

Review

Ca²⁺-Selective Microelectrodes and Their Application to Plant Cells and Tissues

Hubert Felle

Institut für Botanik I der Justus-Liebig-Universität, Senckenbergstrasse 17-21, D-6300 Giessen, Federal Republic of Germany

ABSTRACT

The use of Ca²⁺-selective microelectrodes is difficult because of some basic problems: (a) electrodes with submicron tips may display non-Nernstian slopes; (b) liquid membrane microelectrodes respond only slowly (within seconds) to changes in ion activity; (c) turgid plant cells with tough walls damage the sensitive tip. This article describes concisely recent advances in fabricating Ca²⁺-selective single and double-barreled microelectrodes and their intracellular applications to different plant cell materials. Beveling the electrodes, mixing the sensor components with polyvinylchloride, insulation of the hydrated glass, and stabilization of the tips with inert materials are considered the basic concepts to circumvent most difficulties. It is concluded that the Ca²⁺-electrode can be a useful tool in plant physiology, but in spite of recent progress this technique remains experimentally demanding.

Since many cellular processes, especially those of signal transduction, are accompanied or require transient changes in cytosolic free calcium, the knowledge of its activity before, during, and after such processes is very important (5). However, measuring free Ca²⁺ is rather difficult, experimentally demanding, and prone to many pitfalls. Since cytosolic free Ca²⁺ is involved in several equilibria with fast exchange rates and is regulated to a very low submicromolar level, the relevant techniques to detect Ca²⁺-activity have to meet a variety of strict criteria with respect to selectivity, detection limit, and response time. Basically, there are four groups of techniques suitable to either detect intracellular free calcium or to measure its membrane transport: vesicles, protoplasts, and isolated vacuoles may best be investigated by using either photoproteins (e.g. aequorin) or metallochromic indicators such as arsenazo III, quin 2, fura, indo, and the like. On the other hand, single cells with walls or tissue cells are best investigated with Ca²⁺-sensitive microelectrodes. A fourth method comprises the classical [⁴⁵Ca²⁺] isotopic techniques which are in principal suitable for most types of cell material but, because of ion exchange properties of cell walls, they may find their best applications with wall-free preparations. Whereas the fluorescent dyes meet their limits in cell loading and calibration, the application of the microelectrodes faces three major difficulties: (a) the response of the sensor to submicromolar calcium activities is limited by the electrical

behavior of the very thin hydrated glass tips; (b) tough cell walls may cause breakage; (c) turgor will push the resin back into the shaft and thus make the electrode insensitive toward changes in calcium activity.

In spite of such profound problems there are encouraging reports from different laboratories on recent advances in the fabrication of Ca²⁺-selective electrodes and their successful application to several plant species.

BASIC DESIGN OF A Ca²⁺-SELECTIVE MICROELECTRODE

The principles, design, and applications to mainly animal systems of a wide variety of liquid membrane electrodes have been described in great detail by Ammann (1).

Basically, a calcium selective microelectrode is built like other liquid membrane electrodes (as described in detail by Felle and Bertl, 7): an ordinary glass micropipette, suitable for impalement of the respective cell, is prepared and made hydrophobic (silylation) on the inside for the purpose of keeping the lipophilic sensor cocktail in place. This cocktail is placed within the first 50 to 100 μm from the tip, the remainder is filled with the Ca²⁺ reference solution. Upon contact with the aqueous phases the lipophilic sensor forms a membrane selective for the respective ion. After suitable equilibration such electrodes are ready for extracellular use but not for the impalement of plant cells (see below). Since these electrodes have high impedances of ≥10¹¹ ohms, only amplifiers with input impedances of 10¹⁵ ohms as well as leakage currents of less than 10⁻¹⁴ A are suitable. An ion-selective electrode, intracellularly applied, always measures both electrical voltage across the respective membrane (membrane potential) and ion activity; therefore, a second (voltage-) electrode has to be placed within the same cell to obtain continuous information on the membrane potential. This latter signal is to be subtracted from the signal of the ion-selective electrode to reveal the net activity of the ion in question.

Two types of Ca²⁺-selective liquid membrane microelectrodes have been developed. One type, using membrane solutions, is based on electrically charged lipophilic esters of phosphoric acid and was presented in 1976 by Christoffersen and Johansen (6). In the same year Oehme *et al.* (13) developed a membrane solution which contained a synthetic neutral carrier (ETH 1001) as the ion-selective component. Both microelectrodes exhibit a high preference of Ca²⁺, over Na⁺ and K⁺, but

the neutral carrier electrode shows a much better selectivity of Ca^{2+} over Mg^{2+} and H^+ .

SOME PROBLEMS WITH CALCIUM MICROELECTRODES

Slope

It has been reported that Ca^{2+} microelectrodes with submicron tip diameters, based on ETH 1001, yield over-Nernstian slopes between pCa 4 and 6 (1, 9, 10, 14). Apart from the apparent 'violation' of thermodynamics, this is a serious problem, because the response to submicromolar calcium activities is then considerably smaller and the detection limit is unsatisfactory. Tsien and Rink (15) were able to improve considerably the response of calcium electrodes with submicron tips by the addition of PVC¹ to the cocktail. The improvement was interpreted to be due to the elimination of electrical shunts caused by the hydration of the extremely thin glass (membrane) near the tip of the microelectrode. Whereas for unknown reasons microelectrodes filled with the sensor ETH 1001 may yield near-Nernstian slopes even without PVC (1), PVC treatment is mandatory when the ETH 129 is used.

Beveling the submicron tips is another way to reduce this problem; this procedure takes part of the hydrated glass from the tip and at the same time produces a larger liquid membrane area. However, this procedure is time consuming and often leads to exhaustive electrode waste.

Turgor Problem

In general, liquid membrane microelectrodes are not applicable to turgid plant cells without stabilization. Cell turgor pushes the sensor back into the shaft and thus makes the electrode insensitive to changes in ion activity. This problem has first been successfully circumvented by Bertl *et al.* (3), in fabricating pH-sensitive microelectrodes, but is described in more detail in Felle and Bertl (7). The idea is based on insulation of the glass using PVC (14, 15). PVC as an inert material can also be used to stabilize an electrode by placing a perforated (electrically conducting) plug of PVC into the very tip, which in most cases protects the sensor from being damaged upon impalement or moved during measurement. The amount of applied PVC is critical; too much of it will lead to a complete plugging of the tip. After such treatment considerably slower response times of the electrode ($t_{1/2} = 5\text{--}20$ s) have to be taken into account (7, 9).

Response Time: Limits and Artefacts

Testing intracellular ion activities is always based on a differential measurement: the signal from the voltage electrode (mainly the membrane potential) is subtracted from the signal of the ion-selective electrode. From the scientific point of view the simultaneous information on the membrane potential is a real advantage, but the problem is that all liquid-membrane microelectrodes respond considerably slower than ordinary voltage electrodes. Whenever there is a fast voltage change this may create a serious measurement problem: due to the inequality in response times the difference between the two traces

will therefore yield an unreal change in ion activity, *e.g.* a fast depolarization will cause an apparent decrease in ion activity and *vice versa* (10). This limits the applicability of such electrodes to situations where the voltage changes are either slow or absent. Furthermore, only relatively slow changes (in the range of seconds) in ion activities can be detected.

CALIBRATION, RECALIBRATION, AND DETECTION LIMITS

Basically, the calibration of a Ca^{2+} -microelectrode is simple and is usually carried out in test solutions with Ca^{2+} set to the respective concentrations with calcium buffers (14). Since K^+ is the most interfering ion and because of its high concentration in the cytosol (usually around 100 mM), these test solutions are supplemented with roughly the amount of K^+ of the respective test cell. Calibration curves of electrodes with different fillings of both sensors and reference solutions ($\text{pCa}_r = 4, 5, 7$) are given in Figure 1. The detection limit for electrodes based on ETH 1001 is just above pCa 7, whereas those electrodes filled with ETH 129 yield detection limits close to pCa 8. The reference pCa does not influence this value. However, these detection limits are not even close to the subnanomolar values reported by Ammann *et al.* (2), a discrepancy which may be due to the PVC treatment, as described above.

Recalibration can also be carried out in most instances, but with liquid membrane electrodes such a procedure has no scientific or analytic value. Even in cases where the cell turgor has pushed back the sensor into the shaft and thus has made the electrode insensitive toward changes in ion activity, it can be observed that upon withdrawal of the electrode from the cell the sensor moves back into place (personal observations). Upon recalibration, such electrodes may again yield good slopes.

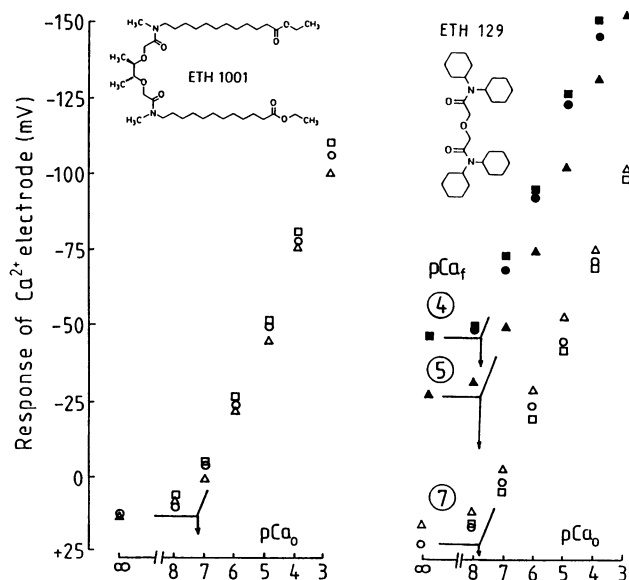


Figure 1. Calibration of Ca^{2+} -selective electrodes filled with the sensors ETH 1001 and ETH 129, and with different calcium reference solutions ($\text{pCa}_r = 4, 5, 7$), as indicated. The operational detection limits are given by the arrows pointing to the respective pCa . The calcium buffers were prepared according to Tsien and Rink (15); the electrodes were fabricated as described in Felle (9).

¹ Abbreviation: PVC, polyvinylchloride.

APPLICATIONS OF THE Ca²⁺-MICROELECTRODE TO PLANT MATERIAL

Due to the difficulty of the method only very few successful applications on plant cells have been reported (Table I). Brownlee and Wood (4) gave calibration curves of a Ca²⁺-electrode based on the sensor ETH 1001. They inserted their electrodes into *Fucus rhizoids* and found relatively high calcium activities at the tips ($2.47 \pm 0.8 \mu\text{M}$), but considerably lower values in subtip regions ($0.28 \pm 0.06 \mu\text{M}$). Removal of external Ca²⁺ caused a steady reduction of cytosolic calcium, whereas replacement of Ca²⁺ caused a faster increase in cytosolic calcium. Treatment with La³⁺ gradually decreased cytosolic free calcium to submicromolar levels. Miller and Sanders (11), using the same sensor, could demonstrate in *Nitellopsis* that high light intensities depleted cytosolic calcium from 390 to 145 nM. Recently, using the sensors ETH 1001 and ETH 129, Felle (8, 9) could demonstrate oscillatory cytosolic Ca²⁺ changes in *Zea mays* coleoptiles after external application of auxin. It could also be shown that in the aquatic liverwort *Riccia fluitans* as well as in *Z. mays* cytosolic pH and Ca²⁺ interact. Changes in external calcium from 0.1 to 10 mM rapidly but transiently increased cytosolic calcium. An increase of cytosolic calcium by about 0.4 pCa units was observed after a vacuolar pH change from 5.1 to 5.35. Miller and Sanders (12), also using ETH 129, fabricated a calcium sensitive microelectrode of dimensions compatible with impalement into fungal cells and with a detection limit of about 10 nM Ca²⁺. They reported 85 ± 24 nM cytosolic calcium in *Neurospora crassa* and found that metabolic uncouplers rapidly increased cytosolic calcium.

DOUBLE-BARRELED Ca²⁺-MICROELECTRODES

Since the detection of intracellular Ca²⁺ is based on a differential measurement and hence requires two electrodes inserted within the same cell (see above), determinations in tissue cells are at present only feasible by the use of double-barreled microelectrodes. The fabrication of such electrodes involves a number of additional problems which are not necessarily typ-

ical for calcium electrodes. Quite unexpectedly, double-barreled Ca²⁺-microelectrodes more frequently display near-Nernstian slopes than single-barreled ones do (personal observation). The reason for this may be that double-barreled electrodes are usually not as sharp as single ones and, as electron micrographs prove, the tip openings of the two barrels are not always at the same level. Therefore, the effective tip orifice is indeed wider as with single barreled electrodes, especially when glass tubing with different diameters was used (7, 9). Although for animal cells there are quite a few reports on intracellular applications of double-barreled Ca²⁺-microelectrodes (1, and refs. therein), to my knowledge there are only three recent reports where such electrodes have been used intracellularly on plant material (Table I). Brownlee and Wood (4) report $2.61 \pm 0.9 \mu\text{M}$ cytosolic calcium for tip regions of *Fucus rhizoids*, but $0.35 \pm 0.15 \mu\text{M}$ for the subtip regions. These values compare well with their results obtained with single electrodes. Felle (8, 9) demonstrated that double-barreled Ca²⁺-microelectrodes can successfully be applied to cells of different plant tissues.

CONCLUSIONS

Applications of Ca²⁺-sensitive microelectrodes in plant cells are few as yet. Although the metallochromic indicators are also hazardous to use, especially as far as calibration and loading are concerned, most investigators seem to prefer these methods. Wherever we are dealing with cell-wall-free systems and/or small cells, it may well be that in the future these dyes will be more successful. The dyes have their advantages in fast response times and permit detection of gradients across the total compartment, whereas the electrodes always yield point measurements. However, as long as the problem of physiological loading of the dyes is not solved in general, there will always be a need for the direct use of Ca²⁺-microelectrodes. Also, the application of double-barreled Ca²⁺-microelectrodes in tissue cells is highly essential for studies of, for example, hormonal actions in plant cells (8). A certain advantage of the ion selective electrodes is that the membrane potential and thus the respective driving force is always monitored simultaneously with the ion activity. This gives the experimenter a brilliant control of the physiological state of the cell. Finally, I would like to emphasize that it is extremely important to combine these methods and compare their results (4, 11).

ACKNOWLEDGMENTS

Financial support was granted by the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. Ammann D (1986) Ion-Selective Microelectrodes. Springer-Verlag, Heidelberg
2. Ammann D, Bühner T, Schefer U, Müller M, Simon W (1987) Intracellular neutral carrier-based Ca²⁺ microelectrodes with subnanomolar detection limit. *Pflügers Arch* 409: 223-228
3. Bertl A, Bentrup W, Felle H (1984) Amine transport in *Riccia fluitans*. Cytoplasmic and vacuolar pH recorded by a pH-sensitive microelectrode. *Plant Physiol* 76: 75-78
4. Brownlee C, Wood JW (1986) A gradient of cytoplasmic free calcium in growing rhizoid cells of *Fucus serratus*. *Nature* 320: 624-628

Table I. Comparison of Cytosolic and Vacuolar Steady State Calcium Activities Measured Directly with Single(s) or Double(d)-Barreled Ca²⁺-Sensitive Microelectrodes in Different Plant Cells

Species/Cell Type	Cytosol	Vacuole	Single (s) / Double (d)		Ref.
			Single (s)	Double (d)	
<i>Fucus serratus</i>					
Tip	$2.47 \pm 0.8 \mu\text{M}$		s		4
	$2.61 \pm 0.90 \mu\text{M}$		d		4
Subtip	$0.28 \pm 0.06 \mu\text{M}$		s		4
	$0.35 \pm 0.15 \mu\text{M}$		d		4
<i>Nitellopsis obtusa</i>	10 ± 395 nM		s		11
<i>Riccia fluitans</i>					
Rhizoid cells	148 ± 39 nM	2.3 ± 0.8 mM	d		9
Thallus cells	127 ± 33 nM		d		9
<i>Zea mays</i>					
Root hairs	179 ± 34 nM		d		9
Root stele	195 ± 44 nM	1.5 ± 0.5 mM	d		9
Coleoptiles	112 ± 31 nM		d		8
<i>Neurospora crassa</i>	85 ± 24 nM		s		12

5. Carafoli E (1987) Intracellular calcium homeostasis. *Annu Rev Biochem* **56**: 395–433
6. Christoffersen GRJ, Johansen ES (1976) Microdesign for a calcium-sensitive electrode. *Anal Chim Acta* **81**: 191–202
7. Felle H, Bertl A (1986) The fabrication of H⁺ selective liquid-membrane microelectrodes for use in plant cells. *J Exp Bot* **37**: 1416–1428
8. Felle H (1988) Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. *Planta* **174**: 495–499
9. Felle H (1988) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: Interaction of Ca²⁺ and pH? *Planta* **176**: 248–255
10. Lanter F, Steiner RA, Ammann D, Simon W (1982) Critical evaluation of the applicability of neutral carrier-based calcium selective microelectrodes. *Anal Chim Acta* **135**: 51–59
11. Miller AJ, Sanders D (1987) Depletion of cytosolic free calcium induced by photosynthesis. *Nature* **326**: 397–400
12. Miller AJ, Sanders D (1989) The role of plasma membrane transport in calcium homeostasis. FESPP-Workshop Physiological and Metabolic Implications of Transport in Plants. Trieste (in press)
13. Oehme M, Kessler M, Simon W (1976) Neutral carrier Ca²⁺-microelectrode. *Chimia* **30**: 204–206
14. Tsien RY, Rink TJ (1980) Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim Biophys Acta* **599**: 623–638
15. Tsien RY, Rink TJ (1981) Ca²⁺-selective electrodes: a novel PVC-gelled neutral carrier mixture compared with other currently available sensors. *Neurosci Meth* **4**: 73–86