Plant Physiol. (1990) 93, 841–845
0032-0889/90/93/0841/05/$01.00/0

Received for publication October 24, 1989
Accepted December 8, 1989

Review

Measuring Intracellular Ca^{2+} Levels in Plant Cells Using the Fluorescent Probes, Indo-1 and Fura-2[1]

Progress and Prospects

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ABSTRACT

Recent advances in the development of methods for measuring cytoplasmic Ca^{2+} levels in higher plant cells are discussed. Emphasis is placed on the new generation of Ca^{2+}-sensitive fluorescent dyes particularly fura-2 and indo-1. These dyes offer many advantages for the measurement of cytosolic Ca^{2+} levels. They can be introduced into cells in a nonintrusive manner, their K_d for Ca^{2+} matches plant cell cytoplasmic Ca^{2+} levels, and shifts in their emission (indo-1) or excitation (fura-2) spectra following Ca^{2+} binding permit accurate quantitation of Ca^{2+} activities. Examples of cytoplasmic Ca^{2+} levels measured in plants with fura-2 and indo-1 are presented, and the prospects for applying more advanced technologies to fluorescent dye measurement are discussed.

Interest in measuring cellular Ca^{2+} concentration has burgeoned with the discovery of the number and complexity of cellular processes that either regulate or respond to the level of cytoplasmic Ca^{2+}. Attempts to measure cytosolic Ca^{2+} in plants using techniques developed for animal cells have confronted difficulties imposed by the architecture of the plant cell. These include the cell wall, cell turgor pressure, and the large vacuolar compartment. Nevertheless, a number of techniques for measuring cytoplasmic Ca^{2+} have now been successfully applied to plant cells. Microelectrodes, metallochromic-dyes, photoproteins, and fluorescent probes have all been employed, albeit in a limited number of cell types, to establish that cytosolic Ca^{2+} in plant cells is maintained at low levels. Application of these same methods has yielded evidence for the widely held, but until now little supported, belief that changes in cytoplasmic Ca^{2+} correlate with or are signals for altered cellular function in plants (see, for example, ref. 5). These discoveries parallel those in animal cells and underscore the importance of developing methods for measuring cytoplasmic Ca^{2+} suitable for a wide range of plant cells. Although no single method is likely to work for all plant cells, the fluorescent probes indo-1 and fura-2 developed by Grynkiewicz *et al.* (12) appear to be a versatile and widely applicable tool for measuring cytosolic Ca^{2+} in plants.

[1]The authors' contributions to the work described in this review were supported by grants from the National Science Foundation and the U.S. Department of Energy.

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CHARACTERISTICS OF THE TSIEN Ca^{2+} PROBES

The family of fluorescent probes developed by Tsien represented a significant step forward in the technology of Ca^{2+} measurement. Tsien's major innovation was the rational design of dyes that addressed the specific requirements for Ca^{2+} measurement inside of cells (12 and references therein). These requirements were to produce a dye with an affinity for Ca^{2+} that matched normal cytosolic Ca^{2+} levels, that was highly selective for Ca^{2+} over other ions present at higher concentrations in cells, and that underwent an easily detectable change upon binding to Ca^{2+}. These requirements were substantially met in the first of Tsien's fluorescent probes, Quin2.

Indo-1 and fura-2 (Fig. 1), the next generation of dyes, were developed from Quin2 and have a number of properties that make them even better indicators of cell Ca^{2+} (12). Both indo-1 and fura-2 have a high selectivity for Ca^{2+} over Mg^{2+}, K^+, and Na^+. Moreover, the apparent K_d for Ca^{2+} binding is around 200 nM for both dyes, a value very close to levels of cytoplasmic Ca^{2+} in plant cells. Perhaps the greatest advantage of indo-1 and fura-2 over other Ca^{2+} probes, including Quin2, is that Ca^{2+} levels can be gauged from shifts in their fluorescence spectra. Ca^{2+} binds to indo-1 or fura-2 with a simple 1:1 stoichiometry and causes the fluorescence emission (indo-1) or excitation (fura-2) spectrum to shift to lower wavelengths (Fig. 1). As a result, Ca^{2+} levels can be accurately estimated from the ratio of fluorescence at two wavelengths (12). This is superior to dyes that change intensity only at a single wavelength, because fluctuations in the optical properties of the experimental system can be distinguished from actual changes in Ca^{2+} levels (4, 12).

DYE LOADING AND COMPARTMENTATION

Although the physicochemical features of indo-1 and fura-2 make them superior indicators of Ca^{2+} concentration, their utility for measuring cytosolic Ca^{2+} levels requires that the dyes remain in the cytoplasm and are not sequestered in other

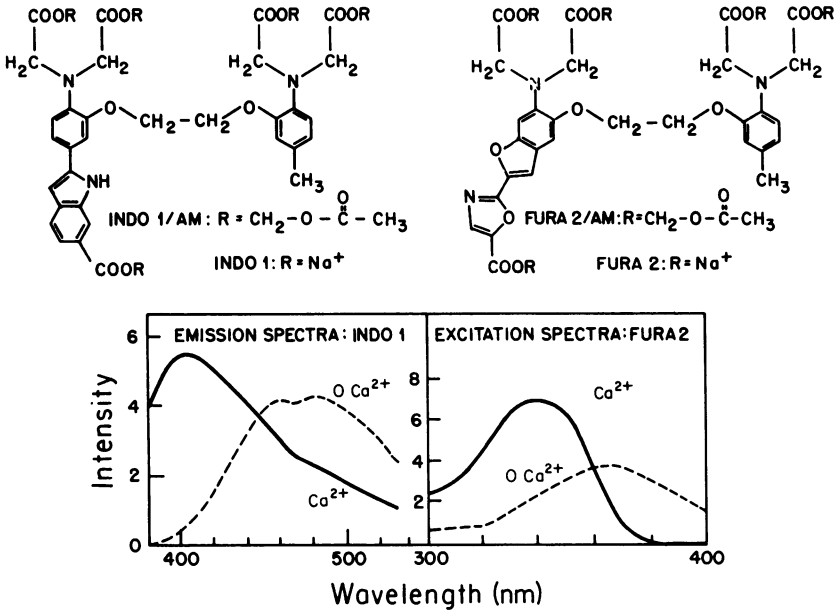

Figure 1. The chemical structures of indo-1 and fura-2, and the effects of Ca^{2+} on the emission (indo-1) and excitation (fura-2) spectra of these dyes. Indo-1 and fura-2 share an EGTA-like tetracarboxylic acid structure but differ in the fluorophor, indo-1 having an indole group and fura-2 a furan group. Indo-1 and fura-2 have five carboxylic acid groups and acetoxymethyl (R = AM) esters of each carboxylic acid have synthesized (indo-1/AM and fura-2/AM). The AM esters of the dyes are Ca^{2+}-insensitive but as pentacarboxylic acids (R = Na^+) the dyes are Ca^{2+}-sensitive. The fluorescence spectra of the Ca^{2+}-sensitive forms of these dyes shift to lower wavelengths when the dyes bind Ca^{2+} relative to the spectra of the Ca^{2+}-free form of the dyes (0 Ca^{2+}). For indo-1 the excitation wavelength was 356 nm, and for fura-2 emission was measured at 450 nm. Redrawn with permission from Grynkiewicz *et al.* (12).

cellular compartments. Much of the initial work using indo-1 or fura-2 in plant cells has addressed the loading and sequestering of these dyes.

Dye Loading

In designing suitable probes for measuring cellular Ca^{2+} levels, Tsien took into account the problems encountered in loading impermeant molecules into the cytoplasm. Tsien's goal was to develop dyes that could be introduced into cells by nonintrusive methods. It was reasoned that methods such as microinjection that involve impaling the plasma membrane could bring about changes in cytosolic Ca^{2+} levels unrelated to the normal physiology of the cell. Furthermore, microinjection and ionophoretic injection preclude loading large numbers or populations of cells. Tsien achieved non-intrusive loading of the fluorescent Ca^{2+} probes by masking the acidic, Ca^{2+}-binding side groups on the dye molecule with a hydrophobic AM[3] group (Fig. 1) This ester linkage rendered the dye insensitive to Ca^{2+} but allowed it to permeate the plasma membrane. Accumulation of dye in the cytoplasm relies on cleavage of the AM ester linkage by cytoplasmic enzymes, rendering the dye Ca^{2+}-sensitive and trapping it inside the cell.

Although this elegant method of dye loading has worked well for many animal cells and a few plant cells (3), it has not proven to be widely applicable to most plant cells. Two reasons have been advanced to explain the inability of plant cells incubated in AM esters of Quin2, indo-1, or fura-2 to accumulate the Ca^{2+}-sensitive form of the dye. It has been suggested that either plant cells lack the necessary esterases to cleave the AM linkage, or that extracellular esterases reduce the concentration of the AM form before it can accumulate in the cell. An alternative explanation is provided by our observation that in barley aleurone protoplasts indo-1/AM loads into the cell but is incompletely hydrolyzed, whereas the single AM linkage in the pH-sensitive dye BCECF/AM is rapidly cleaved (4). This suggests that aleurone esterases are sterically hindered from cleaving all of the AM linkages in indo-1, where four of the five ester groups are present at one end of the molecule (Fig. 1). In the case of indo-1/AM, partial hydrolysis of the five ester groups could be expected and has been reported in both plant (15) and animal cells. Incompletely hydrolyzed indo-1/AM is not Ca^{2+}-sensitive, however.

Since loading of the AM forms of indo-1 and fura-2 has not always been successful, alternative methods have been devised to achieve their accumulation in the cytoplasm. Electroporation, microinjection, and patch-clamp pipettes have been used to introduce the dye. Electroporation has been used to introduce Quin2 into protoplasts isolated from suspension cultured carrot cells and barley mesophyll protoplasts, and some of the first data on Ca^{2+} levels in plant cells were obtained using this method of loading (11). The chief advantage of electroporation over other intrusive methods of dye loading is that it allows measurements of cell Ca^{2+} in a population of protoplasts and the correlation of these measurements with the functioning of the same population (11). The disadvantages of electroporation are common to all intrusive methods, *i.e.* it causes a more or less temporary disruption of the cytoplasmic environment of the cell. However, electroporated cells appear to recover from this method of loading and function normally (11).

Microinjection and ionophoretic injection have also been used to load plant cells with dye. Perhaps the most elegant example of the microinjection method of loading comes from the work of Callaham and Hepler (7) using the stamen hair of *Tradescantia.* Because the stamen hair is made up of a single file of cells interconnected by plasmodesmata, dye injected into one cell is carried by cytoplasmic streaming into adjacent cells. In this organ, therefore, Ca^{2+} measurements can be made on single cells that have not been impaled.

[3]Abbreviation: AM, acetoxymethyl.

Unfortunately, the vacuole of stamen hair cells rapidly sequesters indo-1 and fura-2, so Ca^{2+} measurements must be made quickly after injection of the dye. Despite these limitations, both electroporation and microinjection have proven to be useful for the introduction of dyes into cells.

We have developed an alternative method for loading indo-1 into plant cells (4, 5). This method, which was devised using barley aleurone protoplasts, is nonintrusive and works well with a variety of plant cells and protoplasts. The essential feature of this method is that cells are incubated with the Ca^{2+}-sensitive form of indo-1 at a pH between 4 and 5. The accumulation of indo-1 by barley aleurone protoplasts is markedly pH dependent. Little dye accumulation occurs above pH 5 or below pH 4, and the maximum amount of accumulation, as determined by fluorescence measurements from single cells, occurs at pH 4.5 (4).

This simple technique permits a large number of protoplasts to be loaded and allows measurements of Ca^{2+} to be made either on individual cells (4, 5, 13) or on cell suspensions (1). Moreover, the technique permits walled cells as well as protoplasts to be loaded with indo-1 (A Wrona, personal communication). Another advantage of loading the Ca^{2+}-sensitive form of indo-1 instead of the AM ester is that accumulation in the cytoplasm of potentially harmful by-products of ester hydrolysis is avoided. Hydrolysis of the ester bond in AM forms of the fluorescent probes yields formaldehyde. When indo-1 is loaded, however, only protons are released into the cytoplasm. Our calculations show that the number of protons released following loading of indo-1 into barley aleurone cells will not alter cytoplasmic pH significantly (4).

Dye Compartmentation

Irrespective of the method of dye loading, Ca^{2+}-sensitive dyes must remain within the cytoplasm if they are to be useful for measuring cytoplasmic Ca^{2+} levels. The compartmentation of fura-2, indo-1, and Quin2 within plant cells is highly variable, however. Fura-2, in particular, is rapidly sequestered into the vacuole of a wide variety of plant cells (1, 4, 7). In the barley aleurone protoplast, fura-2 enters the vacuole when the protoplasts are loaded with fura-2/AM, but when aleurone protoplasts are loaded with indo-1/AM, the dye does not enter the vacuole (4). Fura-2 and indo-1 both accumulate in the vacuole of the stamen hairs of *Tradescantia* when the dye is introduced into neighboring cells by microinjection (7). Fura-2 also accumulates in the vacuole of root hairs of *Lycopersicon* and *Brassica* (8). Similarly, Quin2 has been shown to accumulate in isolated vacuoles of barley leaf protoplasts (11). In cells where Ca^{2+}-sensitive fluorescent dyes load into the vacuole, sequestration into the vacuole occurs rapidly, in most instances within 1 to 20 min. Little is known about the mechanism of dye transport into the vacuole.

The sequestration of significant amounts of fluorescent dye in an extracytoplasmic compartment can limit the usefulness of the dye for measuring cytoplasmic Ca^{2+} levels. Thus, total fluorescence from a cell or cell suspension will represent the average Ca^{2+} level in the whole cell weighted by the size of the signal from each of the compartments. This obviously makes unambiguous estimates of cytoplasmic Ca^{2+} problematic at best.

In a few cases, it may be possible to estimate the contribution of the compartmentalized dye to the signal. In barley aleurone, for expample, the amount of dye in the ER can be estimated from an isolated microsomal fraction by using a lumenal ER protein, α-amylase, as an indicator to quantitate the recovery of ER components (6). In general, strategies for minimizing sequestration must be developed. Several different strategies have been developed for animal cells. Sequestration of fura-2 has been prevented or slowed by inhibitors of anion transport such as probenicid (9). Lowering the incubation temperature while loading has also prevented sequestration of dye in some animal cells. Finally, in cases where the dye can be injected, coupling the dye molecule to a high mol wt dextran as has been done with pH-sensitive dyes may prevent sequestration. Although these strategies have apparently not been tried with plant cells, they suggest that methods of preventing sequestration could be developed.

Sequestration of Ca^{2+}-sensitive dyes in the endomembrane system may permit the measurement of the Ca^{2+} concentration there. For example, we have exploited the observation that indo-1 is accumulated in the ER to measure the levels of Ca^{2+} in this compartment (6). When ER is isolated from protoplasts loaded with indo-1, about 2% of the total cellular indo-1 is sequestered within the isolated ER vesicles (4, 6). The dye sequestered in the isolated ER vesicles is Ca^{2+}-sensitive and reports high levels of Ca^{2+} (>3 μM; 6).

DATA COLLECTION AND ANALYSIS

Cells loaded with fluorescent probes can be analyzed in several ways. The fluorescence signal from single cells can be monitored using fluorescence microscopy. Alternatively, the fluorescence signal from a population of cells can be quantitated spectrofluorimetrically (1). In order to take full advantage of the wavelength shift induced by Ca^{2+} binding to indo-1 and fura-2, it is necessary first to measure autofluorescence and second to measure fluorescence from the dye at two wavelengths. Autofluorescence spectra from plant cells frequently have peaks in the range of 350 to 500 nm (3). In weakly loaded cells it is, therefore, particularly important to measure accurately the level of autofluorescence and the rate at which it changes.

The wavelengths of choice for measuring dye fluorescence are the peaks of fluorescence for the dye in its Ca^{2+}-bound and Ca^{2+}-free states. In practice, however, other wavelengths close to the peaks are frequently used. Accurate estimates of cytoplasmic Ca^{2+} from measurements of fluorescence at chosen wavelengths also require calibration. This calibration of the dye response has most frequently been done *in vitro* by measuring the fluorescence of solutions of Ca^{2+} buffers and dye introduced between two coverglasses of a microscope slide. *In vivo* calibration, which is more informative, requires manipulation of the Ca^{2+} concentration in dye-loaded cells using Ca^{2+} buffers and nonfluorescent ionophores such as ionomycin (4, 11). *In vivo* calibration allows the estimation of the binding constant of the dye as well as the calibration of the measurement system. A comparison of *in vivo* and *in*

vitro calibration of fura-2 in algal cells has shown that the dye's affinity for Ca^{2+} is not greatly changed in the cytoplasmic environment from its value in 100 mM KCl (3).

Although measurements of fluorescent signals from a population of cells are informative, the most exciting developments in cytoplasmic Ca^{2+} research have utilized measurements of single cells. A wide range of equipment is now available for collecting, guantifying, and recording the signal from an individual cell. The most powerful instruments allow ratio imaging of the Ca^{2+} levels over the entire cell surface (2, 8). In ratio imaging, the fluorescence intensities at two wavelengths at many points in the cell-image surface are digitized and ratios are calculated. By ascribing colors of the visible spectrum to digitized data, a false color map of Ca^{2+} levels can be overlayed on the cell image. Ratio imaging has so far been applied to only a few plant cells. Imaging of fura-2 fluorescence in tomato and oilseed rape (8) did not reveal marked spatial gradients within the cytoplasm, but Ca^{2+} levels in the cytoplasm could be easily distinguished from those in the vacuole.

RESULTS AND PROSPECTS

Estimates of resting Ca^{2+} levels obtained using fura-2 or indo-1 vary greatly among cell types. Low levels of 30 to 90 nM Ca^{2+}, similar to those reported for animal cells, have been found in root hairs of tomato and oilseed rape (8). In general, however, resting levels of Ca^{2+} reported by indo-1 or fura-2 appear to be somewhat higher, ranging from one hundred to several hundred nanomolar in barley aleurone (5), guard cell (JI Schroeder, personal communication), and corn root (1, 13) protoplasts. These estimates are in agreement with those obtained using microelectrodes in *Riccia* and *Zea* (10) and in *Nitellopsis* (14).

There have also been several recent reports that document changes in cytosolic Ca^{2+} using fluorescent dyes. In barley aleurone, for example, we have shown by measuring the fluorescence ratio from single aleurone protoplasts loaded with indo-1, that resting cytosolic Ca^{2+} can be elevated from 200 to 350 nM by increasing the concentration of Ca^{2+} in the incubation medium surrounding the protoplasts from 10 μM to 20 mM. Lynch *et al.* (13) measured fluorescence from single protoplasts isolated from roots of *Zea* and loaded with indo-1 to show that salt stress raised the level of cytosolic Ca^{2+}. Bittisnich *et al.* (1), using spectrofluorimetric methods to monitor Ca^{2+} levels in populations of protoplasts isolated from barley and corn roots and loaded with indo-1 or fura-2, also showed that salt stress caused a rise in cytosolic Ca^{2+} levels. The fluorescent probes have also been useful for monitoring the effects of ion-channel blockers on cytoplasmic Ca^{2+} levels, thus providing indirect proof of the existence of membrane Ca^{2+} channels, which have heretofore not been characterized by electrophysiological methods in higher plant cells.

The ability to measure spatial and temporal gradients in cytoplasmic Ca^{2+} using the fluorescent probes indo-1 and fura-2 will undoubtedly increase as complementary technologies are developed. The application of ratio imaging, for example, holds the promise of answering long-standing conjectures about the role of spatial gradients in establishing polarity, or sensing external stimuli. The utility of ratio imaging will be further enhanced by recent developments in microscopy that allow optical slices of cells to be analyzed. This technique of optical sectioning, or confocal microscopy, should allow even greater resolution of variation in levels of Ca^{2+} inside plant cells.

Using these new techniques, plant physiologists are poised to answer exciting questions about the role of Ca^{2+} in plant growth and development. Does Ca^{2+} play a role as a second messenger for plant hormones? Does it signal responses to changes in environmental cues such as gravity, light, and temperature? Are nastic movements, such as those that change leaf angle, and the turgor movements that are involved in stomatal opening, regulated by changes in cytosolic Ca^{2+} concentration?

Calcium is thought to be involved in many phases of a plant's growth and development. It is implicated in the movement of cellular organelles such as the spindle apparatus and secretory vesicles and may play a key role in integrating plant cell metabolism. Ca^{2+} is known to play a role in the regulation of photosynthesis and oxidative carbon metabolism, and there is mounting evidence that protein phosphorylation is a Ca^{2+}-dependent process in plant cells. Our ability to measure changes in cellular Ca^{2+} using the new generation of fluorescent dyes should allow plant physiologists to ascertain how Ca^{2+} is involved in the regulation of these processes.

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