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Effect of calcium, its inhibitors, and heavy metals on the growth cycle of peanut cell aggregates

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Abstract

During the normal growth cycle of peanut (*Arachis hypogaea* L.) cells, cultured in suspension medium, cell aggregates of <0.5 mm were formed during the log phase and grew to aggregates of >0.5 mm during late growth phase. Calmodulin rose to its original level during <0.5 mm aggregate formation following an initial 50% drop. Observations by UV microscopy showed that calmodulin. Ca^{2+} was centered in intense fluorescent sites. Calmodulin antagonists and a calcium chelator inhibited <0.5 mm aggregate formation as well as protein accumulation. The chelator suppressed cationic peroxidase isozyme release, while the antagonists had some partial effect on the anionic isozyme. Some heavy metals such as cadmium, mercury, lead and cobalt at low concentrations would allow continued growth of >0.5 but not of the <0.5 populations. At high (1 mM) concentrations these ions caused arrested growth. At low (10 μM) levels and in the presence of 3 mM calcium they had a synergistic effect.

Abbreviations: AOA – amino oxy acetic acid, BCA – big cell aggregates, CPZ – chlorpromazine, EGTA – ethylene glycol bis (β-aminoethyl ether), N,N,N′,N′ – tetra acetic acid, SCA – small cell aggregates, TFP – trifluoperazine

Introduction

Calcium functions as a regulator of plant cell metabolism (Kauss 1987; Gilroy et al. 1987; Allan & Hepler 1989; Pietrobon et al. 1990). In general, the regulatory effects of calcium are believed to be mediated by calmodulin or other calcium binding proteins (Cheung 1980; Piazza 1988).

Calmodulin's relation to the mitotic apparatus is based on its occurrence in the kinetochor microtubules, mitotic spindle and the phragmoplast at cytokinesis in the cell cycle of onion and pea root meristem cells as well as in animal cells (Means et al. 1982; Muto & Miyachi 1985; Wick et al. 1985; Vantard et al. 1985). A quantitative analysis of calmodulin in cultured cucumber cotyledons shows a correlation with root differentiation (Xu et al. 1988). In addition, a correlation in spatial distribution between calmodulin and action during cell wall formation was observed (Goddard & La Claire II, 1991). Young dividing and growing cells of pea seedlings contain more calmodulin than mature and differentiated cells (Muto & Miyachi 1984). These results have been confirmed (Allan & Trewavas 1985; Hernandez-Nistal et al. 1986).

Calmodulin antagonists and calcium chelator show major effects on the physiological cell processes such as secretion, division and differentiation (Chafouleas et al. 1982; 1984).

Ethylene has been correlated with cell division

in large cell aggregates of cultured cells (Mackenzie & Street 1970). It is correlated with levels of Ca^{2+} and negatively correlated with the level of calmodulin (Burns & Evensen 1986; Mattoo et al. 1982).

Studies on the cell wall deposition in spruce hypocotyl cuttings indicated that Ca^{2+} was essential and that *in vitro*, its absence diminished cell wall deposition and made the wall more susceptible to injury (Eklund & Eliasson 1990).

It has been shown that divalent heavy metals such as cadmium may effectively replace calcium to activate calmodulin by nonmetallothionein-bound Cd^{2+} (Chao et al. 1984). Cadmium may also substitute Ca^{2+} (Morishima et al. 1986) as the element in the structure and function of a major cationic peanut peroxidase (van Huystee et al. 1992). As low as 0.001 mM Cd^{2+} fed to algae increased cell growth and chlorophyll *a* content (Reddy & Prasad 1989). Some heavy metal salts may attenuate the soaking injury in *Phaseolus vulgaris* seeds and increase both ethylene production and germination (Small et al. 1991).

The present study examines a possible correlation between calmodulin/calcium and protein synthesis and morphological aspects of the growth cycle of peanut cells over a 14 d culture period.

Materials and methods

Chemicals

EGTA was purchased from BDH chemical Ltd., chlorpromazine and trifluoperazine from Sigma Chemical Co. A calmodulin ELISA kit was a gift from the Nanjing Agricultural University. Monoclonal antibodies (mcAb) were raised against either anionic or cationic peanut peroxidase as described (Hu et al. 1987a; Xu et al. 1990). All reagents were of the highest analytical grade.

Cell culture techniques

Cell suspension cultures derived from peanut (*Arachis hypogaea* L.) seeds were routinely maintained through biweekly subcultures (Hu et al. 1987b).

Following a 14 day culture period, the cells were washed in 0.25 M sucrose in 0.05 M phosphate buffer (pH 7.0) and 10 gm F.W. cells were resuspended in 250 ml fresh medium per flask. One set of 3 flasks was used as control containing 3 mM Ca^{2+} (Hu et al. 1987b); the second and third sets of flasks were brought to 0.05 mM TFP or CPZ in the fresh medium containing 3 mM Ca^{2+}; a fourth set received 6 mM EGTA. In Ca^{2+} substitution experiments cadmium acetate, mercury chloride, lead chloride and cobalt sulphate were added to medium containing 3 mM Ca^{2+} and 6 mM EGTA. The AOA treatment was conducted also in 3 mM Ca^{2+} containing medium.

Cell aggregate sizing

Samples of the culture were taken a couple of days apart and passed through a sieve with pores of 0.5 mm. The small cell aggregate (SCA) fraction was that which passed through the sieve and the big (BCA) was the fraction that remained. The individual fractions were washed with double distilled water, fresh weight was recorded and then used for subsequent analysis.

Protein extraction and determination

Protein and the anionic and cationic peroxidase from spent culture medium and from cells was extracted as described (Sesto & van Huystee 1989; Xu et al. 1990). Protein determinations were carried out by the technique of Lowry et al. (1951).

Determination of calmodulin

The ELISA technique was used as before (Xu & van Huystee 1991). All data were derived from at least three independent experiments.

Evaluation of inhibitor effect on the cell cycle

Peanut cells were incubated with the control medium containing 3 mM Ca^{2+}, or with this medium containing 6 mM EGTA, or with 0.05 mM CPZ or TFP (Kevers et al. 1982) for a 14 d culture period. At sampling the cells were washed three times with control medium and then placed in control medium containing 0.05 mM TFP for a 20 min incubation. When the

calmodulin Ca^{2+} complex had been conjugated and labelled with TFP, cells were plated out on microscope slides, covered with coverslips and exposed to UV radiation for 10 min before being viewed under a UV fluorescence microscope.

Results

Cultured peanut cells pass through a normal growth cycle (Fig. 1A). A lag phase of 2 d is followed by the logarithmic phase and a 2 d stationary phase. The percentage BCA expressed in f.w. decreases to 40% at day 6 of culturing and then rises slowly again to 80% at d 14 (Fig. 1B). The percentage of SCA supplements this (Fig. 1B). The amount of cellular protein in each (SCA & BCA) cell culture fraction is a reflection of these growth curves (Fig. 1C). Calmodulin was found to be 13 $\mu g\ mg^{-1}$ protein at the time of sub-culture and decreased by more then 50% by the end of the lag phase, but then increased again till the maximum percentage of SCA in the culture had been reached (Fig. 1D). It rose from there only very slightly to 13.4 $\mu g\ mg^{-1}$ protein. The data in Fig. 1 suggest that along with the growth cycle of the cell culture there is also a re-arrangement of cells in aggregates. Protein levels in the cells follow this pattern. Whether the rise of the calmodulin in the culture is directly associated with the formation of the SCA is uncertain from these data.

Calmodulin antagonists CPZ and TFP as well as Ca^{2+} chelator EGTA, appeared to prevent release of SCA from BCA (Table 1). Smaller cell aggregates were not formed at the normal rate from the large aggregates (Fig. 1B). There is relatively little variation in the overall content of both fractions throughout the 14 d culture period. Only in the case of incubation with EGTA is there some increase of cell population. It may be that enough Ca^{2+} is stored in the cells to allow for normal growth to occur during one culture cycle.

In comparison to control, cellular proteins were reduced for both fractions when the culture was incubated with CPZ, TFP or EGTA (Table 2). That protein secretion by treated cells lags

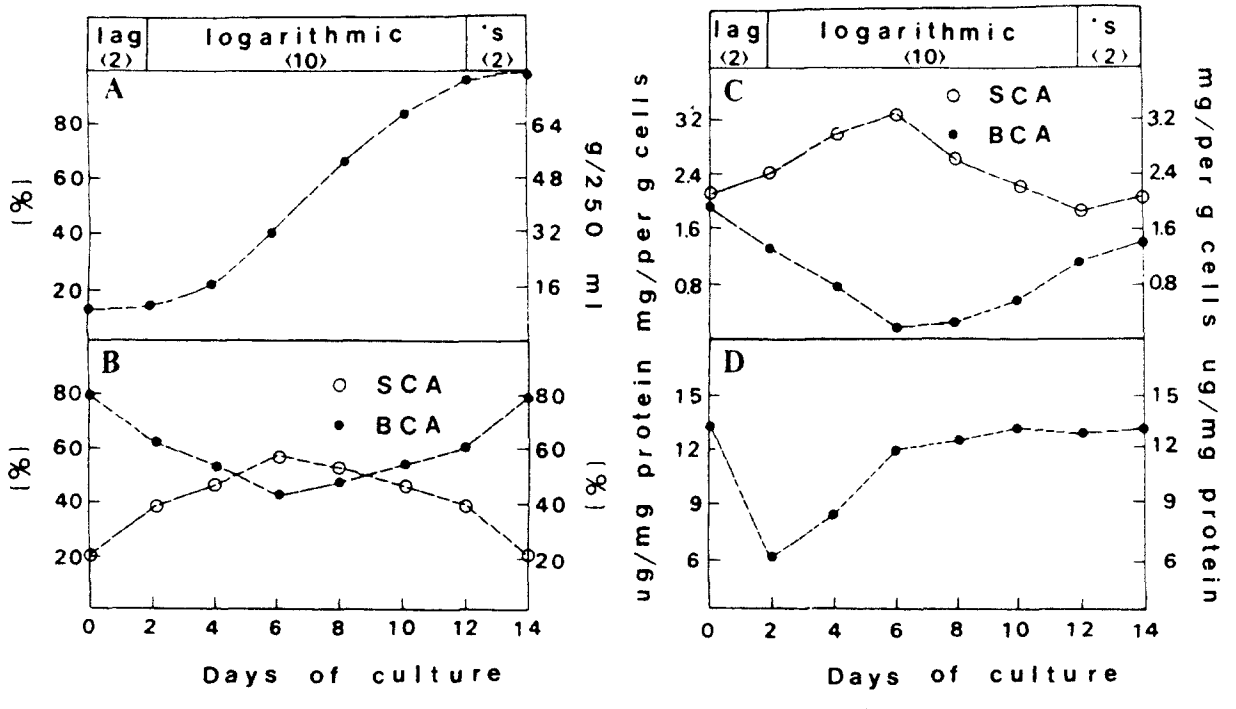

Fig. 1. Growth cycle of peanuts cells cultured in control medium containing 3 mM Ca^{2+}. (*A*) Fresh weight of all cells, (*B*) Proportions of SCA and BCA, (*C*) Cellular proteins from SCA and BCA, (*D*) Calmodulin during cell growth as denoted at the top of each graph.

Table 1. Effects of CPZ, TFP and EGTA on peanut cell growth cycle in suspension cultures.

Culture day	0.05 mM CPZ				0.05 mM TFP				3 mM EGTA			
	BCA	%	SCA	%	BCA	%	SCA	%	BCA	%	SCA	%
2	6.3		2.4		4.7		2.0		11.0		3.3	
		72.3		27.2		70.4		29.6		77.0		23.0
4	7.2		3.1		4.8		2.2		17.3		4.1	
		70.1		30.0		68.3		31.7		80.8		19.2
6	7.6		3.2		5.2		2.4		21.5		5.5	
		70.7		29.3		68.2		31.8		79.8		20.2
8	7.0		3.5		5.2		2.9		22.2		5.9	
		67.0		33.0		64.2		35.8		79.0		21.0
10	5.9		3.3		5.4		2.6		22.9		6.4	
		64.1		35.9		67.5		32.5		78.3		21.7
12	5.1		2.8		5.4		2.7		26.4		5.4	
		64.7		35.3		66.5		33.5		83.0		17.0
14	5.4		2.8		4.8		2.4		26.9		5.1	
		65.0		34.4		66.4		33.6		84.0		16.0

The fw of total cells at subculturing (0) time was 10 ± 1.2 g, while the amounts of BCA and SCA were 8.25 (80.25%) $\pm$ 1.04 and 2.05 (19.95%) $\pm$ 0.32 g in a 250 ml medium per flask, respectively.

Table 2. Effects of CPZ, TFP and EGTA on intracellular protein accumulation.

Day	0.05 mM CPZ				0.05 mM TFP				3 mM EGTA			
	BCA	%	SCA	%	BCA	%	SCA	%	BCA	%	SCA	%
2	1345		1727		1282		1633		2059		2320	
		100		100		100		100		100		100
4	934		1209		913		1255		1837		2176	
		69.4		70		71		76		89.2		93.8
6	683		944		755		1031		1462		1953	
		50.8		54.7		58.9		63.1		71		84.2
8	694		692		629		903		740		1891	
		59.6		40.1		49.1		55.3		35.9		81.5
10	779		602		492		892		409		1788	
		57.9		34.9		38.4		54.6		19.9		77.1
12	733		515		426		845		338		1723	
		54.5		29.8		33.2		51.7		16.4		74.3
14	463		423		338		802		283		1604	
		34.4		24.5		26.4		49.1		13.7		69.1

The amounts of BCA and SCA intercellular proteins at the time of subculturing were: 1890 ± 203 and 2097 ± 182 µg per g fw cells, respectively.

well behind that of control Ca^{2+} medium throughout the culture period (Fig. 2) is a logical consequence.

The influence of the treatments on peroxidase secretion is variable (Table 3). The effect of EGTA on the anionic isozyme was insignificant. However, EGTA effectively prevented the release of the cationic isozyme. Conversely, the effect of the calmodulin antagonists was most pronounced on the anionic isozyme. The effect of these antagonists on the cellular calmodulin level suggests a reduction throughout the logarithmic phase (data not shown).

Next TFP fluorescence was used to determine the sites of the calmodulin. Ca^{2+} in the two populations of cells (Fig. 3). When 5 d cultured control Ca^{2+} cells were incubated with 0.05 mM TFP for an additional 20 min and exposed to UV radiation, the calmodulin sites can readily be seen in the cells of both SCA and BCA (Fig 3A {c-1 to c-3}). It is obvious that fluorescence in the 14 d cultured cells is greatly diminished (Fig

Table 3. Cell growth and secretion as related to Ca^{2+}, EGTA, CPZ and TFP in cultured medium.

Treatment	Increase f.w.		Peroxidase secretion	
	SCA	BCA	Anionic μg mg^{-1}	Cationic μg mg^{-1}
3 mM Ca^{2+}	187.7	85.2	0.144 ± 0.013	197 ± 21
3 mM EGTA	−13.7	9.1	0.177 ± 0.019	-
0.05 mM CPZ	15	−11.9	0.035 ± 0.004	109 ± 27
0.05 mM TFP	8.2	−5.7	0.012 ± 0.002	46 ± 8

The SCA is calculated at 6 days and the BCA increase from 6 to 14 days. The release secretion of peroxidase was measured by ELISA at 14 days expressed mg protein.

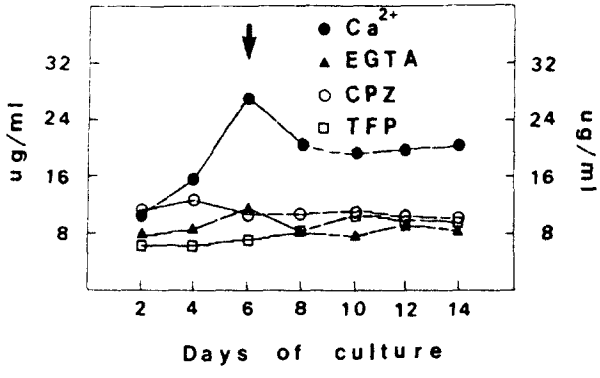

Fig. 2. Effect of control, chelator and calmodulin antagonists on protein release by the cells. Protein at the day 0 was 0.38 ± 0.25 μg. Arrow indicates the sharp rise in protein for control.

3A, c-3) in comparison to that in the cells of the log phase (Fig. 1A). The large aggregates (BCA) have increased in size during the later stages of growth (cf. Fig 1A c-3 & c-2 with c-1). Similar treatment of cells that had been incubated with either EGTA or CPZ or TFP for the cultured period did not show comparable fluorescence (Fig. 3B, C, D). In some cases there was only diffuse fluorescence.

Cell cultures were exposed to divalent metals at two or three concentrations during their culture period to determine the effect on the growth of both population of cells in the culture (Table 4). While exposure to 1 and 0.1 mM of cadmium arrested or severely (40%) reduced growth respectively, 10 μM did not reduce growth. In fact growth was enhanced by 10 μM Cd^{2+} in comparison to control Ca^{2+} (Fig. 1A). The color of the cells changed from white to slightly green.

The incubation of cells with mercury caused growth arrest of all but the BCA population with 10 μM Hg^{2+} (Table 4). The addition of 3 mM Ca^{2+} to the Hg^{2+} incubation medium alleviated the growth arrest particularly for the BCA population of cells at 10 μM Hg^{2+}. However, for the SCA population no growth improvement was noted. The results for incubation of SCA with lead (Pb^{2+}) and cobalt (Co^{2+}) resemble very much the data obtained with mercury (Table 4.).

To examine a potential effect of ethylene on cell growth the cells were grown in a medium with an ethylene induction inhibitor, AOA. The results show that a suppression of ethylene evolution created a loss of SCA formation from the BCA. It also shows that the latter did not proliferate under those conditions except perhaps for the 1 mM concentration.

Discussion

Cells dislodge from the large aggregates during normal cell growth (lag, logarithmic and stationary) phases of cultured peanut cells and then form in turn aggregates in the last phase of the growth period. The calmodulin level, as measured by ELISA, drops by 50% during the lag phase but increases by 100% during the log phase. During this phase the accumulations of calmodulin may be seen by UV microscopy as small centers of high fluorescence. During the stationary phase the fluorescence of these centers diminishes greatly. Whether the calmodulin level is directly related to the variations in cellular and extracellular proteins during the 14 day culture period is not known. However, the addition of either calcium chelator or calmodulin antagonists to the culture medium shows that the fluorescence centers are no longer so distinct. The protein levels are also decreased. This could be a reason for the altered growth pattern of the cell

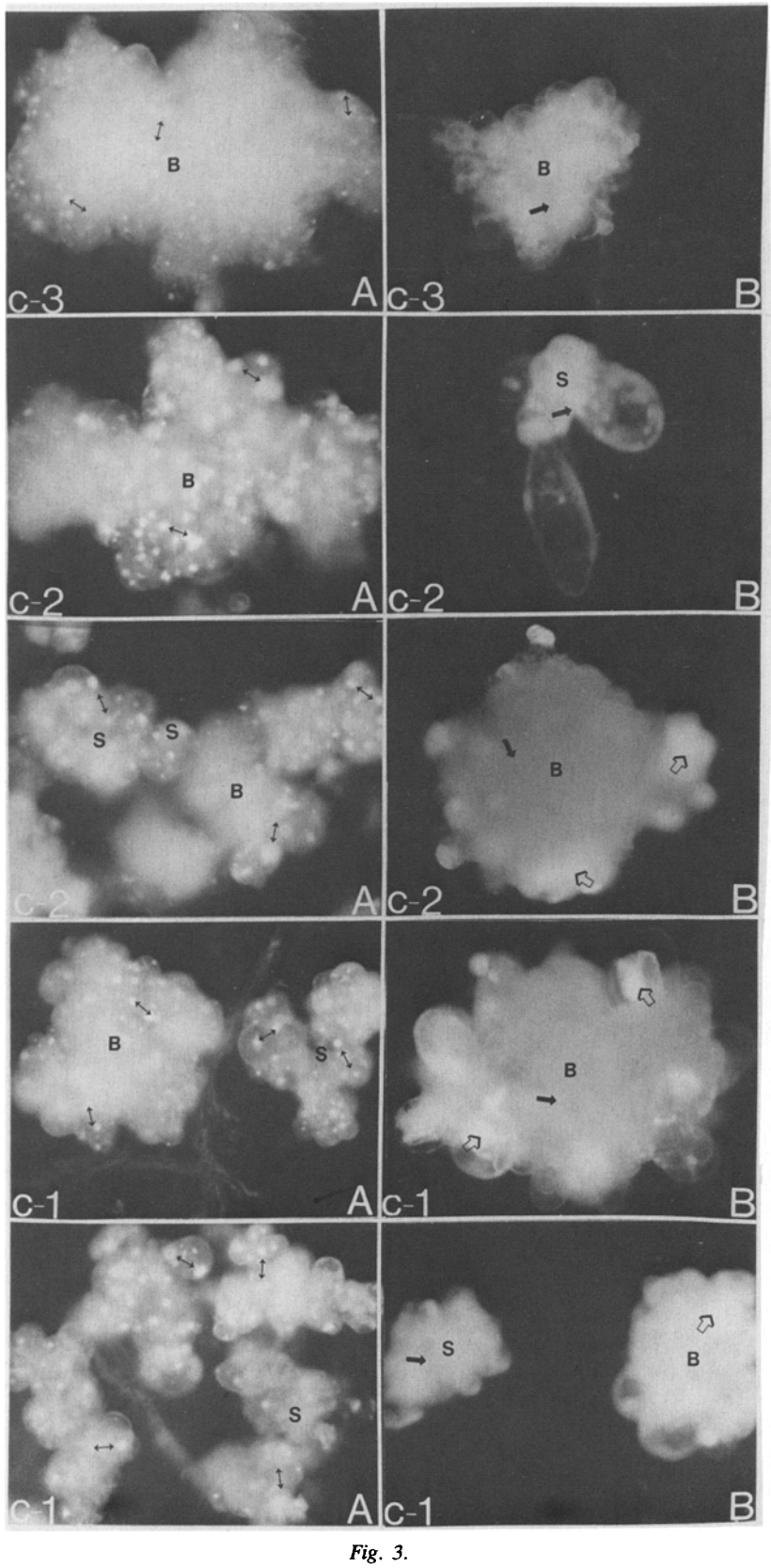

Fig. 3.

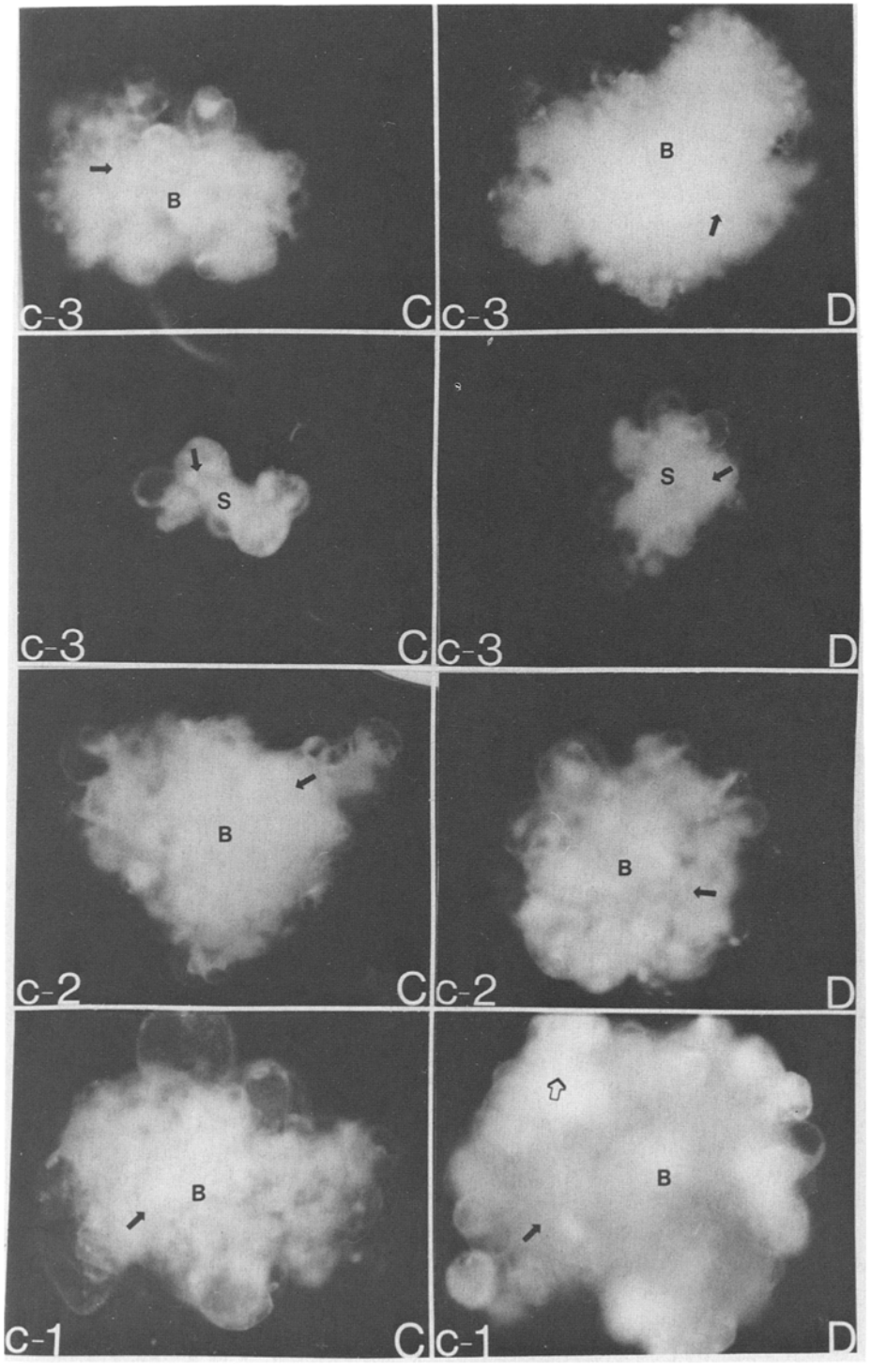

Fig. 3. Fluorescence at sites of calmodulin accumulation. Treatment marked at the righthand are: (*A*) 3 mM Ca^{2+}; (*B*) 3 mM EGTA; (*C*) 50 μM CPZ; (*D*) 50 μM TFP. Sampling time marked at the lefthand are: c-1, 6 d; c-2, 10 d; c-3, 14 d. The S and B on the figs indicate SCA and BCA respectively. Thin arrows indicate putative nuclear sites while broad arrows suggest other accumulation sites. Magnification (*A*, *B*) 90× and (*C*, *D*) 80×.

Table 4. The effect of heavy metal and heavy metal given together with Ca^{2+} (3 mM) and AOA on peanut cell growth cycle in suspension cultures.

Treatment	Days after subculturing fw g/250 ml						
	2	4	6	8	10	12	14
Cd^{2+} (μM)							
1000 BCA	4.8	3.5	3.2	3.0	3.2	3.1	3.2
SCA	3.8	3.3	2.8	2.6	2.3	2.4	2.4
100 BCA	9.2	10.6	11.9	13.4	18.0	27.6	39.7
SCA	7.1	8.2	9.2	10.0	9.9	9.8	10.4
10 BCA	10.3	12.6	22.5	39.3	57.9	78.2	111.9
SCA	8.0	9.1	10.4	9.4	8.5	7.2	7.5
Hg^{2+} (μM)							
1000 BCA	4.4	3.7	2.9	2.5	2.3	2.4	2.4
SCA	1.4	1.3	1.4	1.5	1.3	1.4	1.2
100 BCA	4.7	4.0	3.2	3.1	2.9	2.9	3.0
SCA	1.6	1.4	1.3	1.2	1.3	1.1	1.1
10 BCA	5.2	5.9	7.2	8.4	10.0	12.0	13.3
SCA	1.8	1.7	1.5	1.4	1.2	1.2	1.2
Hg^{2+} (μM)/Ca^{2+}							
1000 BCA	4.7	4.8	4.4	4.5	4.6	5.0	5.2
SCA	1.8	1.7	1.5	1.5	1.4	1.2	1.0
100 BCA	5.6	5.2	5.7	5.5	5.9	6.3	6.9
SCA	1.7	1.6	1.8	1.6	1.5	1.5	1.4
10 BCA	7.9	9.9	12.5	23.6	37.5	48.1	79.6
SCA	2.1	2.1	2.2	1.9	1.7	1.4	1.2
Pb^{2+} (μM)							
1000 BCA	4.1	3.5	2.7	2.4	2.7	2.5	2.9
SCA	1.7	1.2	0.9	1.1	1.1	1.1	1.0
10 BCA	5.1	5.7	6.5	8.2	9.4	10.5	12.4
SCA	1.8	1.5	1.7	1.4	2.0	1.2	1.1
Co^{2+} (μM)							
10000 BCA	3.9	3.7	3.1	2.9	3.0	2.8	2.9
SCA	1.6	1.3	1.0	1.2	1.0	1.0	1.0
100 BCA	4.3	4.8	5.4	6.7	7.4	9.4	11.3
SCA	1.6	1.6	1.3	1.2	1.2	1.0	1.2
AOA (mM)							
10 BCA	7.8	6.9	6.9	6.7	6.1	5.4	4.9
SCA	1.3	1.2	1.3	1.3	1.2	1.2	1.1
5 BCA	7.2	6.6	6.0	5.5	5.0	4.7	4.3
SCA	2.1	2.0	1.8	1.9	2.0	1.9	1.8
1 BCA	8.2	8.9	9.4	9.0	9.2	9.1	8.6
SCA	2.1	2.3	3.6	3.1	2.9	2.2	2.0

aggregates. Similar conclusions have been drawn from calcium experiments with other cells (Hazelton et al. 1979; Tupper et al. 1980; Chafouleos et al. 1982; Saunders & Hepler 1981, 1982, 1983; Muto & Takayasu 1987). The effect of EGTA is pronounced on the suppression of the cationic isozyme release. The calmodulin antagonists have a greater effect on the release of the anionic than on that of the cationic isozyme. This confirms the suggestion that each isozyme has its specific response to the form of calcium found in the cell (Xu & van Huystee 1992).

The effect of AOA is deemed to be on the inhibition of the conversion of s-adenosyl-methionine (SAM) to 1-amino cyclopropane-1-

carboxylic acid (ACC) and thereby prevents ethylene synthesis (Yang et al. 1980). Adding AOA to the incubation medium arrests the increase in F.W. of both SCA and BCA. It implies that ethylene production is associated with cell proliferation (Mackenzie & Street 1970). That both calcium and ethylene have been closely linked to cell growth is well documented (Ferguson 1984; Burns & Pressay 1986; Liu et al. 1990).

Heavy metal effect on calmodulin and its associated processes is well known (Chao et al. 1984; Cheung 1984). In some cases the heavy metal may substitute for the Ca^{2+} in the calcium-binding protein (Morishima et al. 1986; Xu & van Huystee 1992). The replacement of Ca^{2+} by any of these ions is probably related to the ionic radii (Cd^{2+}–0.97 Å; Hg^{2+}–1.10 Å; Pb^{2+}–1.20 Å; Co^{2+}–0.72 Å and Ca^{2+}–0.99 Å). The range of effective ionic radii is 1 ± 0.2 Å (Weast & Astle 1981).

The effect of these ions on the growth of BCA is biphasic in that growth at high dosages is inhibited while at low dosages it is stimulated. Such biphasic responses to heavy metals have been observed calmodulin regulation (Chao et al. 1984; Suzuki et al. 1985), myosin light chain kinase (Mazzel et al. 1984), Ca^{2+}–ATPase (Sotiroudis 1986), cationic peroxidase (Xu & van Huystee 1992) and cell microtubule assembly (Perrino et al. 1986). These heavy metals could have a relatively higher affinity than Ca^{2+} (Morishima et al. 1986) and thereby regulate calmodulin and other Ca^{2+} binding proteins. The effect of Ca^{2+} and Hg^{2+} together is synergistic to that of the sum of the two. The effect of these metals appears to be more toxic on the SCA than on the BCA population. It may depend on the relatively few young cells in the SCA aggregates which may be more easily permeated by the heavy metal ions than the heavy metal diffusion at some depth in the larger BCA aggregates.

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