Dynamic Continuity of Cytoplasmic and Membrane Compartments between Plant Cells

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Abstract. Fluorescence photobleaching was employed to examine the intercellular movement of fluorescein and carboxyfluorescein between contiguous soybean root cells (SB-1 cell line) growing in tissue culture. Results of these experiments demonstrated movement of these fluorescent probes between cytoplasmic (symplastic) compartments. This symplastic transport was inhibited with Ca^{2+} in the presence of ionophore A23187, and also with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Both of these agents have previously been demonstrated to inhibit gap junction-mediated cell-cell communication in animal cells. In a companion experiment, a fluorescent phospholipid analogue, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylcholine (NBD-PC), was incorporated into soybean cell membranes to examine whether dynamic membrane continuity existed between contacting cells, a transport route not existing between animal cells. Photobleaching single soybean cells growing in a filamentous strand demonstrated that phospholipid did exchange between contiguous cells.

CELL-CELL communication between contiguous tissue-forming cells is vital for control and coordination of cellular biosynthetic, bioenergetic, and proliferative activities (11, 17). The movement of low molecular weight molecules between plant cells appears to occur through a cytoplasmic syncytium termed the symplast (8). Morphological investigations have provided evidence that symplastic transport is mediated by specialized trans-cell wall structures called plasmodesmata (8, 19, 27).

All morphological investigations to date suggest that both a cytoplasmic as well as membrane transport pathway may coexist in these structures (6–9, 19, 27, 33). Evidence for the role of plasmodesmata as trans-wall low-resistance pathways includes (*a*) the observation that aqueous channels appear to penetrate through them (7, 8, 19, 27); (*b*) the transport of plant cellular substances between cells is proportional to the plasmodesmatal frequency (7, 8, 19, 27, 29); (*c*) ion transport, as represented by AgCl deposits, is found to colocalize with plasmodesmata (35); (*d*) intercellular transport appears not to exist between cells not connected by plasmodesmata (25); (*e*) plasmolysis, which affects plasmodesmatal organization, alters rates of cell-cell communication (2, 6); and (*f*) there is a strong correlation between the extent of electrical coupling and the frequency of plasmodesmata (9). The data presented in these studies concerns communication of small hydrophilic molecules between cytoplasms. Nothing has been reported on movement of membrane components between cells, although morphological evidence suggests that membrane connections exist between cells (9).

In experiments to be described, we have attempted to use the method of fluorescence redistribution after photobleaching (FRAP)[1] (36) to examine the intercellular movement of both low-molecular weight hydrophilic molecules (carboxyfluorescein [CF], fluorescein [F]) and a fluorescent phospholipid analogue (*N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylcholine [NBD-PC]) between contiguous soybean (*Glycine max* (L.) Merr. cv. Mandarin) root cells (SB-1 cell line) grown in suspension culture. Results of these investigations suggest that plant cell-cell communication may occur not only between cytoplasmic compartments, as observed for animal cells, but also between membrane compartments, a communication pathway that has not been found to occur between animal cells (34) and has not been reported before in plant tissue.

Materials and Methods

Labeling the Cells

Communication experiments were performed with soybean (*Glycine max* (L.) Merr. cv. Mandarin) root cells (SB-1 cell line) grown in 1B5C medium (22) in suspension culture. After 72–96 h of growth, cells were washed with the medium 1B5C and incubated with either carboxyfluorescein or fluorescein diacetate (CFDA and FDA, respectively; Molecular Probes, Inc., Eugene, OR) (10 μg/ml) for 10 min at room temperature. Cells were washed three times in 1B5C medium to remove unincorporated dye. Labeling with NBD-PC (Avanti Biochemicals, Birmingham, AL) was performed as for the CFDA and FDA. The cells were incubated with 50 μg/ml of NBD-PC for 30 min at room temperature, then washed three times with 1B5C medium. All labeled cells were kept on ice up to 4 h before measurement to prevent

1. *Abbreviations used in this paper*: CF, carboxyfluorescein; CFDA, carboxyfluorescein diacetate; F, fluorescein; FDA, fluorescein diacetate, FRAP, fluorescence redistribution after photobleaching; NBD-PC, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylcholine.

dye leakage from the cells into the medium. The dye concentration and labeling conditions did not affect cell viability.

Measurements on the ACAS 470 Workstation

The ACAS 470 Workstation was used to photobleach single cells and cells connected in a filament (31, 36). The labeled cells are placed on a slide which is mounted on the ACAS 470 stage. The automated stage moves the sample in 1.5-μm steps in a two-dimensional grid past a microscope objective that focuses the excitation beam (488 mM) from an argon ion laser to a 1–30-μm diameter beam on the sample. A photomultiplier tube captures the emission intensities at each addressed excitation point. The emitted intensities are then color coded and displayed on a cathode ray tube as false color images of cellular fluorescence. Multiple photobleaching on the same cell did not alter the transport results.

Measurements on a FRAP Instrument

Cells were scanned and photobleached on a FRAP instrument as described (31, 36). A defocused laser beam (width approximately equal to the cell diameter) was employed, resulting in a linear fluorescence intensity profile across the cell; 20 scans were monitored at 15-s intervals for a total monitoring time of 5 min.

Results

Cell–cell communication between the cytoplasmic compartments of plant cells was determined by using fluorescence redistribution after photobleaching (FRAP) in conjunction with the fluorescent low-molecular weight compounds, CF and F (both added to the cells as the diacetylated derivative as described in Materials and Methods). This technique, first employed for the measurement of intercellular communication between mammalian cells (31, 36), is performed in the following manner. Briefly, a laser beam whose width is approximately equal to the plant cell diameter photobleaches the fluorescent probes in a cell contacting other similarly labeled cells. After photobleaching, a redistribution of dye molecules may occur between bleached and unbleached cells. Monitoring this process as a function of time yields a rate constant for dye movement. Photobleaching a single noncontacting cell provides a control that recovery is a function of transport between contacting cells and not the physical consequences of dye reactivation after photobleaching or uptake of dye from the media.

Fig. 1 demonstrates the cellular distribution of CF and F. The acetates have been removed from the acetylated precursors by intracellular esterases; this traps the compounds in the cell over the time course of our measurements (1). A clear difference for CF and F localization is observed in soybean (SB-1) filaments. When cells are labeled and examined in medium at pH 5.5, fluorescein is found to localize predominantly in the cytoplasm and the nucleus (Fig. 1, *A* and *B*), whereas CF at pH 5.5 appears to preferentially localize in the vacuole (Fig. 1, *C* and *D*). This corroborates a report by McCredy et al. (19a) that these two dyes differ in their intracellular localization. Both of these dyes were used to measure intercellular communication (Figs. 2 and 3). Using the ACAS 470 Fluorescence Workstation (see Materials and Methods), a computer-generated false color image of fluorescence distribution is shown in Fig. 2 *a* for CF and in Fig. 3 *a* for F. Photobleaching results in a drop in the fluorescence

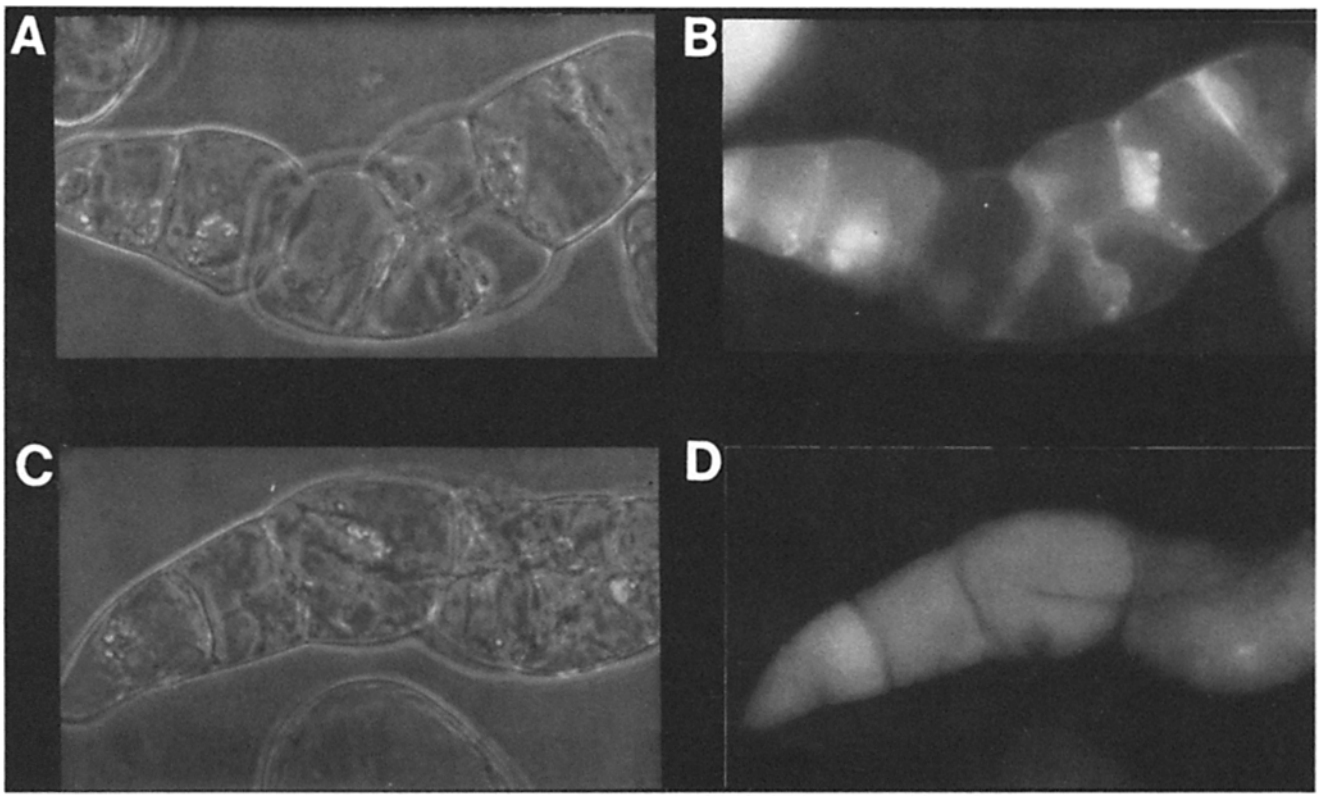

Figure 1. A phase contrast (*A* and *C*) and fluorescence image (*B* and *D*) of soybean tissue culture cells labeled with F (*B*) and CF (*D*). Cells were labeled with a dye concentration of 10 μg/ml for 10 min at room temperature (Materials and Methods), washed, and viewed with phase optics and epifluorescent illumination.

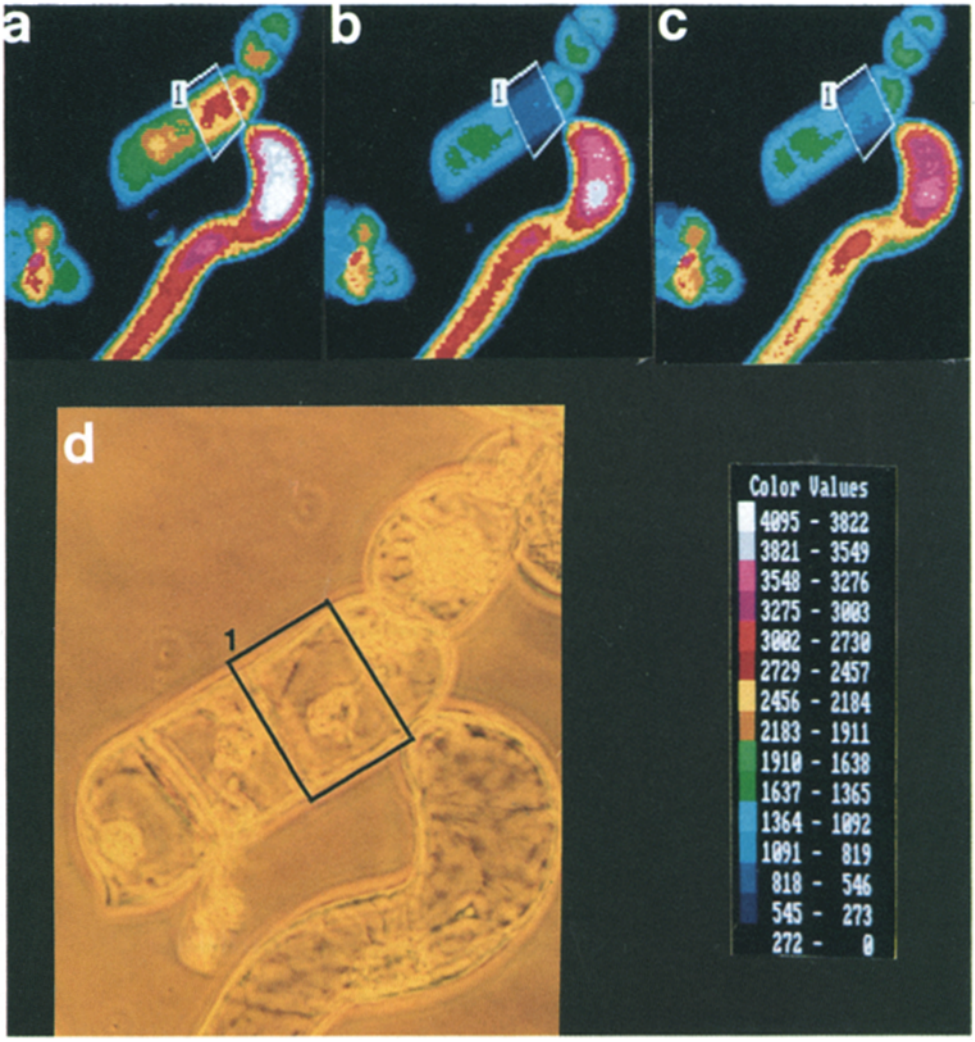

Figure 2. Recovery of fluorescence in photobleached soybean tissue culture cells labeled with CF. Labeling of the cells was performed as described in Materials and Methods. The ACAS 470 Workstation was used to photobleach the cells and record fluorescence distribution as false color images visualized on a cathode ray tube. Before photobleaching, there is a strong fluorescence intensity (*a*) that is reduced after photobleaching the cell located in the polygon (*b*). (*c*) Fluorescence intensity in the filament 10 min after photobleaching. (*d*) Phase view of the cells under analysis. Box denotes the photobleached cell. The color code presented relates color to an arbitrary numerical scale of fluorescence intensity.

intensity in the bleached cell and some bleaching in contacting cells (Fig. 2, *a* and *b*, and Fig. 3, *a* and *b*). Recovery is demonstrated to occur for both CF and F (Figs. 2 *c* and 3 *c*), although the extent of recovery for CF is less than F. This correlates with the observation (see Fig. 1) that a high concentration of CF resides in the vacuole and may not be readily available for exchange between cells. Single isolated cells containing CF or F did not recover after photobleaching (data not shown). Fig. 2 *d* is a phase view of the SB-1 cells presented as pseudo-color images in Fig. 2, *a–c*.

Subsequent analytical transport experiments were performed on a FRAP instrument as described (14, 36). Cells labeled with the cytoplasmic localized dye F were photobleached with a defocused laser beam, the width of which was approximately equal to the cell diameter and the laser beam was run across the cell. The redistribution of fluorescence data was analyzed according to the equation: $F(-) - F(t)/F(-) - F(o) = e^{-K_1 t} + e^{-K_2 t}$, where $F(-)$, $F(o)$, and $F(t)$ are fluorescence signals before photobleaching, after, and at time t (26). A sample recovery curve for dye transport between cells is represented in Fig. 4 *A*. Note that the recovery is represented by a fast component (K_1) and a slower component (K_2). Fig. 4 *B* demonstrates that a photobleached single cell, which should have no recovery, has a fast component (K_1) but no slower component (K_2). These results are interpreted as demonstrating that component (K_2) represents the intercellular flux, whereas component (K_1) represents the rearrangement of bleached and unbleached molecules within the photobleached cell volume. The calcu-

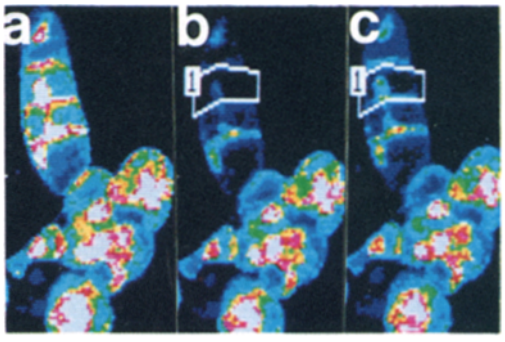

Figure 3. Recovery of fluorescence in photobleached soybean tissue culture cells labeled with fluorescein. The experiment was performed as in Fig. 2. (*a*) Prebleach distribution. (*b* and *c*) The bleached cell immediately and 10 min after photobleaching, respectively.

lated flux rate derived from component (K_2) for intercellular dye transport is 1.46 ± 0.43 × 10^{-3} s^{-1} (50 cells).

Effect of Ca^{2+} and TPA on Plant Cell–Cell Communication

Previous measurements of plant intercellular communication have established that Ca^{2+} may prevent cell–cell transport (5) as initially observed in mammalian cell communication studies (17). SB-1 cells incubated with 5 mM Ca^{2+} and ionophore A23187 (100 μg/ml) demonstrated inhibition of intercellular transport for F (Fig. 4 *C*). Inhibition was also observed at 1 mM Ca^{2+} with ionophore. No inhibition by Ca^{2+} was observed without the presence of ionophore (data not shown). Clearly, the Ca^{2+} effect on transport is more complicated, as can be discerned from the multiphasic response, but nonetheless significant inhibition is observed (compare Fig. 4, *A* and *C*, for slope and extent of recovery). The complex response may represent an effect on a number of potential Ca^{2+} processes, such as movement of dye to the plasmodesmata by cytoplasmic streaming. Previous work on animal cell–cell communication has demonstrated that TPA, a tumor promoter derived from croton oil (12), can inhibit cell–cell communication at ~0.7 ng/ml (36 and references therein). When SB-1 cells were incubated with 0.4 ng/ml TPA for 18 h, subsequent transport measurements demonstrated complete inhibition of intercellular dye transport (Fig. 4 *D*). Partial inhibition was observed for 0.2 ng/ml, whereas no inhibitory effect was observed at 0.05 ng/ml (data not shown). Using 4-phorbol-12,13-didecanoate, a nontumorigenic phorbol ester, we observed no inhibition (data not shown). These results are identical to those reported for the effect of tumorigenic and nontumorigenic phorbol esters on animal cell–cell communication (36 and references therein).

Intercellular Membrane Transport

Morphological investigations of plasmodesmata have provided evidence of membrane continuity between plant cells across the cell wall (8, 9, 27). Both plasma membrane and endoplasmic reticulum have been suggested to traverse the cell wall at sites occupied by the plasmodesmata. In the case of endoplasmic reticulum, the electron micrographs suggest that endomembranes may thread through a central plasmodesmatal channel. To explore this possibility, we labeled soybean cells with fluorescent phospholipid analogue NBD-PC, which has been demonstrated to partition into the plasma membrane and endomembranes of animal and plant cells (15, 21). Fig. 5 *a* shows an ACAS 470 false color image of the cellular distribution of NBD-PC fluorescence. Cells in the filament were arbitrarily selected for photobleaching (Fig. 5 *b*), and fluorescence redistribution was observed (Fig. 5 *c*). The limited extents of recovery observed are most likely related to the incorporation of exogenously added fluorescent phospholipid into noncontinuous membrane compartments, i.e., vacuole, intracellular vesicle, or Golgi membranes. Fig. 6, *a–c*, demonstrates no recovery for a single cell stained with NBD-PC and then photobleached. This again excludes the possibility for this probe that the lipid fluorescence recovery is due to dye reactivation after photobleaching or uptake of unincorporated NBD-PC from the media. A differentiation between fluorescence recovery due to diffusion of membrane lipids along continuous membrane strands or through lipid vesicle-mediated transport, or potentially, some aspect of all of these means is not, as of yet, possible.

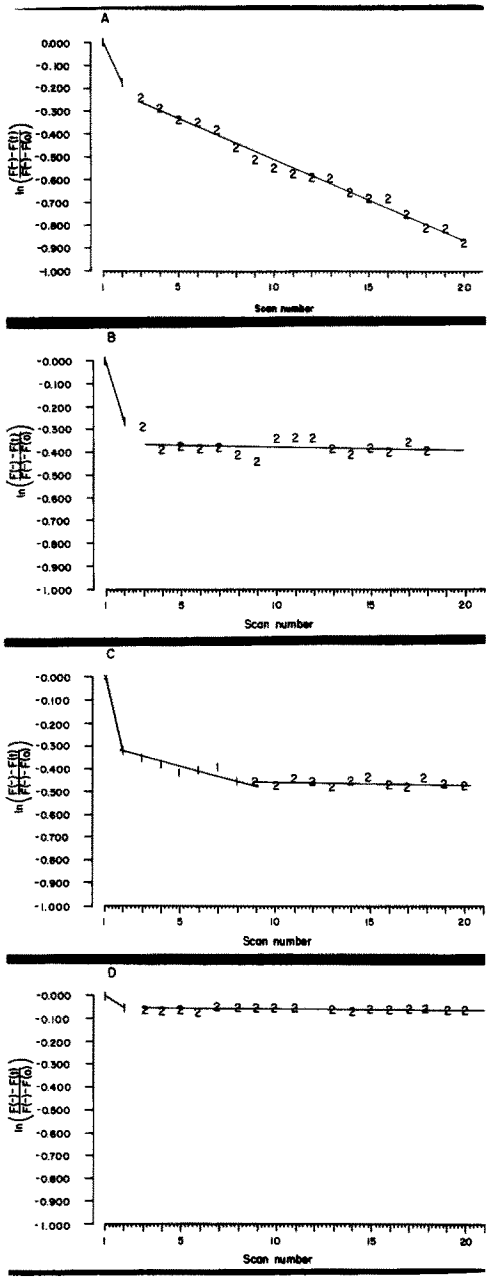

Figure 4. Fluorescence recovery in soybean tissue culture cells containing fluorescein as a function of Ca^{2+} and TPA. (*A*) Analysis of the fluorescence recovery of a control cell within a filament. (*B*) No recovery for a single isolated cell. (*C*) A cell in a filament incubated with 5 mM Ca^{2+} and the calcium ionophore, A23187 (100 μg/ml), for 10 min at 25°C and then put on ice before the measurement. (*D*) The recovery profile of a cell in a filament incubated for 18 h at 25°C with TPA (0.4 ng/ml), labeled with fluorescein, and photobleached. The fluorescence recovery was obtained using a FRAP instrument (14). The laser scans occurred at 15-s intervals, with a total time (scan plus delay) of monitoring for each scan of 5 min.

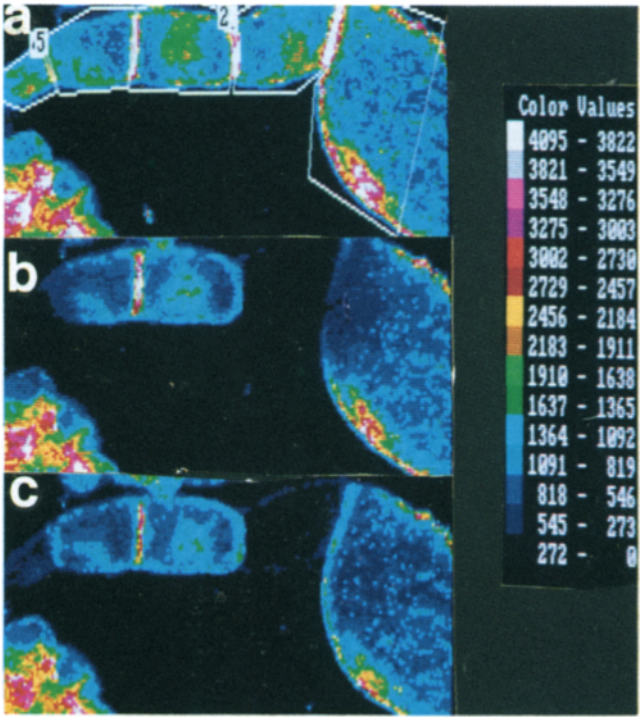

Figure 5. Recovery of fluorescence in photobleached soybean tissue culture cells labeled with the membrane probe, NBD-PC. Using the ACAS 470 Workstation in the same manner described in Figs. 2 and 3, a false color image of the fluorescence distribution of NBD-PC in the cells was determined (*a*). (*b* and *c*) Images immediately after and 20 min after photobleaching, respectively.

Discussion

Symplastic communication between plant cells has been suggested to be the dominant form of plant intercellular communication (8). Morphological evidence has suggested that trans-wall tubular structures, termed plasmodesmata, provide the channel(s) for the intercellular movement of hydrophilic low-molecular weight (≤1,000 mol wt) metabolic, biosynthetic, and signaling molecules (8, 27, 32). In addition, it has been suggested that membrane continuity between cells may occur at the plasmodesmatal sites affording a transport route for hydrophobic compounds and membrane components. In previous attempts to measure cell–cell communication, membrane impermeant dyes were injected into plant cells and the movement of dye from the site of microinjection to the surrounding cells was optically monitored (2, 18, 25, 33). These experiments demonstrated a transfer size limit of ≤1,000 mol wt for model compounds and variations in the inhibitory effect of Ca^{2+} on dye transport as a consequence of differing osmotic conditions (5, 6). A potential difficulty with these microinjection experiments may be inferred from the work of Davies and Schuster (4), who found that the properties of wounded tissues are markedly different from those of intact tissue. A needle breaching the cell wall barrier may, thus, lead to altered cellular responses. In an attempt to circumvent this potential problem, we employed fluorescence photobleaching (31, 36) to measure the transport of fluorescein and carboxyfluorescein. These dyes are introduced passively into the cell as fluorescein and carboxyfluorescein diacetate, which subsequently become deacetylated by intracellular esterases. F and CF were found to transfer between cells with a calculated permeability of $\sim 0.9 \times 10^{-6}$ cm/s. Ca^{2+} (in the presence of ionophore A23187) or the tumorigenic phorbol ester TPA were individually found to inhibit transport. The inhibition of transport mediated by these compounds in plants was previously reported to occur in mammalian cells (36). In an examination of TPA effects on transmembrane signaling in animal cells, evidence was provided that TPA may serve as a diacylglycerol (DAG) analogue stimulating protein kinase C (3, 16). In this context, phosphorylation, albeit cAMP-dependent, has been suggested to be a control mechanism for gap junction permeability (28, 37). Because recent observations in plant tissue have demonstrated Ca^{2+} and phorbol ester activated protein kinase activity (10, 23, 30), it may be reasonable to suggest similar posttranslational modifications of plasmodesmatal proteins through activation of the polyphosphoinositide pathway and the initiation of DAG, IP_3, Ca^{2+} mediated protein kinase activity. Recent preliminary results by Tucker and Rosenbaum (32a) demonstrating that IP_3 inhibits cell–cell communication in plants support this view. The comparable results for TPA inhibition reported for gap junction mediated communication (36) may provide evidence that gap junction and plasmodesmatal mediated transport share similar cellular control features. These similarities reported for transport properties and putative control mechanisms for gap junction and plasmodesmatal intercellular communication may now also be examined in the light of recent evidence presented by Meiners and Schindler (20) that a gap junction homologous protein exists in the cell wall/membrane fraction of soy-

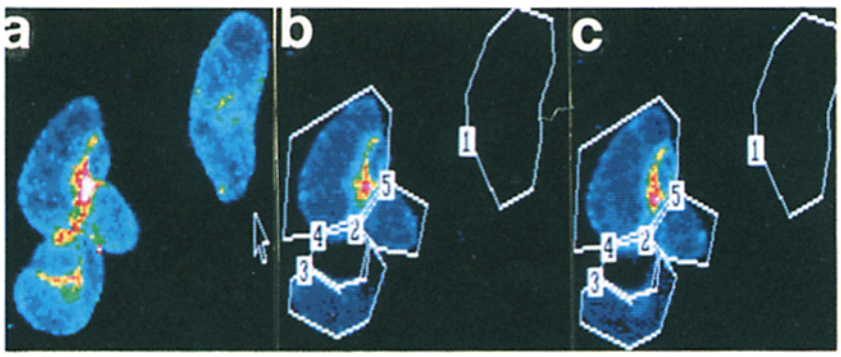

Figure 6. NBD-PC fluorescence recovery in unattached single soybean cells in tissue culture after photobleaching. The experiment was performed as described in Fig. 5. No recovery is observed.

bean plant cells. This has recently been topologically refined to suggest an enrichment of this material in the cell wall (Meiners, S., and M. Schindler, unpublished results). As of yet, plant gap junction homologous protein has yet to be definitively localized to plasmodesmatal structures.

Dynamic Assessment of Plasmodesmatal Frequency

By employing results from our dynamic measurements in conjunction with literature values for wall diameter and plasmodesmatal size, we may attempt to mathematically derive the number of structures required to provide observed transport rates. In our simplified model for transport, we assume cube-shaped soybean cells having an average dimension of 12 μm organized in a filament (two contacting sides per monitored cell). Permeability coefficient (p) is related to a transport rate constant K by: $p = (V/A)K$, where V is the volume of the model cell and A is the cell–cell contact area available for communication through plasmodesmata (24). In the case of carboxyfluorescein transport, we obtained $p \simeq 0.9 \times 10^{-6}$ cm/s ($K = 0.0015$ s^{-1}). Assuming the functional channel diameter is diam $\simeq$ 2.0 nm (obtained by analysis of molecular exclusion [32]), the critical dimension of carboxyfluorescein (0.7 × 1.12 × 1.27 nm) for radial transit through the channel is $\text{diam}_{CF} \sim 1.27$ nm (3a) and the plasmodesmata length is $\Delta X \simeq 255$ nm (average cell wall thickness from Robards [27]), then the area density of channels can be calculated by $n = p\,\Delta X/D_C A_e$ (26), where p is permeability coefficient, ΔX is plasmodesmatal length, and D_C is cytoplasmic diffusion coefficient of CF. D_C is in the range of 0.1–0.2 D_W (13), where D_W is the CF diffusion coefficient in pure water ($D_W = kT/3\pi\eta_W \text{diam}_{CF}$).[2] We thereby calculated $D_C \simeq 5.2 \times 10^{-7}$ cm^2/s. For A_e, the mean effective area of a channel ($\pi\text{diam}^2/4$), correction factor K_1 must be introduced describing the fractional resistance to diffusion in a cylindrical channel relative to that in free cytoplasmic pool. We estimated $K_1 = 12$ for a value of $\text{diam}_{CF} = 1.27$ nm and diam $\simeq$ 2 nm ($\text{diam}_{CF}/\text{diam} = 0.64$) (24), resulting in the expression $A_e = \pi\text{diam}^2/4K_1$. Given the above values, the channel density $n \simeq 169/\mu\text{m}^2$.

The plasmodesmatal frequency in the literature varies from tissue to tissue, but Sauter and Kloth (29) report that in ray parenchyma cells of poplar wood, plasmodesmatal frequency is $\sim 39/\mu\text{m}^2$ in cell rows in the center and $\sim 26/\mu\text{m}^2$ in cell rows at margins of the tissue. Considering this disparity between our calculated result and the observed plasmodesmatal frequency, it may be important to note that Terry and Robards (32) have proposed a controversial model of the plasmodesma consisting of nine channels around the central desmotubule. Using this number, our calculated channel density drops to $\sim 19/\mu\text{m}^2$, in far better agreement with the observations of Sauter and Kloth (29). Interestingly, in the context of results presented by Meiners and Schindler (20), if we assume each channel is approximately the size of a connexon, then six to nine channels could be accommodated around a central desmotubule.

Intercellular Membrane Communication

The cell–cell communication experiments performed with NBD-PC suggest that, unlike contiguous mammalian cells in tissue, plant cells may also communicate through continuous membrane paths. Our data suggest that this, indeed, can occur for phospholipids. Whether membrane proteins or other lipophilic molecules can commute between plant cells through connecting membranes, or whether membrane continuity also implies membrane potential continuity is presently under investigation.

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2. k is Boltzmann's constant, T is absolute temperature; η_w is water viscosity; diam_{CF} is critical dimension of CF taken as Stokes diameter.

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