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Tracheary-element differentiation in suspension-cultured cells of *Zinnia* requires uptake of extracellular Ca^{2+}

Experiments with calcium-channel blockers and calmodulin inhibitors

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Abstract. Tracheary-element (TE) differentiation in suspension cultures of *Zinnia elegans* L. mesophyll cells was inhibited by blocking calcium uptake in three ways: 1) reducing the $[Ca^{2+}]$ of the culture medium, 2) blocking calcium channels with the non-permeant cation La^{3+}, and 3) blocking calcium channels with permeant dihydropyridine calcium-channel blockers. Calcium-channel blockers were effective when added at any time between 0 and 48 h after culture initiation; after 48 h, calcium sequestration and secondary cell-wall deposition began. In contrast, calmodulin antagonists inhibited TE differentiation when added at the beginning of culture, but not when added after 24 h. These results indicate that TE differentiation involves at least two calcium-regulated events: one calmodulin-dependent and occurring shortly after exposure to inductive conditions, and the other calmodulin-independent and occurring just prior to secondary cell-wall deposition.

Key words: Calcium in cell differentiation – Calcium-channel blockers – Calmodulin antagonists – Cell culture and differentiation – Tracheary element differentiation – *Zinnia*

Introduction

Using histochemical methods, we have recently shown that calcium sequestration accompanies the onset of cell-wall thickening in tracheary elements (TEs) developing in suspension cultures of *Zinnia* mesophyll cells (A.W. Roberts and Haigler 1989). One interpretation of these results is that calcium uptake across the plasma membrane and sequestration in intracellular compartments is required for deposition of the patterned secondary wall. However, L.W. Roberts and Baba (1987) have reported that the calcium-channel blocker verapamil does not inhibit TE differentiation in lettuce pith explants.

Differentiating suspension cultures offer advantages over callus or explant cultures for calcium- and calmodulin-inhibitor studies because all cells have equal access to the drugs. An additional advantage of *Zinnia* suspension cultures is that TE differentiation occurs fairly synchronously. This offers the opportunity to investigate the calcium- and calmodulin-dependence of the sequential events in TE differentiation, which include response to cytokinin, gene expression, pattern formation, rearrangement of the cytoskeleton, deposition of secondary cell-wall polysaccharides, lignification and autolysis (reviewed by Fukuda and Komamine 1985).

Calcium-channel blockers from different chemical classes may vary in effect in different organisms and tissues. Each class binds to voltage-dependent calcium-channels at a different receptor site (reviewed by Hosey and Lazdunski 1988). In addition, the ionization state (dependent on the pKa) of calcium-channel blockers can influence their effectiveness (see Hille 1984; Uehara and Hume 1985; Kass and Arena 1989), particularly at the low pH of many plant cell and tissue culture media. The classifications and properties of the calcium- and calmodulin-antagonists used in this study are summarized in Table 1.

In this paper we report that TE differentiation can be inhibited by neutral dihydropyridine calcium-channel blockers. In contrast, verapamil, D-600 and diltiazem, which are charged at culture pH, had no effect on differentiation. By adding inhibitors at various times throughout the culture period we have shown that TE differentiation involves at least two calcium-dependent events; one calmodulin-dependent and one calmodulin-independent.

Abbreviations: BA = N^6-benzylaminopurine; CPZ = chlorpromazine; DIC = differential interference contrast; DMSO = dimethylsulfoxide; IC_{50} = concentration resulting in 50% inhibition; TE = tracheary element; TFP = trifluoperazine; W-5 = N-(6-aminohexyl)-1-naphthylenesulfonamide; W-7 = N-(6-aminohexyl)-5-chloro-1-naphthylenesulfonamide

Table 1. Classification and properties of calcium-channel blockers, calcium-channel activators and calmodulin (CaM) inhibitors used in this study

Drug	Abbrev.	Class	pKa	Function
D600		Phenylalkylamine	8.5	Ca-channel blocker
Verapamil		Phenylalkylamine	6.5	Ca-channel blocker
Nifedipine		Dihydropyridine	~1	Ca-channel blocker
(−)202–791		Dihydropyridine	–	Ca-channel blocker
(+)202–791		Dihydropyridine	–	Ca-channel activator
Diltiazem		Benzothiazipine	7.7	Ca-channel blocker
Chlorpromazine	CPZ	Phenothiazine	–	CaM inhibitor
Trifluoperazine	TFP	Phenothiazine	–	CaM inhibitor
N-(Aminohexyl)-5-chloro-1-Naphthylenesulfonamide	W-7	Naphthylenesulfonamide	–	CaM inhibitor
N-(6-aminohexyl)-1-Naphthylenesulfonamide	W-5	Naphthylenesulfonamide	–	CaM Inhibitor

Material and methods

Culture methods. Mesophyll cells were isolated from the first true leaves of 10–14-d-old seedlings of *Zinnia elegans* L. cv. Envy (Park Seed Co., Greenwood, S.C., USA) and cultured in shell vials (25 mm diameter, 95 mm high) containing 1.75 ml of medium with shaking at 112 rpm on a rotary shaker. Differentiation, as detected by the appearance of cell-wall thickenings seen with differential interference contrast (DIC) microscopy, usually became evident after 50–55 h (all times refer to the time elapsed from the initiation of culture). Differentiation could be detected as early as 48 h by fluorescence microscopy after staining with 0.01% Tinopal LPW (Ciba-Geigy, Greensboro, N.C., USA; CI No. 40622). Additional details of culture conditions have been described in Haigler and Brown (1986) and Fukuda and Komamine (1980a).

Determining extent of differentiation. Cultured cells were scored for percent differentiation after 85–90 h when differentiation was complete. We found no difference in the number of TEs detected with fluorescence microscopy, which is most sensitive for early stages of thickening (Ingold et al. 1987), or DIC microscopy at this time; for convenience, DIC was used for counting mature TEs. Six-hundred cells from each culture were classified and counted as follows: undifferentiated dead cells (D), tracheary elements (T), undifferentiated living cells (L). Percent differentiation was calculated as $[T/(T+L)]\times 100$ and percent cell death as $[D/(T+L+D)]\times 100$. Graphs illustrate mean percent differentiation for a single experiment run in triplicate unless specified otherwise in the figure legend; error bars represent the standard deviation (SD). All experiments were repeated at least twice with similar results. The IC_{50} values (concentration giving 50% inhibition) for dose-response experiments are reported as means of two or three experiments.

Calcium-deprivation experiments. Cells were grown in culture medium in which the added $[CaCl_2]$ was reduced below the standard 1 mM. The free-$[Ca^{2+}]$ in the medium was measured with a Nova 7 Biomedical Ca^{++} Analyzer (Nova; Newton, Mass., USA) after adding cells. In some experiments, $CaCl_2$ was added to calcium-deficient cultures after 48 h.

Drug treatments. Generally, drugs were added at the beginning of culture or just prior to visible secondary wall deposition at 48 h, in order to determine which phases of differentiation were affected. In some cases drugs were added at other times between 0 and 48 h in order to determine the timing of effectiveness with greater resolution.

For La^{3+} treatment, cells grown for 48 h in culture medium containing 20 μM KH_2PO_4 and 0.5 mM $CaCl_2$ were transferred to medium containing 5–100 μM $La_2(NO_3)_3$ and no KH_2PO_4. Lanthanum forms an insoluble precipitate in the presence of PO_4^{-3}, as indicated by Tanimoto and Harada (1986) and confirmed by our observations. Controls were transferred to KH_2PO_4-free medium at the same time. In some experiments, 1–4 mM $CaCl_2$ was added just prior to La^{3+} addition.

Nifedipine (8 mM stock in dimethylsulfoxide (DMSO)) and isomers of 202–791 (8 mM stock in absolute ethanol or DMSO) were added in dim light and the cultures were incubated in the dark to avoid photo-inactivation of these drugs (Morad et al. 1983). Verapamil, D600 and diltiazem were dissolved in culture medium. Controls contained DMSO or ethanol as appropriate.

Calmodulin inhibitors trifluoperazine (TFP), chlorpromazine (CPZ), N-(6-aminohexyl)-5-chloro-l-naphthylenesulfonamide (W-7) and N-(6-aminohexyl)-1-naphthylenesulfonamide (W-5) were dissolved in BA (N6-benzylaminopurine)-free culture medium. When added at t=0, the inhibitors were introduced before the addition of BA to a final concentration of 0.2 mg/l. The W-7 added at the beginning of culture was removed after 24 h by centrifugation and resuspension of cells in inductive (200 mg/l BA) or non-inductive (1 mg/l BA) medium (Fukuda and Komamine 1980a).

Determination of pKa. Verapamil-HCl (10 mM) was titrated with 50 mM NaOH. The pKa value was determined from the titration curve.

Sources of chemicals. Isomers of 202–791 were a gift of Sandoz Ltd. (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

Results

Inhibition of TE differentiation by calcium deprivation. Tracheary-element differentiation was inhibited when the $[CaCl_2]$ (1 mM in normal culture medium) was reduced below 0.5 mM (Fig. 1). When no $CaCl_2$ was added, mean differentiation was reduced about 78% compared to cultures containing 1 mM $CaCl_2$, whereas the percentage of dead cells increased by about 60%. No significant increase in cell death (by the Mann-Whitney U test) was observed when added $CaCl_2$ was reduced to 0.2 mM, while TE differentiation in these cultures was reduced by 20% compared to cultures containing 1 mM $CaCl_2$. Medium to which 0–0.05 mM $CaCl_2$ had been added contained <0.1 mM free Ca^{2+}, the lower

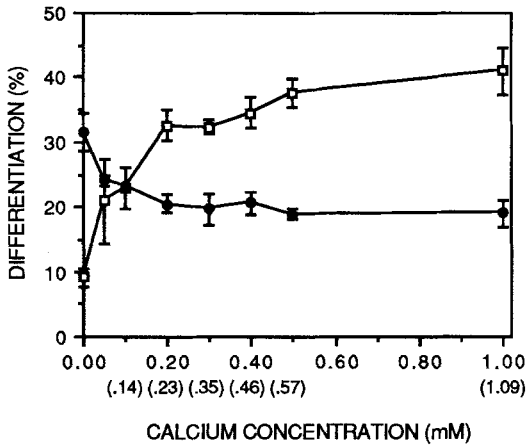

Fig. 1. Inhibition of TE differentiation (□—□) and increase in cell death (●—●) in *Zinnia* cell cultures grown in reduced-calcium medium. The *X-axis* represents [$CaCl_2$] added to the medium; () indicates measured [free-Ca^{2+}]

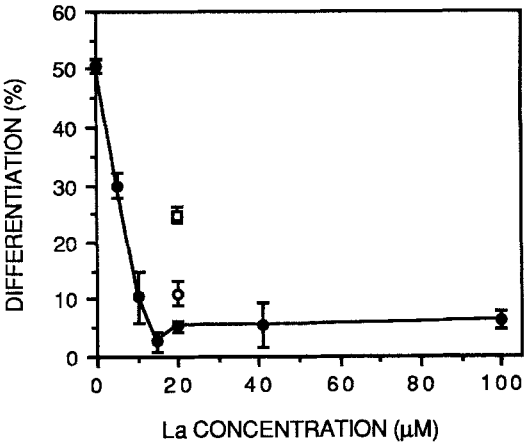

Fig. 2. Inhibition of TE differentiation in *Zinnia* cell cultures by $La_2(NO_3)_3$ added at 48 h. Cells were treated with La^{3+} in the presence of 0.5 mM Ca^{2+} without PO_4^{3+} (●—●). Differentiation percentages for cultures treated with 20 μM La^{3+} in the presence of 1 (○) or 4 (□) mM $CaCl_2$ are also shown

limit of detection of the Nova 7 Ca^{++} Analyzer. Further reduction of free Ca^{2+} with calcium buffers was not pursued because of the increase in cell death already observed. When calcium-deprived cultures (no added $CaCl_2$) were "rescued" by adding 1 mM $CaCl_2$ after 48 h, the mean percent of differentiation was increased from 13.8 to 21.8 ($P<0.005$ by the Mann-Whitney U test).

Inhibition of TE differentiation by lanthanum. Tracheary-element differentiation was inhibited when cells were transferred to phosphate-free medium containing $La_2(NO_3)_3$ and 0.5 mM $CaCl_2$ at 48 h (Fig. 2). Cells transferred at 48 h to phosphate-free medium containing 0.5 mM $CaCl_2$, but no $La_2(NO_3)_3$, differentiated normally. As shown in Fig. 1, the requirement for Ca^{2+} is nearly saturated at 0.5 mM $CaCl_2$. At this [Ca^{2+}], 15 μM La^{3+} resulted in maximum inhibition. Raising

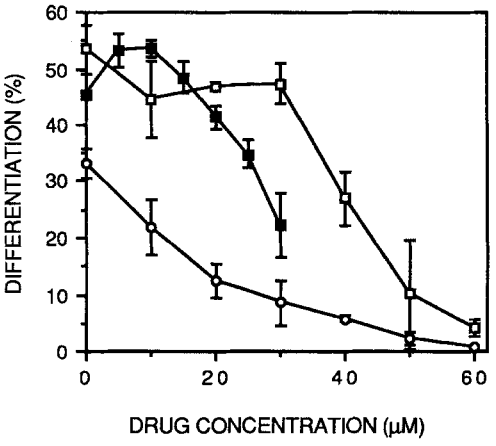

Fig. 3. Inhibition of TE differentiation in *Zinnia* cell cultures by dihydropyridine calcium-channel blockers nifedipine (□—□) and (−)202–791 (○—○) and stimulation by (+)202–791 (■—■). All drugs were added at 48 h. Increase in cell death was insignificant at 30 μM nifedipine and 40 μM (−)202–791; it increased by 32% at 60 μM nifedipine and 24% at 60 μM (−)202–791. (+)202–791 was non-toxic at 10 μM, but a 68% increase in cell death was observed at 30 μM

[La^{3+}] to 100 μM resulted in the same degree of inhibition (approx. 90%) as seen with 15 μM La^{3+}. When La^{3+} was added to cultures containing phosphate, concentrations of 150 μM were required for inhibition (data not shown) in agreement with the results of L.W. Roberts and Baba (1987). Inhibition by La^{3+} added at the initiation of culture was not determined because KH_2PO_4 is required during the first 48 h for normal differentiation (data not shown). When 1 mM or 4 mM $CaCl_2$ was added along with 20 μM La^{3+}, mean differentiation was increased to 11.1% and 24.8%, respectively (Fig. 2; $P<0.05$ by the Mann-Whitney *U*-test).

Inhibition of TE differentiation by organic calcium-channel blockers. The dihydropyridine calcium-channel blockers nifedipine and (−)202–791 inhibited TE differentiation with mean IC_{50} values of 44 μM and 17 μM, respectively, when added to cells at 48 h (Fig. 3). Nifedipine produced a nearly identical dose-response curve when added at the time of culture (data not shown). The calcium-channel-activating isomer (+)202–791 (Hof et al. 1985) stimulated differentiation by about 25% at 5–10 μM (Fig. 3), but was toxic at higher concentrations (nearly 60% increase in cell death at 30 μM). In contrast, the phenylalkylamines, verapamil and D600, and the benzothiazipine, diltiazem, were ineffective when added at 150 μM either at the time of culture or 48 h later (Table 2). Verapamil was also ineffective when applied in the dark (Table 2). Since D600 is most effective at high pH (Triggle 1980), we attempted to culture cells in medium buffered at pH 6.5, but no differentiation was observed under these conditions. Titration of verapamil-HCl indicated a pKa value of 6.5.

Nifedipine (60 μM) and (−)202–791 (50 μM) added at 24 or 47.5 h were not inhibitory if removed 48 h after

Table 2. Percent of control differentiation for *Zinnia* cell cultures treated with the calcium-channel blockers verapamil (in the light or in the dark), D600 or diltiazem at 0 or 48 h or with the calmodulin inhibitors CPZ or TFP at 0, 24 or 48 h. *P*-values were calculated using the Mann-Whitney *U*-test

Drug	Concn. (μM)	Time added (h in culture)	Mean differentiation (% of control)	*n*	*P*
Verapamil	150	0	99.7	6	>0.1
		48	96.1	5	>0.1
Verapamil (dark)	150	0	100.1	6	>0.1
		48	97.2	7	>0.1
D600	150	0	100.3	6	>0.1
		48	101.5	6	>0.1
Diltiazem	150	0	98.1	4	>0.1
		48	99.0	5	>0.1
CPZ	30	0	7.5	5	<0.005
		24	95.4	5	>0.1
		48	89.2	5	>0.1
TFP	12.5	0	9.8	5	<0.005
		24	79.5	5	>0.05
		48	91.4	5	>0.1

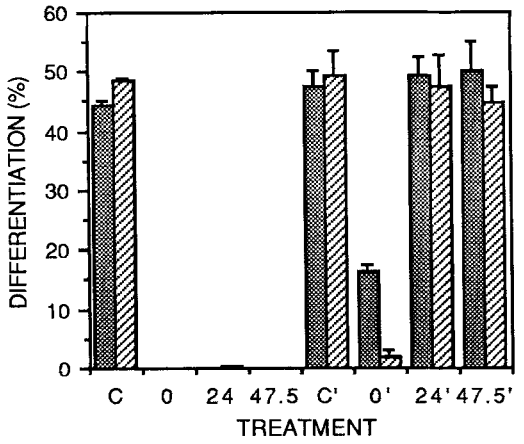

Fig. 4. Differentiation percentages for controls (C) and cultures of *Zinnia* cells treated with 60 μM nifedipine (▨) or 50 μM (−) 202–791 (▨) at 0, 24 or 47.5 h after culturing. Cultures that were washed and resuspended in fresh, inductive medium at 48 h are indicated by '

culturing (Fig. 4). When 60 μM nifedipine or 50 μM(−) 202–791 was added at the time of culture and removed at 48 h, only about 40% recovery for nifedipine and about 5% recovery for (−)202–791 was observed (Fig. 4). Inactivation of nifedipine by ultraviolet light (Morad et al. 1983) was attempted, but 50–60% cell death occurred in nifedipine-treated cultures. The ultraviolet irradiation was not lethal in control cultures containing DMSO, indicating that ultraviolet radiation produced toxic nifedipine derivatives.

When La^{3+} or dihydropyridines were applied at concentrations that did not completely inhibit differentiation, secondary-wall bands in cells that did differentiate appeared thinner than those in control cells (Fig. 5). Tracheary elements that formed in low-calcium medium also had thinner secondary-wall bands. The pattern of wall thickenings, however, was unaltered in either case. When 90-h cultures that had been treated with higher concentrations of La^{3+} or dihydropyridines were observed by light microscopy, the walls of drug-treated cells did not appear to be any thicker than those of cells incubated in non-inductive medium. This indicates that secondary wall deposition, not just patterned wall formation, was inhibited. In contrast, secondary walls in *Zinnia* cells formed in the presence of colchicine clearly appeared thickened with light microscopy even though no pattern was distinguishable (Fukuda and Komamine 1980b).

Inhibition of TE differentiation by calmodulin antagonists. Calmodulin antagonists of the phenothiazine class, CPZ and TFP, inhibited TE differentiation with mean IC_{50} values of 22 μM and 8 μM, respectively (Fig. 6). These values are consistent with the higher anti-calmodulin activity of TFP (Prozialeck and Weiss 1982). Similarly, W-7, of the naphthylenesulfonamide class (Asano and Hidaka 1984), has a higher anti-calmodulin activity and a lower mean IC_{50} (2.5 μM) than W-5 ($IC_{50}=10$ μM, Fig. 7). Because W-7 showed the least variability among replicates, it was chosen for further reversibility and timing experiments. W-7 was used at a concentration of 4 μM, which caused nearly complete inhibition of differentiation with little increase in cell death (Fig. 7).

W-7 was completely inhibitory when added between 0 and 12 h, variably inhibitory when added between 15 and 23 h, and ineffective when added at 27 h or later (Fig. 8). Trifluoperazine and CPZ also caused maximal inhibition when added at the initiation of culture and no significant inhibition (95% confidence) when added at 24 or 48 h (Table 2). We detected no change in the pattern of secondary-wall deposition in cells that developed in the presence of calmodulin inhibitors.

Cells treated with W-7 at the beginning of culture could be "rescued" by washing out the drug with inductive medium after 24 h (Fig. 9). In contrast, when W-7 was washed out with non-inductive medium (containing 0.5% of the inductive concentration of BA) differentiation was not greater than in unwashed W-7 treated cells.

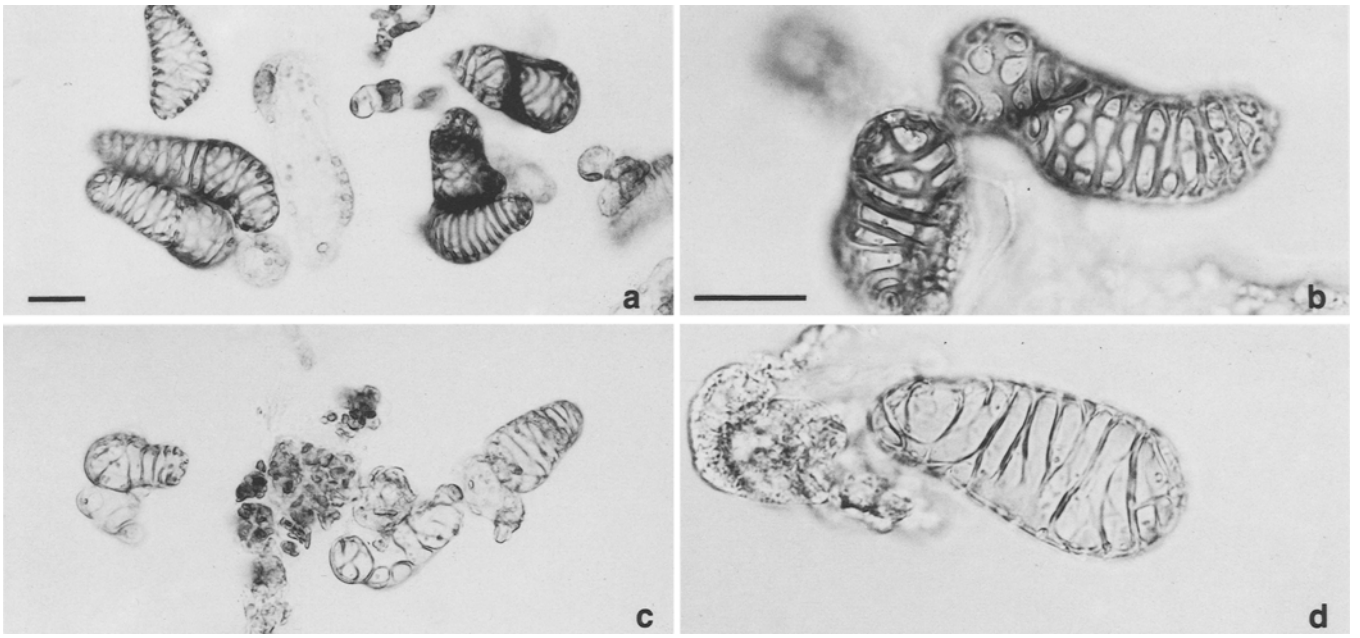

Fig. 5a–d. Control TEs (**a, b**) and TEs that differentiated in *Zinnia* cells in the presence of 40 μM (−)202–791 added at 48 h (**c, d**). Note the thinner appearance of the secondary-wall bands in the (−)202–791-treated TEs. Cells were stained with 1% phloroglucinol in 20% HCl and photographed using a green (546 nm) filter. Bars = 20 μm; **a, c** ×340; **b, d** ×680

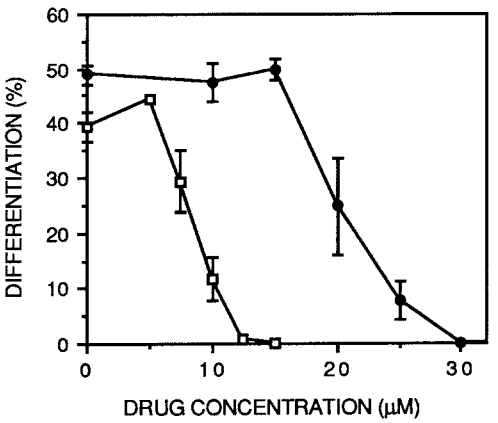

Fig. 6. Inhibition of TE differentiation in *Zinnia* cell cultures by the phenothiazine calmodulin inhibitors trifluoperazine (□—□) and chlorpromazine (●—●) added at the beginning of culture. Increase in cell death was insignificant at 10 μM for both drugs and increased only 20% at 20 μM CPZ. Cell death increased by about 150% at 15 μM TFP and 130% at 35 μM CPZ

Non-drug-treated cells washed with non-inductive medium after 24 h exhibited about 40% of the differentiation in controls at 90 h. The difference between percent differentiation in control and W-7 treated cells washed with non-inductive medium (C/N versus W/N, Fig. 9) was significant at the 99.9% confidence level using the Mann-Whitney *U*-test.

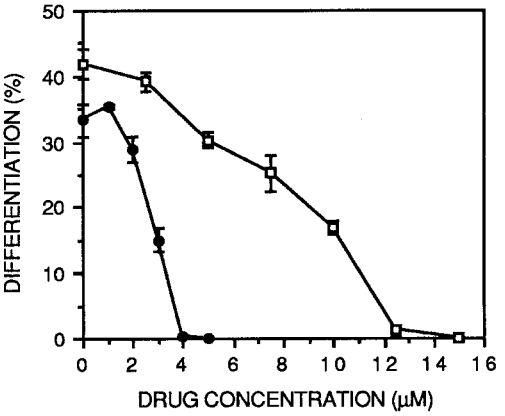

Fig. 7. Inhibition of TE differentiation in *Zinnia* cell cultures by naphthylenesulfonamide calmodulin inhibitors W-7 (●—●) and W-5 (□—□) added at the beginning of culture. Increase in cell death was insignificant at 3 μM W-7 and 12.5 μM W-5; it increased by 63% at 5 μM W-7 and 40% at 15 μM W-5

Discussion

Tracheary-element differentiation requires uptake of extracellular calcium. Inhibition of TE differentiation by dihydropyridine calcium-channel blockers, lanthanum and reduction of extracellular $[Ca^{2+}]$ indicates that uptake of extracellular Ca^{2+} is required for this process in suspension cultures of *Zinnia*. Furthermore, six lines of experimental evidence indicate that the inhibitory effects of these treatments are calcium-specific. *First*, differentiation was partially restored to calcium-deprived cultures by adding $CaCl_2$ at 48 h, just at the onset of visible differentiation. *Second*, La^{3+} does not enter plant cells (Thomson et al. 1973) so it is unlikely to have non-specific effects on cellular metabolism; its action can be attributed to inhibition of calcium uptake (dos Reme-

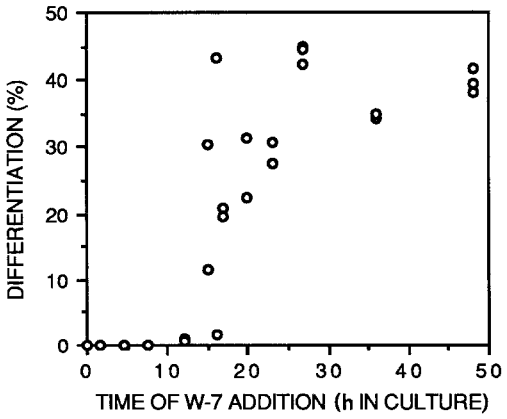

Fig. 8. Inhibition of TE differentiation in *Zinnia* cell cultures by W-7 (4 μM) added at various times. Each *point* represents the percent differentiation for a single culture in one experiment, except for "0" values, which represent three superimposed replicates. The graph is a compilation of data from three separate experiments

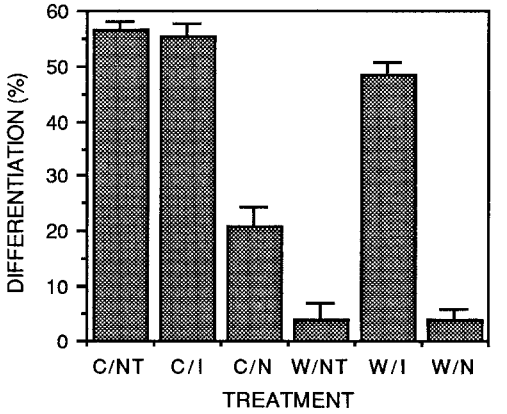

Fig. 9. Control *Zinnia* cells (*C*) or cells treated with 4 μM W-7 (*W*) at the time of inoculation were washed after 24 h with inductive medium (*I*) or non-inductive medium (*N*) or were left untreated (*NT*)

dios 1981). *Third*, the inhibitory effectiveness of La^{3+} was dependent on extracellular $[Ca^{2+}]$. *Fourth*, the inhibitory action of calcium-channel blockers is reversible; normal differentiation proceeded when channel blockers were washed out less than 2 h before the expected onset of TE formation. *Fifth*, the calcium-channel-activating isomer of 202–791 stimulates differentiation at low concentrations; its lethal effects at high concentrations are probably the consequence of toxic levels of Ca^{2+} in the cytoplasm. *Finally*, although calcium-channel blockers are known to have calmodulin-antagonizing activity (Roufogalis 1985), dihydropyridines were effective inhibitors of TE differentiation when added 0–48 h after culturing, whereas calmodulin inhibitors were effective only when added <24 h after culturing.

The failure of D600, verapamil and diltiazem to block differentiation in *Zinnia* cell-suspension cultures and in lettuce pith explants (L.W. Roberts and Baba 1987) does not necessarily contradict the requirement for Ca^{2+} uptake in TE differentiation. The effectiveness of calcium-channel blockers is dependent on their degree of ionization, and therefore on the external pH (Hille 1984; Uehara and Hume 1985; Kass and Arena 1989). Their lack of effect may be explained by the inability of charged drug molecules to penetrate the cell membrane and bind to internal receptors (Hescheler et al. 1982) or their inability to bind external receptors (Kass and Arena 1989). D600, diltiazem and verapamil have pKa values, in the sequence given, of 8.5 (Dörrscheidt-Käfer 1977), 7.7 (Uehara and Hume 1985) and 6.5; therefore they are primarily charged at pH 5.5. As a result, they may not be effective in cells incubated in Murashige and Skoog medium (pH 5.7–5.8; Murashige and Skoog 1962) used by L.W. Roberts and Baba (1987) or *Zinnia* culture medium (pH 5.5; Fukuda and Komamine 1980a). Tanimoto and Harada (1986) reported that verapamil inhibits bud initiation in *Torenia* stems cultured on Murashige and Skoog medium, but 300 μM verapamil was needed for complete inhibition. Lehtonen (1983), using Waris medium (pH 6.0), needed 330–380 μM verapamil for alteration of morphogenesis in *Micrasterias*. In contrast, Ruth et al. (1988) found that 50 μM verapamil induced cell division in *Vittaria* gemmae grown on Knop's medium at pH 5.5. However, since calcium-channel blockers inhibit cell division in other plants (Hepler 1985; Wolniak and Bart 1985), these results are difficult to interpret. To our knowledge, there have been no other reports of the application of verapamil, D600 or diltiazem to plants at low pH. In contrast, the dihydropyridines nifedipine and (−)202–791 are neutral amines (nifedipine has a pKa of approx. 1; Uehara and Hume 1985) and are also very effective inhibitors of TE differentiation in *Zinnia*. We propose that the ineffectiveness of D600, verapamil and diltiazem in inhibiting TE differentiation can be explained by their charged state at low pH. Since *Zinnia* TEs will not differentiate at higher pH, we were prevented from testing this hypothesis.

The concentration of calcium required for maximum TE differentiation (0.5 mM) is high compared to many other calcium-regulated processes in plants (reviewed by Hepler and Wayne 1985), particularly in light of the relatively low concentrations of channel blockers required to inhibit this process. This may be, in part, beause of the high concentrations of Mg^{2+} (1 mM) and Mn^{2+} (0.1 mM) in the *Zinnia* culture medium; both of these ions are known to block calcium-channels (see Hille 1984).

Uptake of Ca^{2+} occurs just prior to visible differentiation and may be related to wall secretion. The role of calcium in the regulation of secretory processes in plant cells has been reviewed recently by Steer (1988). The inhibition of TE differentiation when dihydropyridine calcium-channel blockers and lanthanum were applied just prior to visible secondary wall deposition (48 h in culture) indicates that calcium influx is required for cell-wall secretion in *Zinnia* as well. This is supported by the reversibility of inhibition upon removal of these drugs at 48 h, by the partial "rescue" of calcium-de-

prived cultures when $CaCl_2$ was added at 48 h, and by our previous observation that the amount of sequestered calcium increases just before wall thickening commences (A.W. Roberts and Haigler 1989). The observation that channel blockers prevented the formation of even a non-patterned secondary wall indicates that plasma-membrane calcium influx is necessary for secondary-wall secretion in general and not only for pattern formation in particular. This contention is further supported by the thinner appearance of thickenings in TEs that differentiated in the presence of partially inhibitory concentrations of calcium-channel blockers.

Calmodulin is also suggested to play a role in calcium-mediated secretion in plants (Steer 1988). The lack of inhibition of TE differentiation by calmodulin inhibitors applied after 24 h indicates that calmodulin may not be directly involved in regulating secretion of the secondary cell wall in TEs. The inability of W-7 to alter the onset or pattern of TE differentiation when added late in culture also indicates that calmodulin is not required for cytoskeletal rearrangement, which occurs just prior to secondary wall deposition in these cells (Falconer and Seagull 1985).

Tracheary-element differentiation requires calmodulin. In agreement with results obtained with lettuce pith explants (L.W. Roberts and Baba 1987), calmodulin inhibitors reduced TE differentiation in *Zinnia* cell-suspension cultures. The higher sensitivity of *Zinnia* cultures to TFP and CPZ is probably a consequence of greater access of suspension-cultured cells to the drugs.

Although the non-specific effects of calmodulin inhibitors are widely recognized, Roufogalis (1985) has proposed a set of criteria for testing the specificity of calmodulin inhibition. Our results satisfy four of his six criteria; specifically, inhibition occurs at appropriate doses, inhibitory potency parallels anti-calmodulin potency, the initiation of TE differentiation is calcium dependent (see next section), and inhibition occurs in the absence of increased cell death at low doses. We did not test the stereo-specificity of inhibition, and the regulation of TE differentiation is not characterized well enough to allow us to address the sixth criterion, which requires the elimination of potential non-calmodulin sites of action by the use of other inhibitors.

The specificity of calmodulin antagonism of TE differentiation is also indicated by the confined time at the beginning of culture during which calmodulin antagonists are effective. For many non-specific effects, the drugs would be expected to be inhibitory regardless of the time of addition. The effects of calmodulin on calcium transport, in particular, can be eliminated because calcium-transport antagonists are effective when added at 48 h and calmodulin antagonists are ineffective when added at this time. We do, however, see some cell death (Figs. 5 and 6, legends), which may be indicative of non-specific effects, particularly at the higher doses. Furthermore, so-called calmodulin inhibitors also antagonize other calcium-binding proteins (Moore and Dedman 1982). Several of these have been identified in plants and they may represent additional sites of action for calmodulin inhibitors.

Calmodulin may be involved in the response to cytokinin. In contrast to calcium-channel blockers, calmodulin inhibitors are not effective when added just prior to visible secondary wall deposition. Timing experiments revealed that by 17 h, 50% of the cells determined to differentiate had passed the calmodulin-dependent event(s) and were not inhibited by W-7 (Fig. 9). In this and other TE cultures (Phillips 1987; Tucker et al. 1986), cells are determined to differentiate shortly after exposure to cytokinin. Our results further indicate that calmodulin-dependent event(s) are essential for the determining response to cytokinin. Inhibition of differentiation by calmodulin inhibitors was completely reversed by washing and resuspension in fresh inductive medium at 24 h whereas inhibition was not reversed when cells were resuspended in non-inductive medium at 24 h. Cells not treated with drugs differentiate at about 40% of the control level when transferred to non-inductive medium at 24 h (Church and Galston 1988; this study). The lack of substantial differentiation in any culture when W-7 was added prior to 15 h indicates that the calmodulin-dependent event(s) continue after the first critical response to cytokinin, which is completed by 6–7 h in up to 40% of the cells (Church and Galston 1988). The involvement of calmodulin in cytokinin-regulated developmental processes has been noted previously in other plants (Saunders and Hepler 1983; Saunders 1986; Elliot 1983).

It is probable that the early calmodulin-dependent event(s) are also dependent on calcium uptake. Inhibition by calcium-channel-blocking dihydropyridines added immediately after culturing can be only partially reversed by removing the drugs at 48 h. This indicates that extracellular calcium is required early in differentiation as well as just prior to the appearance of TEs. Differentiating cells do not seem to require extracellular calcium between 24 and 48 h as supported by the complete reversibility of dihydropyridines present throughout this time.

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References

Asano, M., Hidaka, H. (1984) Biopharmacological properties of naphthalenesulfonamides as potent calmodulin antagonists. In: Calcium and cell function, vol. 5, pp. 123–164, Cheung, W.Y., ed. Academic Press, New York

Church, D.L., Galston, A.W. (1988) Kinetics of determination in the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. Plant Physiol. **88**, 92–96

Dörrscheidt-Käfer, M. (1977) The action of D600 on frog skeletal muscle: facilitation of excitation-contraction coupling. Pflügers Arch. **369**, 259–267

dos Remedios, C.G. (1981) Lanthanide ion probes of calcium-binding sites on cellular membranes. Cell Calcium **2**, 29–51

Elliott, D.C. (1983) Inhibition of cytokinin-regulated responses by calmodulin binding compounds. Plant Physiol. **72**, 215–218

Falconer, M.M., Seagull, R.W. (1985) Xylogenesis in tissue culture: taxol effects on microtubule reorientation and lateral association in differentiating cells. Protoplasma **128**, 157–166

Fukuda, H., Komamine, A. (1980a) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol. **65**, 57–60

Fukuda, H., Komamine, A. (1980b) Direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in culture of single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol. **65**, 61–64

Fukuda, H., Komamine, A. (1985) Cytodifferentiation. In: Cell culture and somatic cell genetics of plants, vol. 2. pp. 149–211, Vasil, I.K., ed. Academic Press, New York

Haigler, C.H., Brown, R.M., Jr. (1986) Transport of rosettes from the Golgi apparatus to the plasma membrane in isolated mesophyll cells of *Zinnia elegans* during differentiation to tracheary elements in suspension culture. Protoplasma **134**, 111–120

Hepler, P.K. (1985) Calcium restriction prolongs metaphase in dividing *Tradescantia* stamen hair cells. J. Cell Biol. **100**, 1363–1368

Hepler, P.K., Wayne, R.O. (1985) Calcium and plant development. Annu. Rev. Plant Physiol. **36**, 397–439

Hescheler, J., Pelzer, D., Trube, B., Trautwein, W. (1982) Does the organic calcium channel blocker D600 act from inside or outside on the cardiac cell membrane? Pflugers Arch. **393**, 287–291

Hille, B. (1984) Ionic channels of excitable membranes. Sinauer Associates, Inc., Sunderland, Mass., USA

Hof, R.P., Ruegg, U.T., Hof, A., Vogel, A. (1985) Stereoselectivity at the calcium channel: opposite action of the enantiomers of a 1,4-dihydropyridine. J. Cardiovasc. Pharmacol. **7**, 689–693

Hosey, M.M., Lazdunski, M. (1988) Calcium channels: molecular pharmacology, structure and regulation. J. Membr. Biol. **104**, 81–105

Ingold, E., Sugiyama, M., Komamine, A. (1988) Secondary cell wall formation: changes in cell wall constituents during the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. Plant Cell Physiol. **29**, 295–303

Kass, R.S., Arena, J.P. (1989) Influence of pH_0 on calcium channel block by amlodipine, a charged dihydropyridine compound. Implications for location of the dihydropyridine receptor. J. Gen. Physiol. **93**, 1109–1127

Lehtonen, J. (1984) The significance of Ca^{2+} in the morphogenesis of *Micrasterias* studied with EGTA, verapamil, $LaCl_3$ and calcium ionophore A 23187. Plant Sci. Lett. **33**, 53–60

Moore, P.B., Dedman, J.R. (1982) Calcium-dependent protein binding to phenothiazine columns. J. Biol. Chem. **257**, 9663–9667

Morad, M., Goldman, Y.E., Trentham, D.R. (1983) Rapid photochemical inactivation of Ca^{2+}-antagonist shows that Ca^{2+} entry directly activates contraction in frog heart. Nature **304**, 635–638

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497

Phillips, R. (1987) Effects of sequential exposure to auxin and cytokinin on xylogenesis in cultured explants of Jerusalem artichoke (*Helianthus tuberosus* L.). Ann. Bot. **59**, 245–250

Prozialek, W.C., Weiss, B. (1982) Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. J. Pharmacol. Exp. Ther. **222**, 509–516

Roberts, A.W., Haigler, C.H. (1989) Rise in chlorotetracycline fluorescence accompanies tracheary element differentiation in suspension cultures of *Zinnia*. Protoplasma **152**, 37–45

Roberts, L.W., Baba, S. (1987) Evidence that auxin-induced xylogenesis in *Lactuca* explants requires calmodulin. Environ. Exp. Bot. **27**, 289–295

Roufogalis, B.D. (1985) Calmodulin antagonism. In: Calcium and cell physiology, pp. 148–169, Marmé, D., ed. Springer, New York Heidelberg Berlin

Ruth, J.B., Kotenko, J.L., Miller, J.H. (1988) Role of asymmetric cell division in pteridophyte differentiation. II. Effect of Ca^{2+} on asymmetric cell division, rhizoid elongation, and antheridium differentiation in *Vittaria* gemmae. Am. J. Bot. **75**, 1755–1764

Saunders, M.J. (1986) Cytokinin activation and redistribution of plasma-membrane ion channels in *Funaria*. A vibrating-microelectrode and cytoskeleton-inhibitor study. Planta **167**, 402–409

Saunders, M.J., Hepler, P.K. (1983) Calcium antagonists and calmodulin inhibitors block cytokinin-induced bud formation in *Funaria*. Dev. Biol. **99**, 41–49

Steer, M.W. (1988) The role of calcium in exocytosis and endocytosis in plant cells. Physiol. Plant. **72**, 213–220

Tanimoto, S., Harada, H. (1986) Involvement of calcium in adventitious bud initiation in *Torenia* stem segments. Plant Cell Physiol. **27**, 1–10

Thomson, W.W., Platt, K.A., Campbell, N. (1973) The use of lanthanum to delineate the apoplastic continuum in plants. Cytobios **8**, 57–62

Triggle, C.R. (1980) Site of action of D-600 in guinea-pig ileal longitudinal muscle. J. Physiol. **305**, 82P

Tucker, W.Q.J., Wilson, J.W., Gresshoff, P.M. (1986) Determination of tracheary element differentiation in lettuce pith explants. Ann. Bot. **57**, 675–679

Uehara, A., Hume, J.R. (1985) Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. J. Gen. Physiol. **85**, 621–647

Wolniak, S.M., Bart, K.M. (1985) Nifedipine reversibly arrests mitosis in stamen hair cells of *Tradescantia*. Eur. J. Cell Biol. **39**, 273–277

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