Methodological aspects of pressure loading of Fura-2 into Characean cells

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Abstract

Four different fura-2 compounds were tested for the application in Characean cells (fura-AM; fura-C₁₈; fura-K₅; fura-dextran; MW = 10 kDa). It is demonstrated that Characean cells impose special problems when cytosolic pCa has to be measured with fluorescent ratio dyes. Fluorescence (λₑₓ = 340 nm) from the dye which had diffused from the cytosol to the huge central vacuole with millimolar Ca²⁺ concentrations overrides the signal from the cytosol and makes Ca²⁺-quantification difficult. This can be avoided by pressure injection of fura-dextran. Because of inhomogeneities in dye concentration or in thickness of the cytoplasmic layer, cytoplasmic streaming causes high noise or pretend oscillations in pCa if data are obtained by subsequent image grabbing. In addition, vesicles filled with high concentrations of dye may sometimes be expelled into the vacuole during the loading procedure enhance this effect. These sources of inhomogeneities can be minimized by loading fura-dextran via the neighbouring cell. The slow loading procedure through the plasmodesmata takes 1-10 h. It results in a more homogeneous distribution of the dye. The operation of the new method is illustrated by the measurement of Ca²⁺-transients during action potentials, the temperature dependence of the fluorescence signal in vivo and in vitro and the butyrate-induced elevation of [Ca²⁺]c.

Fura-AM was found not to be well suited for use in algal cells. Fura-C₁₈ has toxic effects and induces clotting of the cytoplasm. In addition, some aspects of the properties of dextran-derivates are discussed.

Key words: Manual pressure microinjection, fura-2, characean cells, fluorescence ratio imaging, temperature dependence.

Introduction

The role of Ca²⁺ in intracellular signal transduction in plant cells is discussed for many phenomena as oscillations of membrane potential (McAinsh et al., 1995; Amtmann et al., 1992; Thaler et al., 1987, 1989; Förster et al., 1989; Felle, 1988), growth (Herrmann and Felle, 1995; Sievers et al., 1991), intracellular signal perception (Allen and Sanders, 1994; Schulz-Lessdorf and Hedrich, 1995), gravistimulation (Sievers et al., 1984), osmoregulation (Katsuhara and Tazawa, 1992; Okazaki et al., 1987), action potentials (Hodick and Sievers, 1986, 1988; Lühring and Tazawa, 1985; Tazawa and Shimmen, 1980; Kikutama and Tazawa, 1983; Lunevsky et al., 1983; Thiel, 1995; Homann and Thiel, 1994; Thiel et al., 1993; Fromm, 1992; Fromm and Spanswick, 1993; Fromm and Bauer, 1994, Shiina and Tazawa, 1988, Williamson and Ashley, 1981, 1982; Callaham and Hepler, 1991) and others. This has focussed much interest on the measurement of cytosolic pCa.

Besides internal perfusion of Characean cells (Lühring and Tazawa, 1985; Shiina and Tazawa, 1988) and indirect approaches like measuring pCa changes via the velocity of cytosolic streaming (Plieth and Hansen, 1992), there are two main methods for measuring cytosolic Ca²⁺ activity directly. Ion-selective microelectrodes provide the benefit of simpler calibration in absolute terms (Felle et al., 1992, Herrmann and Felle, 1995; Miller and Sanders, 1987), whereas the application is easier in the case of fluorescent indicator dyes. They are also more suitable for monitoring spatial distribution (Reiss and

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Abbreviations: APW, artificial pond water; CTC, chlorotetracycline; fura-AM, fura-2-acetoxymethylester; fura-C₁₈, fura-2-C₁₈ pentapotassium salt; fura-K₅, fura-2-pentapotassium salt.

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Nobiling, 1986; Reiss and Herth, 1978, 1979) and enable a faster temporal resolution.

Special problems have to be encountered when the huge characean cells (length up to some centimetres, diameter up to 1 mm) have to be loaded. Loading of dyes which penetrate lipid membranes from the bathing medium like CTC is easy. However, CTC is not only sensitive to pCa but also to pH and pMg (Caswell, 1979; Schneider et al., 1978, 1983; Gupta and Berkowitz, 1989; Plieth, 1995). Furthermore, it adheres preferably to membranes, and thus its usage is restricted to the observation of pCa changes in the vicinity of membranes (Plieth, 1995; Tirlapur and Willems, 1992; Caswell and Hutchison, 1971; Carvalho, 1978; Meindl, 1982; Polito, 1983).

In contrast Fura-2, which distributes very well in the cytosol, cannot enter the cell via the plasmalemma.

AM-ester loading (Kao, 1994; Thomas and Delaville, 1991; Tsien, 1981) does not work well in algal cells. Very often the esterases required for the cleavage of the AM-linkage are not present in plant cells (Bush and Jones, 1987, 1990) or external esterases in the cell wall or the apoplast reduce the concentration of the AM-form (Graziana et al., 1993; Gilroy et al., 1986, 1990, 1993; Cork, 1986a, b). Additional problems are that

1. the dye may move into the vacuole and other compartments of the cell (Almers and Neher, 1985; Cobbold and Rink, 1987; Connor, 1993; Oparka and Hawes, 1992; Roe et al., 1990; Rathore et al., 1991)

2. the hydrolysis of the AM-ester leads to a release of formaldehyde and acetic acid (Negulescu and Machen, 1990) which interfere with cellular metabolism (Spray et al., 1984)

3. the AM-form of the dye is not water soluble and needs additional compounds for solvation (i.e. DMSO) which may lead to toxic effects (Poenie et al., 1986; Bright et al., 1987a, 1989b)

4. during the AM-cleavage some calcium insensitive intermediates are generated. They have different spectral properties (Scanlon et al., 1987; Hightsmith et al., 1986; Elliott and Petkoff, 1990).

Nevertheless, some authors got reliable results by means of using AM-ester loading of plant cells (Linberg, 1995; Read et al., 1992; Irving et al., 1992; Elliott and Petkoff, 1990; Gehring et al., 1990a; Brownlee et al., 1987; Wang et al., 1991; Shacklock et al., 1992).

Electrophoretic loading was used by Hodick et al. (1991) for Chara rhizoids. However, in the case of internodal cells, electrophoretic loading takes too much time because of the huge volume of these cells. In addition, the dextran derivates which have to be used in order to avoid uptake into the vacuole (see below), have a 10- to 70-fold higher molecular weight as, for example, Indo-1 used by Hodick et al. (1991).

In the studies presented here, pneumatic pressure loading as applied by Golnick et al. (1991) in Amoeba proteus was found to be suited best for loading characean cells with fura-dextran. A syringe operated with an elbow lever is used for a smoother control of the pressure.

Some methodological aspects and improvements of pressure loading of fura-2 into characean cells are reported, especially loading of fura-dextran via neighbouring cells. The loading via the neighbouring cell avoids the impaling of the plasma membrane of the investigated cell which disturbs the normal pCa (Bush and Jones 1990).

Materials and methods

**Plant material**

*Nitella flexilis* L. was purchased from R Kiel (Frankfurt) and *Chara corollina* was a gift of Dr D Gradmann, Göttinigen. The algae were grown in the lab in APW (0.1 mM KCl, 1.0 mM NaCl, 0.5 mM CaCl₂) on a layer of old beech leaves covered by a layer of sand at room temperature (22°C) and at an irradiance of 5–10 W m⁻² for 14 h d⁻¹. The pH was adjusted to pH 7.0 by additions of HCl when it increased beyond pH 7.5.

**Preparations of cells**

For the experiments, individual or pairs of two adjacent internodal cells of 5–15 mm length were dissected and transferred to a Petri dish. The cells were pressed to the bottom with needles sticking in silicon rubber at the bottom of the Petri dish as described by Plieth and Hansen (1992) or Plieth (1995).

**Dye loading apparatus**

Pressure injection of the dye was done by means of a glass capillary (AR-type, Hilgenberg, Malsberg, Germany) drawn on a puller for glass microelectrodes (Microelectrode-Puller HCE, Hugo Sachs, March-Hugstetten, FRG). Electron micrographs showed that the internal diameter was slightly more than 1 μm. The pipette holder was similar to that used for patch experiments. The pipette which also served as conductor for the electrical measurements was filled with 10 μl of the injection fluid (double-distilled water with 1 mM of the fluorescent dye). The air-filled pipette holder was mounted on a hydraulic manipulator (Narishigi, Model MO 303, Tokyo, Japan). It was connected via a polyethylene tube (internal diameter 1.4 mm) to a big laboratory-made injection syringe. This air-filled syringe made from aluminium had a diameter of 30 mm, and the travel of the piston was 80 mm. The piston was operated by an elbow lever as shown in Fig. 1. The lever with a length of 40 cm gave good control over the application of up to 2500 kPa to the injection pipette. Especially in the high pressure range, the lever can easily be controlled.

**Injection into cytoplasm**

In Characean cells, a huge vacuole (100–500 μm) is surrounded by a thin cytoplasmic layer (2–10 μm) beyond a rigid cell wall. This renders the insertion of the tip of the pipette into the cytoplasmic layer difficult. The problem can be overcome by using electric control in addition to the visual inspection during insertion. For this purpose, the pipette was connected to an amplifier (lab-made) which records the electrical potential ($V_m$ in Fig. 1).
The advancement of the pipette was observed through an inverted microscope (Diaphot, Nikon, Düsseldorf) and loss of the sac to the vacuole. The final cytosol (Figs 2, 3), and low enough to prevent bursting or dissection of the membrane potential, BE = bath electrode, \( V_m \) = Registration of electrical plasmalemma potential.

During pressure injection, a cytosolic sac was built at the tip of the electrode (Fig. 2). This corresponded to the normal behaviour also observed in the case of microelectrodes used for electrical measurements. Here, the sac was inflated because the dye was pressed out of the pipette. Its extension into the vacuole had to be observed under the microscope. Now the benefit of the elbow lever of the injection syringe became important because this construction enabled a very fine manual adjustment of the pressure just in the region of the turgor pressure (Nitella 700-1200 kPa).

**Calibration**

The in vitro calibration was done with rectangular glass capillaries (50 x 1000 \( \mu \)m, WS005, VitroDynamics, Rockaway, NJ, USA) and standard Ca\(^{2+}\) solutions (Calibration Kit, C-3722, Molecular Probes, Haugland 1992) containing 3 \( \mu \)M fura-dextran. An example for a calibration curve is shown in the inset of Fig. 8. The saturation of Fura-2-dextran above 1 \( \mu \)M needs a comment. Fura-dextran has a higher dissociation constant (\( k_d \approx 350 \text{nM} \), varying from lot to lot, Haugland, 1992) than the pure pentapotassium salt Fura-K\(_5\) (\( k_d \approx 140 \text{nM} \); Lattanzio, 1991). Thus it is not surprising that Fura-dextran saturates above 1 \( \mu \)M Ca\(^{2+}\) (i.e. \( \text{pCa} < 5 \)).

The pressure had to be kept slightly below the internal turgor pressure in order to make the dye flow gently into the cytosol (Figs 2, 3), and low enough to prevent bursting or dissection and loss of the sac to the vacuole. The final concentration of the dye in the cytosol was estimated to be 3-6 \( \mu \)M from the comparison of the fluorescence of the cell and of the calibration capillaries.

The measurement of pCa by fluorescence was done with a set-up similar to that of Fenton and Crofts (1990) with an inverse microscope (Diaphot, Nikon, Düsseldorf, FRG) mentioned above and an ImproVision image grabbing system (Coventry, UK). Briefly, a filter wheel (Ludl-Electronic, ImproVision, Coventry, UK) selected alternatively two wavelengths, 340 nm and 380 nm (interference filters from Ealing Electro-Optics, Holliston, MA, USA), from the light of a Xenon lamp (XBO100W OFR, Osram). A half-translucent mirror (DM400, Nikon) reflected the exciting beam into the objective (20 x or 40 x) focused on the plant cell. The induced fluorescence was collected by the same objective and went straight through the mirror and a bandpass filter (BA510, Nikon) to the CCD camera (ISIS-M XTI, Photonic Science, Robertsbridge, Sussex, UK). The picture of the camera could be analysed with a commercial image analysis programme (IonVision, Vers. 1,5, ImproVision, Coventry, UK).

The camera sensitivity and the intensities of the 340 nm beam and the 380 nm beam had to be aligned to appropriate values.

The camera was adjusted to an adequate sensitivity before the cell was loaded. Adjustment was done in such a way that the background grey levels were below 5 (256 is the maximum) for both signals. Thus, background subtraction was not required after the experiment because the background level could be neglected.

Grey filters were selected to align the excitation intensities, after the dye had been loaded and the equilibration of the dye had been checked by eye. Mostly, the 380 nm signal was higher than the 340 nm signal. The stronger signal was reduced by a grey filter until the grey levels of the brightest spots were below 256 (maximum) minus the increase which was expected to occur during the experiment. The grey level for the other signal was adjusted to give a ratio around 1 (0.5 to 1.2). A finer adjustment was not possible because of the limited availability of grey filters.

Even when full care was taken during the injection procedure an absolutely homogeneous distribution of the dye within the cell could not be achieved. The clouds of different dye concentrations or different thickness of the endoplasmic layer moved around the cell with the streaming of the cytoplasmic layer. They could be easily visualized by the CCD-camera (Fig. 4).

**Results and discussion**

Four different fura-2 compounds were compared, fura-AM, fura-C\(_{18}\), fura-K\(_5\), fura-dextran (10 kD).

**Fura-AM and fura-C\(_{18}\)**

Fura-AM is designed to penetrate the cell membrane directly and to be hydrolysed by esterases in the cytoplasm. Thus it does not require injection. However, in contrast to animal cells, no signal was found in the algal cells within 30 min after fura-AM was given to the bathing medium. In most cases fluorescence was not found after washing indicating the absolute absence of fura in the cell. Only in some cases, a fluorescence signal was observed. A detailed inspection under the microscope, however, showed that the light came from epiphytes (bacteria and fungi), but not from the Chara cell.

Usually injection of fura-C\(_{18}\) killed the cells after 2-3 h (no resting potential, no streaming, plasmolysis). Thus, fura-C\(_{18}\) seemed to be toxic at least in characean cells. As in the case of the other dyes, the presence of fura-C\(_{18}\) inside the cytosol was tested by an action potential.
Fig. 2. Injection sac and distribution of the dye by cytoplasmic streaming. Fluorescence image of the tip of a micropipette during injection of fura-dextran into a Chara cell ($\lambda_{ex} = 400$ nm; $\lambda_{em} = 510$ nm) is depicted. The pseudocolours indicate the concentration of the dye. At the tip a cytoplasmic sac is built which is filled with dye. The loaded cytoplasm is taken with the cytoplasmic streaming along the endoplasm layer and thus distributed in the cell.

Fig. 3. Temporal development of the distribution of the dye by cytoplasmic streaming. The fluorescence images show the injection of fura-dextran into a Nitella cell ($\lambda_{ex} = 400$ nm; $\lambda_{em} = 510$ nm). The pseudocolours indicate the concentration of the dye. The dye is distributed within the cell by the cytoplasmic streaming. Images were taken at intervals of c. 10 s.
immediately after injection. When an electric action
potential was elicited by a current through the Petri dish,
the peak of the electrical action potential coincided with
that of the fluorescence ratio (340/380), thus showing the
transient elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$. Fura-C$_{18}$ is a lipophilic
$\text{Ca}^{2+}$ indicator and rapidly associates with membranes
(Etter et al., 1993). As it induced clotting in these
experiments, its performance was much worse than that
of CTC, which also adhered to membranes, but did not
induce clotting. The presence of CTC in the cytosol was
concluded from experiments, where loading via the neigh-
bouring cells had been used routinely in other studies on
the light-effect on cytosolic pCa (unpublished results).

Fura-K$_5$ and Fura-dextran were studied in more detail
as described now.

*Injection of fura-K$_5$ into single cells*

Directly after the injection of the dye into a cell, recording
started with a sampling rate of one 340/380-pair per
2 min. The 340 nm and the 380 nm-images were taken
alternatively with an interval of 0.6 s. From the grabbed
images, an area of 100 x 200 $\mu$m was selected which was
located completely in the cytosol of the alga. Two observa-
tions were important,

1. slight or no increase of the mean value of the 340 nm
   signal and a strong decrease of the mean value of the
   380 nm signal were observed within about 3 h
   (Fig. 5A, B). The ratio increased steadily (Fig. 5C, D).

2. periodic fluctuations in the fluorescence signals
   (Fig. 5A, B) and in the ratio (Fig. 5C, D).

The decrease of the mean values of the 380 nm fluores-
cence signal resulted from the movement of the dye into
the vacuole as had been observed also by Clarkson et al.
(1988), Oparka and Hawes (1992), Oparka (1991), and
Read et al. (1992). This was indicated by the behavior of
the ratio (Fig. 5C, D). The penetration of the dye into
the vacuole with millimolar $\text{Ca}^{2+}$ concentrations (DuPont
et al., 1990; Felle, 1988) made the high $\text{Ca}^{2+}$ signal from
the vacuole override that from the cytosol. The effect
seemed to be stronger in Chara (Fig. 5B, D) than in
Nitella (Fig. 5A, C).

The periodic fluctuations after injection indicated the
circular movement of the dye or of the inhomogeneities
mentioned above (Fig. 4) with the cytoplasmic streaming.
The decrease of the periodic fluctuations was supposed
to result mainly from the increasing influence of the signal
from the vacuole, as suggested by the behaviour of the
oscillations in the case of fura-dextran shown below
(Fig. 6).

*Injection of fura-dextran into single cells*

In the case of fura-dextran, the oscillations could persist
for hours (Fig. 6A, Chara), indicating that there were
inhomogeneities which moved around with cytosolic stream-
ing as shown above (Fig. 4). In Fig. 6B (Nitella), the
oscillations can scarcely be detected because of the high
noise. However, the maximum peaks keep a constant

Fig. 4. Inhomogeneous dye distribution in the cytoplasmic layer. The pseudocolour presentation of the fluorescence ($\lambda_{ex} = 400$ nm; $\lambda_{em} = 510$ nm) indicates the concentration of the dye.
Fig. 5. Fura-K$_4$ fluorescence observed after dye injection into Chara (A, C) and Nitella (B, D). The traces in (A) and (B) show the fluorescence induced by 340 nm (open circles) and by 380 nm (closed circles). In (C) and (D) the ratios are given. The sampling rate was 1 pair per 2 min. The interval between the 340 nm signal and the 380 nm signal was 0.6 s. The increase in the ratio shows the displacement of the dye from the cytoplasm into the vacuole.

The temporal distance of about 12 min which coincides with the period of cytosolic streaming. The periodicity can also be seen in the ratio (Fig. 6C, D).

The origin of the noise in Fig. 6 was not related to the sensitivity of the recording system. Also its occurrence in the ratio may not be taken as evidence that there were inhomogeneities of pCa moving around. Instead it was found to originate from the subsequent grabbing of the images at 340 nm and 380 nm. As the interval between the sampling of the two signals was 0.6 s, the inhomogeneities of the cytoplasmic layer or of the dye concentration had moved about 30 μm (velocity of cytosolic streaming is about 50 μm s$^{-1}$, Plieth and Hansen, 1992). Figures 5A and C show that there are parallel shifts in the 340 nm and in the 380 nm signal. The inhomogeneities in pCa should result in opposite shifts of the signals evoked by 340 nm and by 380 nm.

This kind of noise would not occur if double-emission technique (e.g. Indo-dyes) would be applied, and if the two fluorescence images were taken simultaneously with two cameras.

A test of this hypothesis about the origin of the noise was provided by records where the streaming stopped. Stopping could be induced by an action potential (Fig. 7), or by loading the cells with butyrate (Fig. 8).

Figure 7 shows the response of fura-dextran fluorescence signals to an action potential initiated by a current (3 V cm$^{-1}$, 0.2 s, DC) through the Petri dish. In Fig. 7A, the fluctuations in the 340 nm and in the 380 nm signal are in parallel. At this time, there was normal streaming in the cell. The action potential leads to deflections of different signs in the two traces, indicating the increase of cytosolic Ca$^{2+}$-concentration (Callaham and Hepler, 1991; Plieth, 1995). The noise before the action potential is to a lesser extent also observed in the ratio (Fig. 7B). The inset in Fig. 7B shows that the noise gets much smaller after the action potential, when the streaming is still at rest.

The effect of streaming on the noise gets even more obvious in Fig. 8. The addition of 32 mM butyrate stops streaming. It is known to induce an acidification of the cytosol (Sanders et al., 1981; Sanders and Slayman, 1982; Fisahn et al., 1986), but it also causes a shift in pCa. The experiment of Fig. 8 belongs to a series which was
designed to test whether the changes in pCa can be explained by coupling to a butyrate-induced effect of pH on a H⁺/Ca²⁺ cytosolic buffer-exchange system (Plieth, 1995; Plieth and Hansen, unpublished observations). The bar in the top of Fig. 8A has two dark regions (stopping of streaming). The first one belongs to the stopping (and increase of [Ca²⁺]_{cyt}) resulting from the first of the two action potentials initiated after the addition of butyrate. Here also the noise was lower. The fluorescence ratio was converted to Ca²⁺ concentrations by means of the calibration curve shown in the inset. The second stoppage is initiated by the second AP and prolonged by the action of butyrate.

The cell used for the experiment in Fig. 8 had many inhomogeneities, and thus the noise was high in the 380 nm and 340 nm traces (Fig. 8A) and in the ratio (Fig. 8B) under streaming conditions. The noise became very low when the addition of 32 mM butyrate stopped streaming. It recommenced when butyrate was washed out, pCa had reached the original value and streaming started again.

The conclusion from the above experiments is that pressure injection of fura-dextran into Characean cells suffers from the following drawbacks

1. often the injecting electrode penetrates into the vacuole,
2. oscillations and noise as shown by Figs. 5–8 render the significant determination of small changes in pCa difficult.

One origin of the noise has been described above. Another one is related to injection artefacts described above. Sometimes minor parts or the whole sac at the tip of the injection pipette are lost to the vacuole. These vesicles filled with high dye concentration and obviously also with high Ca²⁺ concentration from the pipette solution (distilled water without EGTA) move around with the cytosolic streaming and cross the area of interest as very bright (340 nm) light balls resulting in strong and long spikes of the fluorescence signal. This occurs especially if the injection pressure is too high. This problem was found to occur frequently in autumn and gets more rare in spring and summer.
Injection of fura-dextran into neighbouring cells

In order to overcome the problems mentioned above, a new loading procedure was tested. Two adjacent internodal cells were laid into the Petri dish, and one cell was loaded as described above.

Figure 9 shows the transfer of 10 kDa fura-dextran from the loaded (source) cell into the neighboring (sink) cell via the plasmodesmata. The fura-2 fluorescence was measured in the sink cell. The loading process was slow, but the curves are smooth (indicating an uniform loading of the whole cytosol). The oscillations shown in Figs 5 and 6 are absent. As a result of the better dye distribution, the noise of the ratio is much lower. The noise of the ratio is still high during the first hour (Fig. 9B). However, this results from the low dye concentration as indicated by the delayed increase of the 340 nm and 380 nm signals in Fig. 9A.

The increase of the fluorescence curves in Fig. 9 was fitted to a simple exponential ($F(t) = A(1 - \exp(-t/\tau))$). The time-constants of loading varied between 1 h and 10 h (mean value 3.1 ± 2.7 h, 5 experiments). Sometimes loading is preceded by a lag time of 0–90 min (Fig. 9A). This could be explained by a closure of the plasmodesmata (Jacob et al., 1995) due to the wounding shock during impalement of the injection pipette into the source cell.
Pressure loading of fura-2

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Fig. 10. Temperature dependence of the fura-fluorescence signals in vivo in Nitella (B) and in vitro (C). 340 nm signal = open circles and 380 nm signal = closed circles. From the fluorescence signals (A, B) the ratios (C, D) are calculated.

In general, this technique was often applied in intact plant tissues to demonstrate transport phenomena within the symplast. Dye transport from cell to cell through plasmodesmata depends on the molecular weight of the dye (Cleland et al., 1994; Palevitz and Hepler, 1985; Robards and Lucas, 1990; Terry and Robards, 1987; Tucker, 1990). Characean plasmodesmata are able to permeate molecules with a weight up to 45 kDa (Kikuyama et al., 1992, 1993). This is explained by the lack of desmotubuli in characean plasmodesmata (Robards and Lucas, 1990). Thus it is not a surprise that the dextran derivates of fluorescent dyes with MW= 10 kDa are easily transported from cell to cell as demonstrated in Fig. 9.

Temperature dependence

A remaining problem of the measurements in characean cells is the tendency of spontaneous firing of action potentials if the cell is subject to a stimulating treatment. This is illustrated by experiments dealing with the temperature dependence of the fluorescence ratio in vitro and in vivo.

In vivo experiments were done in Nitella. The temperature of the medium was changed by the following procedure. Ice-cold APW (0 °C) or warm APW (40 °C) was added to the bathing medium. The fluorescence of the dye was monitored during warming or cooling by equilibration with room temperature (c. 22 °C). Temperature was measured with a NTC resistor connected to a lab made instrument (Plieth and Hansen, 1992).

For the in vitro experiment, rectangular glass capillaries (50 x 1000 μm, W5005, VitroDynamics, Rockaway, NJ, USA) were filled with EGTA/KCl solution containing 100 nM of free Ca²⁺ (Calibration Kit, C-3722, Molecular Probes, Haugland 1992) and 3 μM fura-dextran. They were laid into the Petri dish beneath the microscope. The temperature was changed by letting pre-warmed or pre-cooled capillaries equilibrate with room temperature.

In vitro (Fig. 10A) and in vivo (Fig. 10B), the 340 nm signal seems to be quite insensitive to temperature, whereas the 380 nm signal decreases with increasing temperature. Both signals were fitted with straight lines. Because of this, the increase of the ratio is described by a hyperbolic function

\[ r(T) = \frac{aT + b}{cT + d} \]

with \( a \) and \( b \) determined from the 340 nm line and \( c \) and \( d \) from the 380 nm line, respectively. Fig. 10C shows the slight dependence of the ratio of the in vitro experiment at a Ca²⁺ level of 100 nM in the temperature range of 0 °C to 37 °C. In vivo (Nitella, Fig. 10D), a similar curve was found, but the increase was smaller. The in vivo curve is much more noisy. This was caused by spontaneous action potentials mentioned above probably initiated by the change in temperature, which could not be avoided during the whole series of experiments. A similar problem became obvious in Fig. 8. There were two action potentials at the beginning of the butyrate treatment.
Conclusion

Fura-dextran turned out to be the component which is best suited for cells with large vacuoles like characean cells, because it stays in the cytosol even in experiments which last for hours.

However, in the case of characean cells, also the Ca\(^{2+}\) measurements by means of this dye suffer from peculiar problems related to the magnitude of these cells and the existence of the huge vacuole. The vacuole is the cause of inhomogeneities seen in Figs 4–8 because of two effects, varying thickness of the flowing cytoplasmic layer and sometimes the existence of dye-loaded vesicles expelled to the vacuole during the loading procedure. Even though the observed region of interest is small (100 × 200 μm), these inhomogeneities cannot be excluded by selecting a homogeneous part of the cell. Cytoplasmic streaming brings the inhomogeneities to all parts of the cell. Because of this, loading via the neighbouring cell is recommended. The distribution of the dye is more uniform and the occurrence of expelled vesicles is restricted to the non-observed cell.

The inhomogeneities mentioned above are a typical problem of Characean cells. Pressure loading with the set-up in Fig. 1 was also applied to *Eremosphaera viridis*. In these cells (diameter about 150 μm) the problem of travelling inhomogeneities did not occur. The cells are much smaller, the equilibration in these cells is much better and cytosolic streaming does not in- and export inhomogeneities into the region of interest during the experiment.

Figures 5 and 6 may suggest that using a dye with double emission would provide a more effective suppression of the inhomogeneities, because the two signals are measured simultaneously, but this raises problems (‘spill over’, Morris *et al.*, 1994) in the case when two different dyes are simultaneously applied (i.e. for pH and pCa\(^{2+}\) measurements).

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