presence of ATP as well as in the presence of ATP together with TFP.

reversible, *i.e.* addition of the drug did not lead to irreversible damage of the vesicles (Fig. 7); (d) the relative efficiency of TFP and related compounds in *Commelina* reflects their activity as CM-antagonists in other, and even nonplant, systems (15, 24). Our data suggest that TFP, in addition to stimulating release of intravesicular $^{45}Ca^{2+}$, mobilizes a pool of membrane-associated Ca^{2+}. Both, plasma membrane vesicles loaded with Ca^{2+} in the presence of ATP (Fig. 6C, trace a) as well as nonloaded vesicles (Fig. 6B, trace a), released arsenazo III-reactive ions when TFP was added after the vesicles had been depleted from free intravesicular Ca^{2+} by A23187. This fraction therefore cannot represent free intravesicular Ca^{2+}. However, arsenazo III is not absolutely selective for Ca^{2+} and reacts with other ions such as Mg^{2+}, although much weaker (selectivity of Ca^{2+} over Mg^{2+} 4000: 1 [6]). Nevertheless, the lack of absolute specificity of the dye for Ca^{2+} made further controls necessary. These were established using vesicles incubated, at the same time (a) in $^{45}Ca^{2+}$ without ATP (to exchange Ca^{2+} for $^{45}Ca^{2+}$), (b) in the presence of an excess of Mg^{2+} (to prevent $^{45}Ca^{2+}$ from assocating unselectively with negative charges at the vesicle surface) as well as (c) in the presence of A23187 to release all free, intravesicular Ca^{2+}. Such vesicles bound significant amounts of $^{45}Ca^{2+}$ and, upon treatment with TFP, they released this fraction (Fig. 8). Most notably, W-5 and W-7 were also effective, but at different levels. The sequence of effectiveness, TFP ≈ W-7 < W-5 was very similar to that found for $^{45}Ca^{2+}$ release from vesicles loaded with $^{45}Ca^{2+}$ in the presence of ATP (*cf.* Fig. 8 with Fig. 3).

As a working hypothesis from these findings, we suggest that the TFP-induced release of intravesicular Ca^{2+} is, in fact, a consequence of the depletion of the membrane-bound pool of Ca^{2+}. Having made detergent-like effects of TFP unlikely (see above), our data indicate the presence of Ca^{2+} channels in the plasma membrane of *C. communis*, however, which are in a Ca^{2+} inactivated state. This explains why vesicles are so tightly sealed for Ca^{2+}. Inactivation might take place already during preparation of the vesicles. Breaking down the cell structures presumably liberates large amounts of Ca^{2+} from storage compartments and the cell walls. This might quickly inactivate the channels. Inactivation, on the other hand, might also be a consequence of the loading of the vesicles with Ca^{2+}. Calcium is usually applied at ≥10 μM concentrations, *i.e.* well above the normal cytoplasmic Ca^{2+} level which is in the range of 150 to 400 nM(14–16). Inactivation of Ca^{2+} channels by Ca^{2+} is a common regulatory mechanism in animal cells (for review, see refs. 25, 26) and usually aids in the termination of a Ca^{2+} message.

The site of action of TFP and W-7 at the plasma membrane remains uncertain. The sequence of activities shown (Figs. 3 and 8) roughly reflects their CM antagonistic effect in other systems (15, 24). However, at least for TFP, we have shown that exogenous Ca^{2+}-CM does not counteract TFP action (Fig. 5). Similarly, the Ca^{2+}-ATPase of *C. communis* L. was unaffected by added CM (17) while in some other plants it was stimulated by CM (10, 32, 35, 40). In pea, it has been shown recently that the plasma membrane contains large amounts of tightly bound CM which cannot, *e.g.* be washed off with EGTA (8). It is not known, if the situation is similar in *C. communis*, but this is the subject of an ongoing study. Alternatively, a CM-independent action of TFP and W-7 would be possible, including a direct interaction of the compounds with Ca^{2+} bound to membrane lipids and release of Ca^{2+} occluded by the Ca^{2+} pump itself (7, 25).

In an earlier study, Gilroy *et al.* (15) have shown an increase in free cytosolic Ca^{2+} in carrot protoplasts induced by W-7

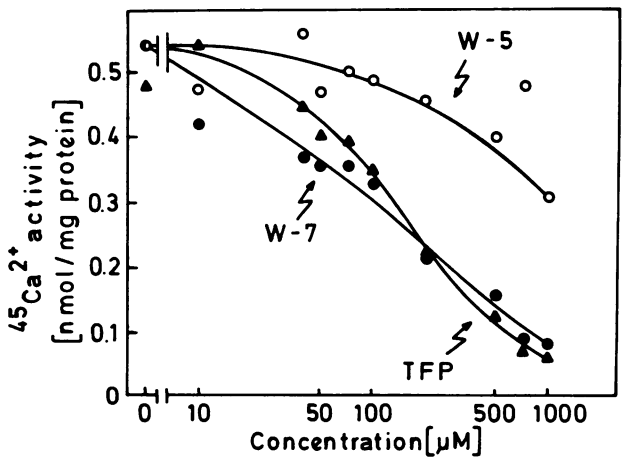

Figure 8. Effect of TFP, W-5, and W-7 on the release of $^{45}Ca^{2+}$ from plasma membrane vesicles preincubated in $^{45}Ca^{2+}$ in the absence of ATP. To remove freely exchangeable Ca^{2+}, the preincubation was carried out in the presence of 2 mM Mg^{2+}, and 5 μM A23187 was added. After an incubation for 10 min, residual $^{45}Ca^{2+}$ associated with the vesicles was determined.

and TFP, but to a lesser extent by W-7 in concentrations comparable to those used in the present study. Gilroy *et al.* attributed this effect to an inhibition of CM. Our data suggest, that an induction of Ca^{2+} influx either alone, or together with an inhibition of Ca^{2+} efflux, might be involved in the TFP and W-7 induced rise of cytoplasmic Ca^{2+} levels in this system.

It is now becoming increasingly clear that plant plasma membranes possess Ca^{2+}-influx systems with channel properties as well as efficient Ca^{2+}-efflux pumps. Inactivation of the influx pathway by a rise in cytoplasmic free Ca^{2+} is indicated from our study while other studies suggest an activation of the efflux pump by high cytoplasmic levels of Ca^{2+}. These two elements would provide an autoregulated switch which would terminate a Ca^{2+} message and restore low cytoplasmic Ca^{2+} levels. Tightly membrane associated CM (8), or a similar, TFP and W-7 sensitive site, could mediate these events. Direct evidence for an involvement of CM in *Commelina* is, however, still lacking (17).

The *Commelina* system will now allow a more detailed cell-free study of Ca^{2+}-influx systems with channel-like properties at the plasma membrane. It will be important to elucidate mechanisms of regulation of Ca^{2+} flux other than the Ca^{2+} inactivation seen in this study. The conditions of our experiments suggest that the Ca^{2+} channel in *C. communis*, after removal of the Ca^{2+} inactivation by TFP, is open when the membrane is depolarized. This again resembles the properties of many voltage-gated, Ca^{2+}-inactivated Ca^{2+} channels is animal cells (24, 26).

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