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Distribution of membrane-bound calcium and activated calmodulin in cultured protoplasts of sunflower (*Helianthus annuus* L.)

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Cultured protoplasts, isolated from the hypocotyl segments of seedlings of *Helianthus annuus*, exhibit rapid changes in intracellular-bound calcium and calmodulin (CaM) activation, in response to auxin (IAA, 10^{-5} M) treatment. Activities of bound calcium and CaM have been localized photomicroscopically, using specific fluorochromes – chlortetracycline (CTC) and trifluoperazine (TFP), respectively. Bound calcium accumulation is followed by an increase in Ca^{2+}–CaM activity. Bound calcium initially shows preferential accumulation in the nucleus, within 2 min of incubation of protoplasts in IAA-containing medium. The fluorescence gradually increases along the plasmalemma. Ca^{2+}–CaM activity shows similar but later (within 10 min of incubation) distribution in the cultured protoplasts. In the multicelled bodies, however, Ca^{2+}–CaM activity appears to be preferentially localized in the meristematic region, whereas bound calcium shows more uniform pattern of distribution. The percentage of protoplast populations exhibiting the above-stated changes in the distribution of bound calcium and calmodulin activation, varied between 70 and 85 in different experiments and their repititions. This indicates the important role of calcium and calmodulin activation in the manifestation of polarity.

SUNFLOWER has proved to be a relatively difficult plant for protoplast culture. A number of genotypes have been investigated[1]. Particular attention has been paid to the first stage of protoplast culture, so that the steps for further development can be optimized. Divisions in cultured protoplasts, their oriented growth and subsequent differentiation are believed to be under the control of ionic fluxes[2]. Intracellular calcium is involved in a large number of physiological processes and many external stimuli result in changes in intracellular concentration and compartmentalization of calcium ions and calmodulin[3,4]. There is increasing evidence that Ca^{2+} participates in the initiation and maintenance of polarity in plant cells[4] . In the light of these observations, we have undertaken a study of the distribution of bound calcium during the initial stages of protoplast culture in sunflower, together with an analysis of the distribution of activated calmodulin (Ca^{2+}–CaM complex) because of its dominant role in the regulation of calcium metabolism and cell division.

Monitoring intracellular free Ca^{2+} poses many problems in plant cells and the success of loading the specific fluorochrome depends on the plant in question and also the fluorochrome being used. Chlortetracycline (CTC), which has been used in the present work to localize intracellular calcium, has a good cell permeability and easily loads into plant cells[5,6] but it localizes membrane-bound calcium. Activated calmodulin (Ca^{2+}–CaM) can be detected by the use of a group of CaM inhibitors (phenothiazines, such as trifluoperazine; TFP)) which bind specifically with activated calmodulin forming a Ca^{2+}–CaM–phenothiazine complex[7,8]. TFP has been used in the present work to study the distribution of activated calmodulin in the cultured protoplasts.

Hypocotyls from 7-day-old *in vitro*, dark-grown seedlings were used for aseptic, enzymatic isolation of protoplasts. Hypocotyl segments (1 gm fw) were sliced and incubated in 5 ml of enzyme solution in plastic steriplates for 16 h in dark at 30 ± 2°C. The mixture was shaken gently for 10 min at the end of incubation and filtered through 80 µm stainless steel mesh. The protoplasts thus released were pelleted by centrifugation at 80 g for 5 min and washed thrice in the isolation medium (IM). This procedure results in a protoplast population free from cell wall debris. The composition of IM and enzyme mixture are as follows: IM (gm l^{-1}): NaCl 18; KCl 0.4; $CaCl_2 \cdot 2H_2O$, 6.13; MES 0.7 (pH adjusted to 5.6).

Enzyme mixture: Macerozyme R10 0.2%; cellulase TC 0.1%; pectinase boerozyme 0.5%. The individual enzymes (Serva Fine Chemicals Co, Germany) were dissolved in IM. Glassware used for protoplast isolation were sterilized by autoclaving at 15 lbs psi for 15 min. The enzyme mixture was filter-sterilized using sterilized filter assembly (pore size: 0.22 µm).

The viability of isolated protoplasts was tested after 5 min of incubation in 0.01% fluorescein diacetate

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(FDA). The protoplasts were examined under UV, using a photomicroscope (Zeiss, Germany) equipped with a FITC excitation filter. The viable protoplasts fluoresced bright green in the presence of FDA. In order to determine the time of incubation for optimum protoplast yield, hypocotyl slices (1 gm fw per 5 ml of enzyme mixture) were incubated in dark at 30 ± 2°C for 8, 10, 12, 14, 16 and 24 h, respectively. The yield of purified protoplasts was determined using a haemocytometer after the routine steps of washing. For culture, the pelleted and purified protoplasts were resuspended in hormone-free liquid medium (LB medium; modified from Guilley and Hahne[9]). The mixture was poured into multiwell steriplates as drops and immersed in liquid LB medium. In order to determine the protoplast density for optimal plating efficiency, cultures were raised in agar droplets containing the following five protoplast densities: 6×10^4, 12.5×10^4, 25×10^4, 50×10^4 and 10^5 protoplasts per ml. All protoplast cultures were initially incubated in dark for 48 h and subsequently transferred to light (4.3 watt m^{-2}). The modified LB medium[9] consists of the following components (mgs $^{-1}$ l): $CaCl_2$ 440; $MgSO_4$ 738; KH_2PO_4 68; H_3BO_3 6.2; $MnSO_4$ 0.17; $ZnSO_4$ 0.28; $CoCl_2$ 0.024; $CuSO_4$ 0.0025; Na_2MoO_4 0.024; myoinositol 100; thiamine 1; pyridoxine 1; sucrose 20,000; mannitol 80,000; MES 700. pH of the medium was adjusted to 5.6 before autoclaving.

In order to observe rapid changes in intracellular bound calcium and calmodulin activation due to auxin (IAA) treatment, protoplast preparations were treated with 10^{-5} M IAA (filter-sterilized) in IM (containing 41.6 µM $CaCl_2$) for various durations, viz. 2, 5 and 10 min. After treatment, the protoplasts were pelleted by centrifugation at 80 *g* for 2 min, washed in IM (minus calcium) and resuspended in a drop of CTC (2×10^{-4} M) dissolved in IM (minus calcium). The bright yellow fluorescence was observed using a Zeiss fluorescence photomicroscope (BP 355-425/DM 455/LP460). Controls consisted of protoplasts incubated in minus IAA medium and in medium containing calcium ionophore A23187 (10 µM) in the absence or presence of IAA. For localizing the activated calmodulin, agar droplets containing the cultured protoplasts were treated with TFP (5×10^{-5} M dissolved in IM). The preparations were observed for reddish-yellow fluorescence due to the complex of TFP with Ca^{2+}–CaM complex photooxidized under UV, using Zeiss fluorescence photomicroscope (BP 365/DM 400/LP420). With both the fluorescent probes, observations were recorded within 1 min of incubation in the fluorochrome. The results were photographed on Fujicolor negative film (ASA 400).

Optimum protoplast yield (11×10^4 protoplasts gm^{-1} fw) was obtained after 16 h of incubation in the enzyme mixture (Figure 1). An incubation of 24 h results in the isolation and subsequent bursting of protoplasts. Based on these observations, all subsequent experiments have been undertaken with 16 h of incubation.

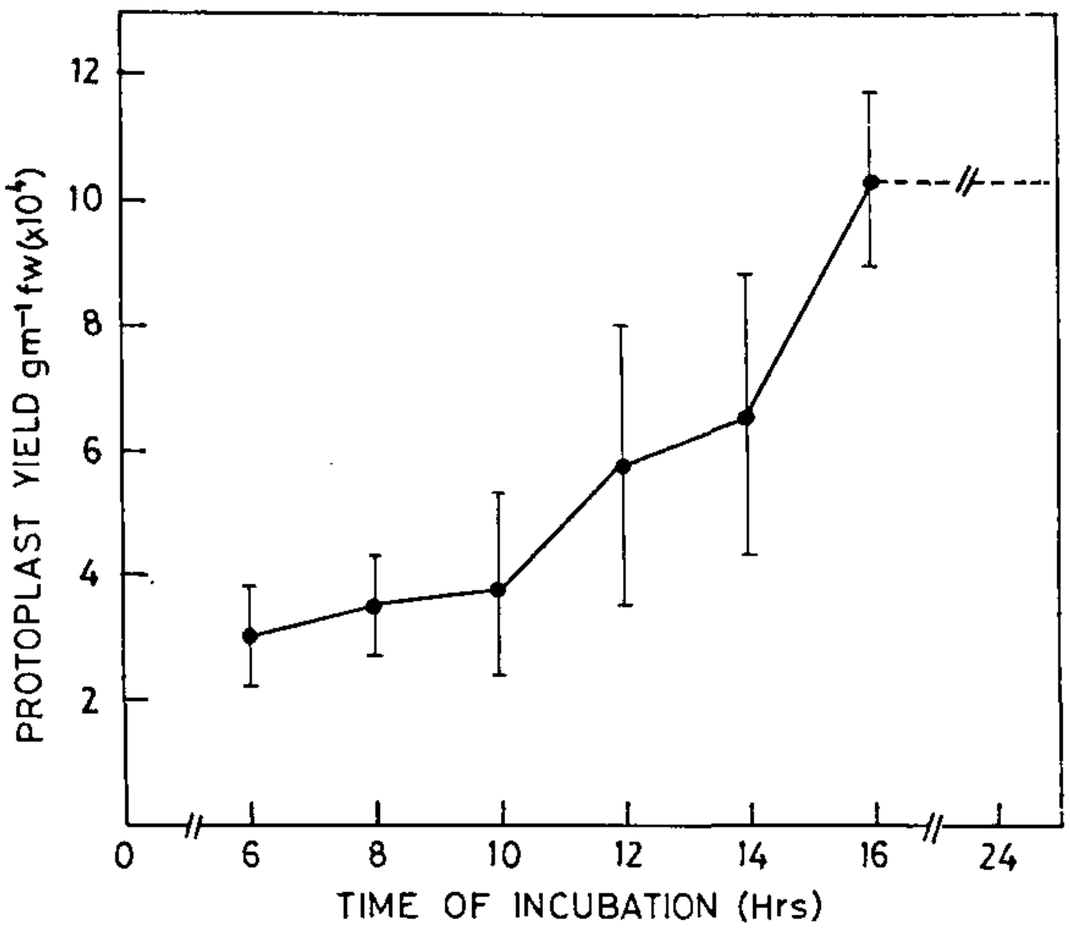


Figure 1. Protoplast yield from the hypocotyl segments of 7-day-old, dark-grown seedlings of *Helianthus annuus* L. as a function of time of incubation in the enzyme mixture. Data represent mean and standard errors from three observations.

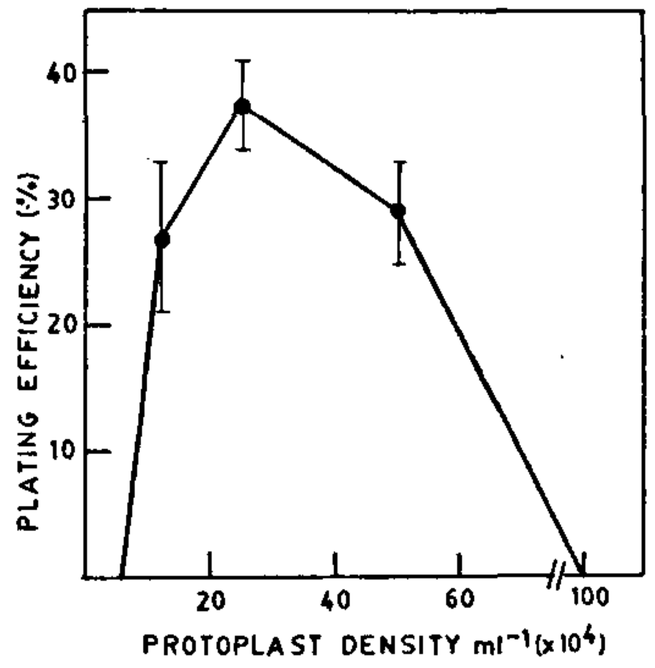
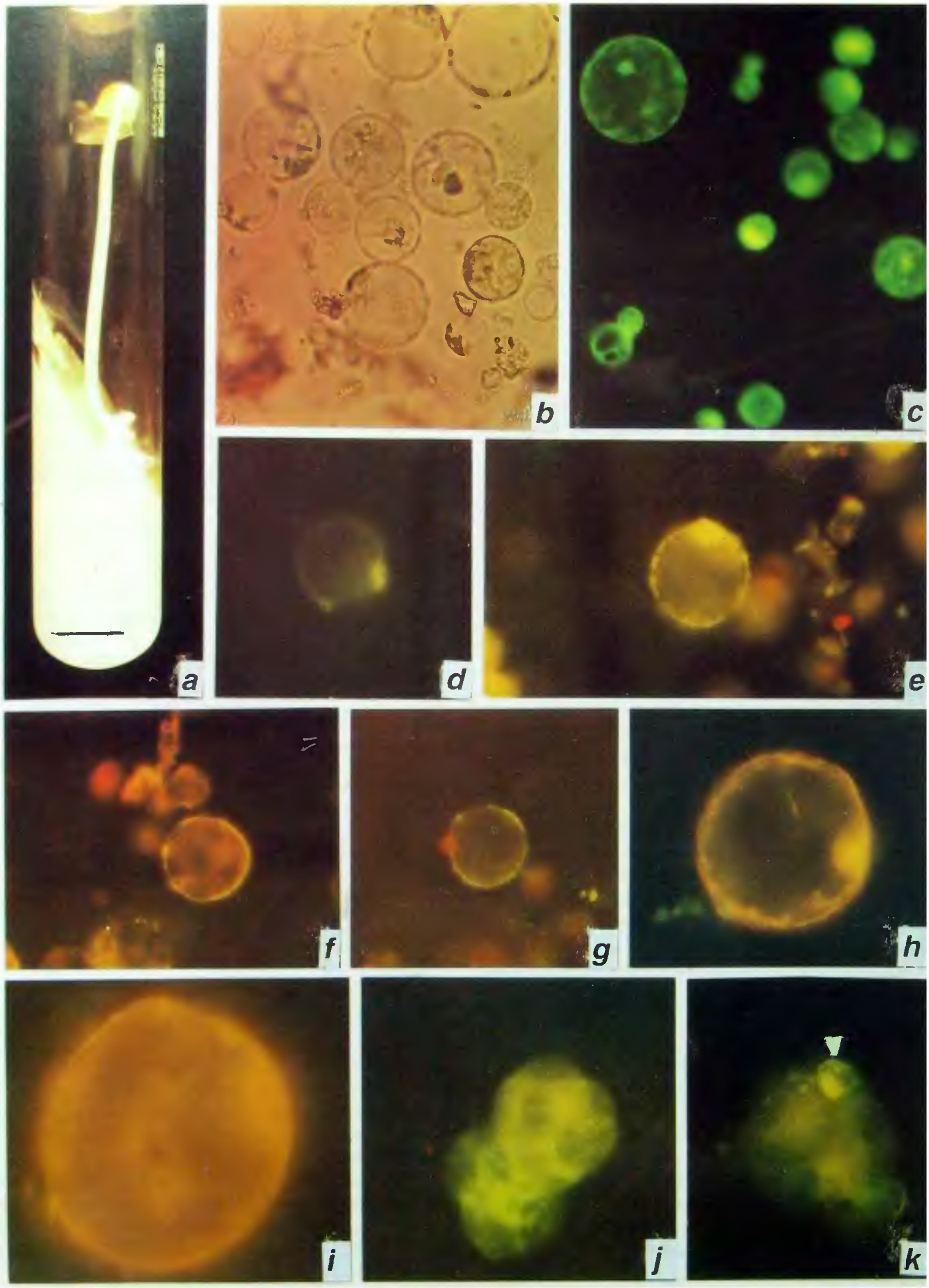


Figure 2. Effect of protoplast density on plating efficiency (%). Data obtained after 8 days of protoplast culture in agar (0.5%) prepared and bathed in LB medium. Data represent mean and standard errors from three observations.

Growth condition of the donor plant affects the yield of protoplasts. Hypocotyl slices from dark-grown seedlings (raised on MS medium) result in slightly better protoplast yield ($11 \pm 2 \times 10^4$ protoplasts gm^{-1} fw) when compared to those isolated from seedlings grown in light conditions mentioned earlier ($8 \pm 3 \times 10^4$ protoplasts gm^{-1} fw). A protoplast density of 25×10^4 ml^{-1} has been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found to be optimum for plating efficiency whereas the extreme populations do not promote any division (Figure 2). The protoplasts isolated from seedlings raised on MS medium and maintained at 25 ± 2°C in dark exhibit heterogeneity with regard to size, degree of vacuolation and presence of chloroplasts/plastids (Figure 3 *a*, *b*). Viability of the protoplasts is not affected significantly by light/dark conditions and it varies between 80 and 85% (Figure 3 *c*). IAA (2×10^{-6} M) remains ineffective in inducing protoplast divisions whereas at 10^{-5} M, it induces divisions in 18–20% protoplasts within 24 h. Further experiments on the localization of intracellular bound calcium and activated calmodulin have been done on protoplasts subjected to IAA treatment (10^{-5} M).

Since the fluorescence signal of CTC associated with membrane-bound calcium develops immediately upon exposure to UV and also decreases fast as a result of photobleaching, the observations were recorded within 1 min of irradiation. A longer exposure (5–7 min) leads to bursting of protoplasts. Calcium tends to accumulate within the protoplasts subjected to IAA treatment (10^{-5} M). IAA treatment for 2, 5 and 10 min results in an increase in fluorescence with time due to Ca^{2+}–CTC complex formation when compared with controls (–IAA; Figure 3 *d*). The fluorescence is localized more around the nucleus after 2 min of incubation and it also spreads along the plasmalemma (Figure 3 *e*). In 10 minutes, the fluorescence exhibits general cytoplasmic distribution (Figure 3 *f*). The effect of IAA is nullified by the co-incubation with calcium ionophore A 23187 (10 μM) for a period of 5 min (Figure 3 *g*).

The Ca^{2+}–CaM–TFP complex within the viable protoplasts results in an intense fluorescence immediately after UV irradiation. Prolonged exposure to UV leads to photobleaching of protoplasts and consequent decrease in the fluorescence intensity after 2–3 min. Observations were, therefore, recorded within 1 min of irradiation. Fluorescence development for Ca^{2+}–CaM complex was not as rapid as that for calcium. No signal could be detected in controls (–IAA) and in protoplasts incubated in the presence of IAA up to 2 min. In the protoplasts subjected to IAA (10^{-5} M) treatment for 10 min, fluorescence due to activated calmodulin is mainly concentrated along the plasmalemma and the nucleus (Figure 3 *h*). In contrast to this, bound calcium tends to show faster localization along the plasmalemma and also the cytoplasm (Figure 3 *e*). Longer incubation of protoplasts in IAA solution (20 min), however, results in an overall distribution of Ca^{2+}–CaM complex along the plasmalemma, nucleus and cytoplasm (Figure 3 *i*), as also observed in the case of bound calcium (Figure 3 *f*). But the observation is delayed when compared to bound calcium. Multicelled bodies formed as a result of protoplast divisions in IAA-containing medium, show a homogeneous distribution of bound calcium throughout the cells (Figure 3 *j*). Although both calcium and calmodulin activities show a general distribution all through the cells, Ca^{2+}–CaM activity is intense in the apical region (Figure 3 *k*). This is in contrast to the relatively uniform distribution of CTC-bound Ca^{2+} (Figure 3 *j*).

The present investigations reveal that hypocotyl slices from dark-grown seedlings result in a better yield of viable protoplasts than from light-grown seedlings and the viability varies between 75 and 85%. Earlier work has shown that sunflower protoplasts cultured in liquid medium produce colonies which fail to develop into calli[10]. Solidified medium provides better conditions for the development of colonies leading to compact microcalli formation. Plating efficiency of the protoplasts has been found to be affected by the density of the protoplast populations as well as the prevailing nutritional conditions. A varying proportion of the cultured protoplasts produce spherical microcolonies which usually develop into very compact structures, often with some apparent polarity. These structures do not surpass 20–30 cell stage and subsequently become necrotic or develop into microcalli[9]. Colonies from all types of genotypes so far investigated (including the present one), that are formed in agarose/agar, are either unorganized or dense, bipolar structures[9].

Incubation of protoplasts in IAA-containing medium results in marked changes in CTC and TFP fluorescence, indicating the mobilization of bound calcium and activation of calmodulin, respectively. CTC fluorescence is an indication of the amount of membrane-bound calcium. Earlier work using electron microscope, has shown an increase in the number of endoplasmic reticulum (ER) profiles in the outer, cytoplasm-rich cells from carrot proembryogenic masses[11]. The increased CTC signal in the present investigation might be in correlation with these results since ER is known to sequester up to millimolar levels of calcium[2]. The decrease in bound calcium signal in the presence of calcium ionophore A 23187 is due to the binding of Ca^{2+} to the ionophore at

Figure 3. Localization of intracellular-bound calcium and Ca^{2+}–CaM complex in auxin (IAA)-treated protoplasts of *Helianthus annuus* L. ***a***, 7-day-old, dark-grown seedling raised on MS medium; ***b***, Isolated protoplasts as observed in visible light (× 400); ***c***, Viable protoplasts as observed in UV (× 400); ***d–f***, Control (–IAA) medium. ***d***, Fluorescence due to calcium CTC complex, after incubation in control medium (–IAA), ***d***, 10^{-5} IAA for 2 min ***e***, and 10 min ***f***, (× 400); ***g***, 5 min treatment with calcium ionphore A23187 (× 400); ***h***, Fluorescence due to Ca^{2+}–CaM complex after subjecting the protoplasts to IAA (10^{-5}M) for 10 min (× 600); ***i***, Protoplasts showing homogenous distribution of Ca^{2+}–CaM complex after 20 min incubation in +IAA medium (× 1000); ***j***, Bound calcium distribution in multicelled bodies (× 400); ***k***, Ca^{2+}–CaM distribution in multicelled body (× 400).

relatively low concentrations of extracellular calcium (41.6 μM). Present observations indicate a preferential accumulation of activated calmodulin in certain specific regions of the multicelled bodies, with the onset of polarity by auxin treatment. IAA has earlier been reported to promote calcium release from the membranes of mungbean and soybean hypocotyls and pea epicotyl[12]. IAA has, however, no effect on calcium uptake in wheat protoplasts[13] and hypocotyl segments of *Cucurbita pepo*[14]. Later work has, however, suggested calcium as a second messenger in early auxin action whereby IAA has been found to rapidly increase the intracellular free calcium levels in the cytoplasm[15–17].

The elevation of $[Ca^{2+}]$ in response to a signal may be uniform throughout a cell or a group of cells or it may be highly localized in certain specific regions of the cell. In many cellular systems, the calcium signal occurs as a wave, beginning at a discrete initiation site and then moving across the site[18]. The present work on *Helianthus* protoplasts shows that bound calcium is initially localized around the nucleus and later, as a result of continued auxin action, it tends to be preferentially localized along the plasmalemma as well. This may have something to do with the activation of calcium-binding sites along the plasmalemma and intracellularly. Intracellular calcium concentration can be increased rapidly by the transient opening of plasmamembrane calcium channels or by the release of calcium ions sequestered in ER. Both mechanisms are activated by the binding of extracellular signals to the plasmamembrane receptors. The elevated $[Ca^{2+}]$ within the cells binds to calmodulin, leading to an activation or inhibition of the target proteins.

Attempts to localize calmodulin during mitosis, using affinity-purified antibodies, have shown dense staining along the spindle poles, suggesting that calmodulin is involved in mitotic functions[19]. The present observations also show similar preferential localization of calmodulin along the nucleus, indicating its possible involvement in mitotic functions. Many other cellular functions have also been assigned to calmodulin through a combination of localization, biochemical and genetic approaches[20]. From the present comparison of fluorescence due to bound calcium and activated CaM in the protoplasts of *Helianthus*, it appears that membrane-associated Ca^{2+} may not be exclusively bound to CaM. Similar observations were also made in the proembryogenic cell masses of carrot[4]. Greater accumulation of fluorescence due to CaM in certain specified zones of the multicelled structures may indicate an important role for CaM in the manifestation of polarity. Calmodulin might act as a mediator of calcium flux across the membranes and ultimately as a sensor of cytoplasmic calcium transient[2]. Non-uniformity in the fluorescence due to bound Ca^{2+} and CaM in the protoplasts of *Helianthus* further indicates the possible participation of other calcium-binding proteins in relation to the calcium-dependent processes. Monitoring intracellular free Ca^{2+} poses many problems in plant cells and the success of loading the specific fluorochrome depends on the plant in question and also the fluorochrome being used. Attempts are underway to look into this aspect in the cultured protoplasts of *Helianthus*.

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