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MEASUREMENT OF FREE CALCIUM CONCENTRATION IN LIVING CELLS

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Abstract - Changes in the concentration of free calcium ions, in the cytoplasm, $[Ca^{2+}]_i$, are used to trigger or regulate a wide variety of cellular functions. Methods for measuring the $[Ca^{2+}]_i$ are thus important tools for investigating cell activation. Two factors make these measurements difficult. Compared with the free concentrations of the potentially interfering cations, H^+, Mg^{2+}, Na^+ and K^+, $[Ca^{2+}]_i$ is very low being around 10^{-7} μM at rest and rising perhaps to 10^{-5} μM on maximum activation. The detection systems thus need excellent sensitivity and selectivity. Secondly, cells are very small so that considerable ingenuity or manual skill is needed to introduce the detection system into intact cells without, wrecking them. The two main approaches have been either to make calcium-selective microelectrodes small and sharp enough to impale single cells, or to introduce into cytoplasm, by various means, indicators which give an optical signal, absorbance, fluorescence or intrinsic luminescence dependent on, and thus reporting $[Ca^{2+}]_i$. This brief account focuses mainly on microelectrodes and a newly developed fluorescent indicator that can be incorporated non-disruptively into populations of cells of any size.

INTRODUCTION

Several recent accounts have detailed methods for measuring calcium and other ion activities in living cells (e.g. Refs. 1-6). These articles are largely directed at biologists who have formulated the problems they wished to study. This article is not intended as a general survey of techniques but rather to indicate to those with a more chemical background why biologists might want to make measurement of calcium in living cells, what are the special difficulties involved and certain recent developments in this field. I have chosen to discuss the measurement of cytoplasmic free calcium, $[Ca^{2+}]_i$ because, 1) many of the problems are common to all ions, though Ca^{2+} has some special features, 2) this is the ion which I have most personal experience, and 3), calcium turns out to have an important regulatory role in almost every type of cell and has widespread importance as an intracellular messenger in many aspects of cell activation. Cell functions regulated by changes in $[Ca^{2+}]_i$ include: contraction of muscle, secretion of hormones, release of neurotransmitters, key events in both sperm and egg at fertilization, the regulation of membrane permeability, and the activity of many different enzyme systems (Refs. 7 and 8).

It may be asked, why one should focus on the free Ca^{2+}, when the ions that actually influence cellular process are those bound to the target proteins. The reason is that the binding of Ca^{2+} to its site of action is some function of the free-concentration and a major way that cells alter the amount of Ca^{2+} bound to these sites is by altering $[Ca^{2+}]_i$. It is also possible to alter the amount of Ca^{2+} bound to critical sites even if $[Ca^{2+}]_i$ stays constant, by altering the Ca-affinity of those sites. But to establish this point one still needs to know what is happening, or not happening, to $[Ca^{2+}]_i$.

ION DISTRIBUTION ACROSS THE CELL MEMBRANE

Cells are bounded by a thin plasma membrane only about 3 nm thick which separates the cytoplasm from the extracellular medium. The ionic composition of these two compartments is very different as is shown in Table 1 which gives typical values for extracellular cytoplasmic-free concentrations of some of the most important ions. It can be seen that the cell sits in a solution mainly made up of NaCl and $NaHCO_3$, while in the cytoplasm the major cation is K^+. The main intra-cellular anions are polyvalent organic phosphates, e.g. ATP, and proteins which bear a net negative charge at cytoplasmic pH . In addition to the large concentration differences for Na^+, K^+ and Ca^- there is an approximately 10^4-fold inward gradient for Ca^{2+}. In most cells there is a 'membrane potential' of several tens of millivolts, cytoplasm negative with respect to the external medium. The normal distribution of ions is maintained by the special properties of the membrane, which presents a hydrocarbon diffusion barrier to hydrophilic solutes, with specific and selective transporter proteins for certain solutes, i.e. it has selective permeability. Some solutes therefore disribute across the membrane much more rapidly than others. Additionally there are various forms of active transport that can produce a net transfer against the electrochemical gradient, the energy for which is directly or indirectly provided by hydrolysis of ATP. Examples of these active transporters are the sodium pump which usually transports 3 Na^+ out and $2K^+$ in for each ATP hydrolysed, and an ATP-fueled calcium pump which pumps out of the cell the calcium which leaks in. Cells also contain many types of subcellular organelles including mitochondria, secretory granules, various forms of endoplasmic reticulum and lysosomes. These organelles are in turn bounded by membranes and have an internal composition that can be very different from that of cytoplasm. The nucleus is bounded by a membrane with pores large enough to permit the transfer of RNA and so its ionic composition is probably in equilibrium with that of the cytoplasm. Some organelles are able to accumulate and sequester calcium and are specialised to release it into the cytoplasm on appropriate stimulation.

TABLE 1. Typical free concentrations of ions in extracellular fluid and cytoplasm. Values for cytoplasm differ somewhat in different cells, particularly the value for Cl^-. The bulk of the anions in cytoplasm are polyvalent organic phosphates and proteins.

	Extracellular	Cytoplasmic
Ca^{2+}	10^{-3} M	10^{-7} M
Na^+	1.4×10^{-1} M	1×10^{-2} M
K^+	4×10^{-3} M	1.4×10^{-1} M
Mg^{2+}	5×10^{-4} M	10^{-3} M
H^+	4×10^{-8} M	8×10^{-8} M
HCO_3^-	2.4×10^{-2} M	1.2×10^{-2} M
Cl^-	1.1×10^{-1} M	varies with cell type

The distribution of calcium is well arranged for its role as a regulator of cytoplasmic processes. A stimulus can rapidly and substantially increase the free concentration by causing an increase in the normally very low calcium permeability of the plasma membrane. Only a small net influx is required to give a large proportional change in $[Ca^{2+}]_i$. A large increase in $[Ca^{2+}]_i$ can also be achieved by discharge of calcium sequestered in intracellular organelles. After a stimulated increase in $[Ca^{2+}]_i$, the resting state can be regained by pumping the additional calcium out of the cell across the plasma membrane and resequestering discharged calcium into the relevant intracellular organelles.

THE TECHNICAL PROBLEMS

Table 1 shows the first problem. A calcium detection system useful inside cells needs excellent calcium sensitivity and selectivity to pick out 10^{-7}M Ca^{2+} from equivalent concentrations of H^+ and very much larger concentrations of Mg^{2+}, Na^+ and K^+. Additionally one has to consider possible interference from other substances in cytoplasm.

The next problem is that cells are very small. The largest mammalian cells are about 60 um across, the smallest only a few um at their maximum diameter. Delivery of the detection system into the cytoplasm without wrecking the cells therefore presents a formidable challenge. Yet, even though cells are so small, $[Ca^{2+}]_i$ need not remain uniform throughout the cytoplasm. The diffusion of Ca^{2+} in cytoplasm is sufficiently slow that localised additions of Ca^{2+} can result in marked concentration gradients (Refs. 9 & 10). For instance a brief increase in calcium permeability of the plasma membrane may allow an influx of Ca^{2+} that results temporarily in a "shell" of raised $[Ca^{2+}]_i$ just under the membrane but no change in the centre of the cell. Moreover, the influx of calcium may be exceedingly brief lasting only a millisecond or so, and so to detect it an extremely rapidly responding system would be necessary. Quantifying such rapid temporal and spatial $[Ca^{2+}]_i$ transients is presently beyond the available techniques, though there has been some success in following more slowly developed spacial gradients with the photoprotein aequorin (Refs. 10 & 11) as mentioned below. Fortunately many cells have either developed special mechanisms to provide a relatively uniform distribution of elevated $[Ca^{2+}]_i$, as is the case in many muscle cells, or have responses that last seconds or minutes when the spatial gradients in the cytoplasm are unlikely to be maintained.

The major methodologies that have successfully measured $[Ca^{2+}]_i$ fall into two main groups. 1. Ion selective electrodes of adequate performance have been made sufficiently small and sharp to impale single cells. 2. Indicator substances of various classes that give some form of optical signal can be incorporated by various means either into single cycles or into populations of cells. Very recently a series of NMR probes have been developed that show some features of the optical probes, and potentially can provide additional information (Ref. 12).

CALCIUM-SELECTIVE MICROELECTRODES

Ion-selective electrodes capable of measuring the activities of each of the cations listed in Table 1 have been available for many years, though only relatively recently have calcium-sensitive-electrodes with the requisite selectivity being developed. The challenge to biologists has been to retain the selectivity, stability and speed of response of such electrodes while minaturising them sufficiently to permit successful impalement of living cells. Membrane potential has been routinely measured in many cell types (though not the very smallest) for many years by means of glass micropipettes drawn out to have tips of external diameter 0.1-1 μm, and filled with a conductive electrolyte, often concentrated KCl (Ref. 13). These are inserted into cells by means of micromanipulators that allow controlled movement of only a fraction of a micron at a time. To make ion-selective microelectordes, the tips of such micropipettes need to contain an appropriate ion-selective membrane. This is most often a complex mixture of organic components, colloqually known as a liquid ion exchanger or sensor, which has the appropriate ion selective properties. Sometimes the mixture is formed into a PVC gel. Only a few microns of the electrode is inserted into the cell, so it is vital that only the very tip of the electrode has ion selective properties, since most of the length of the electrode is exposed to the extracellular solution (see Fig. 1).

As with any ion-selective electrode system one has to have an "indifferent" electrode to give the reference potential. This is often another similar microelectrode simply filled with electrolyte inserted under microscopic control into the same cell. Better, in some respects, is to have a double-barrelled combination microelectrode in which the tip of one barrel contains the ion-selective sensor and the other simply electrolyte. One can then be assured that the indifferent electrode records the appropriate reference potential, necessarily at the same point as the tip of the ion-selective barrel. However, double-barrelled, ion-selective microelectrodes can be especially tricky to make.

With the two electrode tips in the same cell changes in the membrane potential, which are often associated with cell activation, should not effect the differential potential that records the ion activity, since the potential on both reference and ion-selective electrodes will be changed by the same amount, with respect to a second indifferent electrode in the external bathing medium. The general arrangement for making such recordings inside cells is shown highly diagramatically in Figure 1.

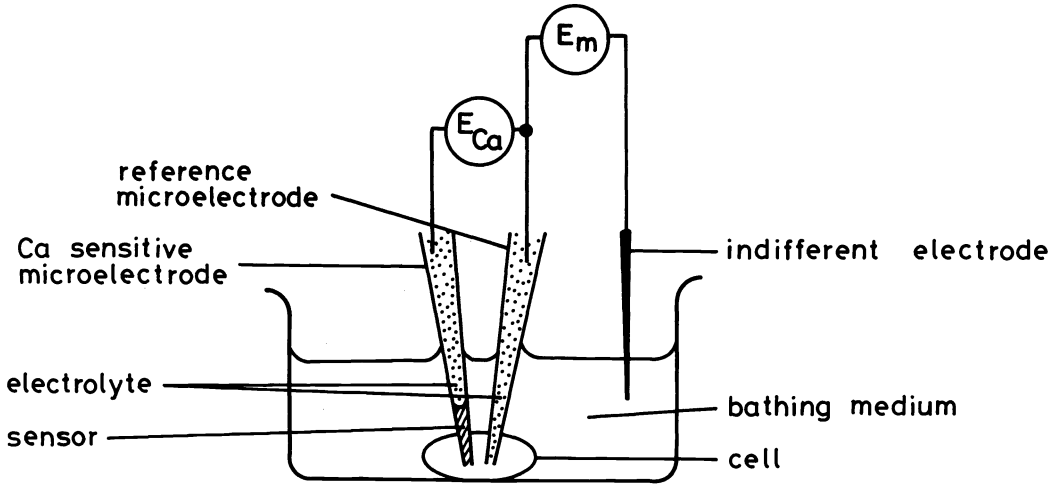


Fig. 1. Shows, schematically the arrangements measuring $[Ca^{2+}]_i$ with a Ca-sensitive microelectrode. The cell, its membrane, and the electrodes are drawn only very roughly to scale. To give some idea of the dimensions involved, the electrode tip might be 0.5 microns in outer diameter and the cell 30 microns across. $[Ca^{2+}]_i$ is obtained by measuring the differential signal, E_{Ca}, with both electrodes in the cell, w.r.t. to the E_{Ca} in the bathing medium which has a known $[Ca^{2+}]$. The E_{Ca} in calibration solutions are also mesured w.r.t. to bathing medium so that E_{Ca} inside the cell can be lined up on the calibration plot to give $[Ca^{2+}]_i$.

Practical details of the contruction and use of ion-selective microelectrodes are given in an excellent handbook by R.C. Thomas (Ref. 13). (This book also describes the ingenious methods developed by Thomas for incorporating a recessed tip of H^+ or Na^+ sensitive glass into the tip of an outer micropippette to produce a glass membrane H^+ or Na^+-selective microelectrode). Further details concerning the construction and use of calcium microelectrodes are given in Refs. 14-17. I shall outline here just a few aspects of making and using Ca-selective microelectrodes.

The sensors

Two main classes of sensors were initially developed for use in PVC membranes in macro electrodes, and show selectivities approaching those needed for measuring $[Ca^{2+}]_i$; those based on organophosphates (see e.g. Refs. 18 & 19) and those based on neutral ligands (Ref. 20). Microelectrodes with organophosphate sensors in their tips show adequate selectivity against H^+, Na^+ and K^+ (Refs. 21-23). The organophosphate sensors suffer Mg^{2+} interference that limits their ability to quantify $[Ca^{2+}]_i$ less than about 0.5 μM (Ref. 16). These sensors also appear to give an excessively high electrical resistance in micropipettes with tips less than 1 μm o.d. and have not been very widely used. The neutral ligand

sensors show adequate selectivity against Na, K^+ and H^+ and have negligible interference from Mg^{2+}. It is also possible to make and use such electrodes with tips finer than 1 micron (Refs. 16 & 17). Many more workers now use this type of electrode. The sensor may be either liquid or gelled in situ with PVC. Several different recipes have been tested but the original liquid sensor formulation of Oehme et al (Ref. 24) is now commercially available. It consists of 10% neutral ligand (ETH1001), N,N'di[11-(ethoxy carbonyl) undecyl)]-N,N'-4,5-tetramethy-3,6-dioxaoctan 1,8-diamide; 1% hydrophobic salt, Na tetraphenylborate; and 89% solvent, (o-nitrophenyl)octylether, NPOE. We found an improvement in performance when the sensor is gelled in situ in the electrode tip with 12-14% PVC and also some benefit from the use of a more hydrophobic salt (Refs. 14 & 16). The formulation of one of our electrodes of this type is given in the legend to Fig. 2.

Siliconization

It is obviously vital to avoid electrical leaks in parallel with the sensor. This is achieved by siliconizing the glass surface prior to filling the tip so that a good electrical seal is made between the glass surface and the hydrophobic sensor. It is important to achieve good siliconization without reaction products blocking the very fine opening. We do this by drying the glass pipettes at 200°C and then exposing them to the vapour of tri-N-butylchlorosilane (Refs. 15 & 16).

Electrode resistance

The impedance of ion-sensitive microelectrodes is typically 10-100 giga-ohms (10^{10} to 10^{11} ohms) and so electrometers with a very high input impedance and very low, stable bias current are required. Since it is difficult to reduce the capacity of the electrode system to less than a few pF the electrical time constant of the system will be 10-1000 msec, so that very rapid response times are not to be expected. In fact at very low $[Ca^{2+}]$ the electrode responses may be diffusion limited giving yet slower responses (Refs. 14, 16).

Size and sharpness

In general, the smaller electrode tip, the better it will impale a cell. However when the tip gets smaller than about 1 um the performance of the electrode tends to deteriorate, possibly because the unavoidable shunt pathway across the very thin glass of tip becomes larger relative to the interfacial resistance of the exposed sensor (Ref. 16). One way to improve matters is to sharpen the tip by grinding it an angle to produce a bevelled end. We bevel microelectrodes on a revolving plate of agar gel with fine diamond dust embedded on its surface (Ref. 15).

Performance

Fig. 2 shows the calibration of a bevelled calcium-selective microelectrode with a tip of about 0.5 microns. The response to $[Ca^{2+}]_i$ is shown against a background of 125 mM K. The response is Nernstian down almost to 10^{-7} M and there is a usable signal down to about 10^{-8} M. The relatively slow response time to changing $[Ca^{2+}]_i$ in the range of cytoplasmic free Ca^{2+} is evident. The response to $[Ca^{2+}]_i$ is almost as good as that seen in the best neutral-ligand macroelectrodes.

All methods of measuring $[Ca^{2+}]_i$ have to be calibrated against calcium-buffer standard solutions and, in practice, the reference standards in the cytoplasmic range are Ca-EGTA buffer mixtures. These give solutions of known free Ca^{2+} concentration, and we prefer not to try to calculate an activity coefficient for Ca^{2+}. One reason is that there is no generally agreed single method for making such calculations and that various different values are used in the literature for the activity coefficient (Refs. 2, 3 & 4). A given differential signal measured inside a cell can be compared with the calibration trace and we then report $[Ca^{2+}]_i$ as the free concentration appropriately read off a calibration plot. This is the way in which biologists have usually calibrated their signals from calcium-selective microelectrodes and almost always done so for other measuring systems. Properly, one might say that the calcium activity measured inside the cell was the same as the activity in a particular free calcium concentration in the calibrating conditions. In practice one expects that the ionic strength in the calibration solutions will match that inside the cell and that usually the intracellular ionic strength will not change significantly. In a sense, we use the free concentration in the calibrations to give a standard state that is more relevant to biology than

one which defines unity activity coefficients as those existing at infinite dilutions, i.e. in distilled water. (A fuller discussion of a biologists view of activity coefficients is given in Ref. 4).

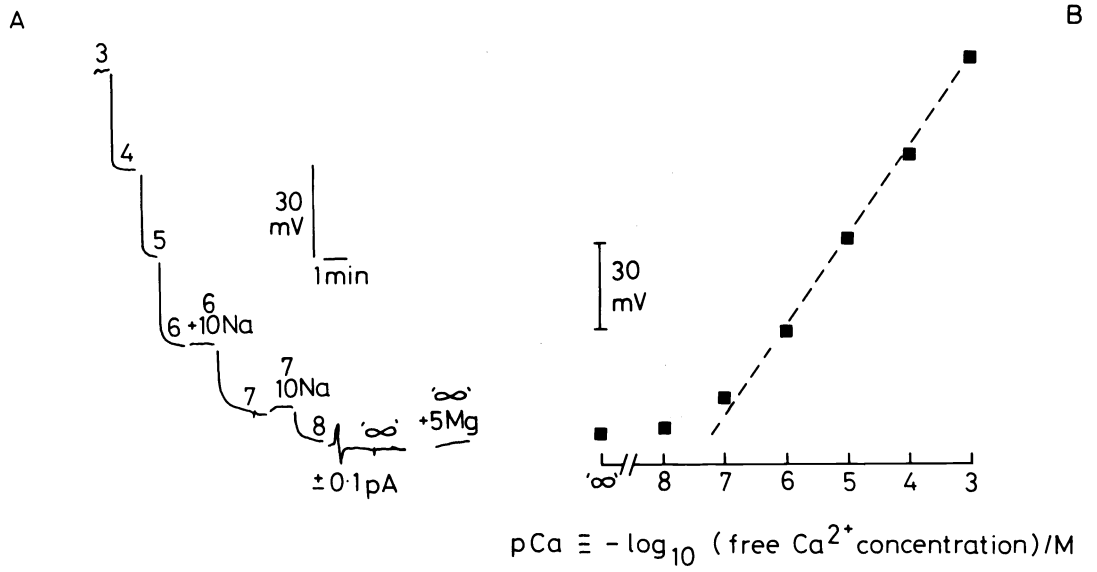


Fig. 2. Calibration plot Ca-selective microelectrode with a tip outer diameter is about 0.5 m. The sensor composition was 10% neutral ligand ETH 1001; 5% of the hydrophobic salt, tetraphenylarsorium tetrakis (p-biphenylyl) borate; 15% PVC; 70% solvent, o-nitropheynyloctyl ether (Ref. 16). On the left is shown the E_{Ca} in calibration solution with a background of 125mM K^+. The numbers above the time indicate the pCa, (defined here as $-\log_{10}$ free Ca concentration/M). The pCa3 and 4 solutions were unbuffered, the pCa5, 6, 7, 8 and " ∞ " were buffered with NTA, MEDTA, EGTA, EGTA and EGTA respectively. The detailed composition of these solutions is given in Refs (16 & 17). pCa " ∞ " denotes a solution with 10 mM EGTA and no added Ca^{2+}. Note that the response to 10 mM Na is tested at pCa 6 and 7. This is a typical value for $[Ca^{2+}]_i$ and it can be seen that at pCa7 a few mV interference from Na^+ has to be taken into account. Intracellular (Mg^{2+}) is around 1 mM and the minimal effect of 5 times this concentration indicates that no correction is needed. The ±0.1 pA shows where this current was passed down the electrode to test its resistance, which was about 7×10^{10} ohms.

Merits and drawbacks

Calcium-selective microelectrodes can give a well calibrated measurement of $[Ca^{2+}]_i$, down to and below the resting level, in a single identified cell. This cannot presently be said of any other technique. The electrode samples from just one point which might be advantageous if one could place the tip of the electrode at the place of interest. Point sampling can be disadvantageous, in that localised transients may be missed entirely. The technique is technically very demanding and requires special skills with special apparatus. Some cells are too small or fragile to survive impalement with ion-sensitive electrodes, and there is always the risk of impalement damage to the cell.

OPTICAL INDICATORS

A quite different approach is to somehow introduce into the cytoplasm an indicator substance giving an optical signal related to $[Ca^{2+}]_i$. Three major classes of substance have been used, bioluminescent calcium-activated photoproteins, bis-azo metolachromic indicators and tetracarboxylate Ca-chelator dyes

Photoproteins

A number of marine creatures show bioluminescence based on macro-molecules which emit blue light at a rate highly dependent on the free Ca^{2+} concentration, but not requiring any other co-factors. The energy for the light emission is stored within the molecule itself and once it reacts with Ca^{2+}, it can give no further signal. This means that it is essential to keep $[Ca^{2+}]$ very low while handling the photoprotein, and that the signal has to be calibrated in terms of fractional light-emission, as the indicator is continually depleted during the measurement. The best known and characterised is aequorin which is obtained from the jelly fish Aequorea. Since aequorin normally operates inside the cells of this organism it is no surprise that its light emission is stimulated in the range of $[Ca^{2+}]$ expected for activated cells. The emission is very weak at normal resting levels and relatively insensitive to changes in $[Ca^{2+}]_i$ around the resting level. Once [Ca2+] is in the range 0.5-10 μM, light emission becomes a very steep function of $[Ca^{2+}]$ with the emission proportional roughly to the $[Ca^{2+}]$ to the power 2.5. Of the cations shown in Table 1 only Mg^{2+} is likely to produce significant interference. A measure of cytoplasmic free Mg^{2+} is therefore needed for a proper calibration of aequorin signal. However free Mg^{2+} is probably rather stable in cells, and so observed changes in signal can reasonably be interpreted as a change in $[Ca^{2+}]_i$, even if free Mg^{2+} is not actually measured.

A great deal of important information concerning the role of Ca^{2+} in cell activation has been obtained with aequorin and several accounts describe both the achievements and the technical details of the use of this photoprotein (e.g. Refs, 2, 25 & 26). The steep power function together with the relative ease of detection of very low levels of light emission by photon counting give aequorin an unrivalled combination of sensitivity and dynamic range. It has been possible to examine localized changes in $[Ca^{2+}]_i$ in certain rather large cells by using image intensification techniques (e.g. Refs. 10 & 11). The response of aequorin is very much faster than that of Ca-sensitive microelectrodes though it is still not quick enough to follow the fastest $[Ca^{2+}]_i$ transients. As a rough guide it seems that the time constant for an increase in light emission at room temperature is about 10 msec.

A major technical problem is delivery into the cytoplasm. Aequorin is a large, complex protein and totally impermeant across cell membranes. It has usually been got into cytoplasm by microinjecting it into individual cells. This is just about as difficult as using ion-selective microelectrodes, and virtually impossible in many small cell types. Other methods of incorporation into cytoplasm have been tried with some success. For example, photoproteins can be incorporated into red blood cell "ghosts" by lysing the cells in the presence of photoprotein and then making them reseal thus trapping some of it inside. Special procedures employing a virus protein can then make these photoprotein-loaded "ghosts" fuse with another cell type and so deliver the photoprotein into its cytoplasm (Ref. 27). In a few cell types (other than red blood cells) it has been possible to make the membrane leaky enough for aequorin to get into the cytoplasm, and then to restore the normal impermeability of the membrane thus trapping some aequorin in the recovered cells (Ref. 28). Disadvantages of these methods include the fact that there are not at present applicable to many types of cell and that they involve rather drastic perturbations of either cell membrane, or cell contents, or both.

Bis-azo dyes

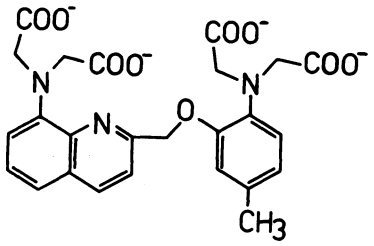
A number of dyes that have been routinely used by analytical chemists as metalochromic indicators for various heavy metals have recently been tried as indicators of $[Ca^{2+}]_i$. Most work has been done with arsenazo III, but antipyrolazo III and dichlorphosphonazo III have also been looked at. These dyes respond to $[Ca^{2+}]_i$ with altered absorbance spectrums in the range of 570-720nM. However, their relatively poor affinity for Ca^{2+},

their complex stoichiometry of binding, and vulnerability to interference by H^+ and Mg^{2+} make them very far from ideal indicators for $[Ca^{2+}]_i$. That data has been obtained from living cells containing these compounds might be thought to represent a triumph of instrumentation and perserverance over the poor indicator properties of these substances. These problems, and the highly sophisticated technicology that has been used in attempts to overcome them are considered in a number of recent reviews and papers (e.g. Refs. 1, 2, 3 & 29). It is worth pointing out that in most intracellular applications the dyes have been used to detect rapid $[Ca^{2+}]_i$ transients but rarely have the signals been calibrated in terms of actual free Ca^{2+} concentration.

These dyes share with aequorin the difficulties of delivering membrane impermeant probes into the cytoplasm of intact cells. Advantages of these dyes over aequorin include a somewhat faster response to changing $[Ca^{2+}]$, and an essentially linear response to $[Ca^{2+}]$ in the cytoplasmic range.

TETRACARBOXYLATE INDICATOR DYES

It is evident that each of the methods discussed above has significant drawbacks and limitations, both in properties of the $[Ca^{2+}]$ detection of system and in difficulties of delivering the system into the cytoplasm of a wide range of cell types. An important advance has recently been achieved by Tsien who has designed and synthesised a new series of calcium chelators and indicators together with a method for non disruptively incorporating them into populations of cells of any size (see Refs 30 & 31).



quin2

Fig. 3. Structure of the fluorescent Ca indicator, quin2.

The indicators

These new calcium chelators show a high affinity for Ca^{2+} with simple 1 to 1 stoichiometry, high selectivity for Ca^{2+} and low affinity for Mg^{2+} and H^+. They also show large absorbance, and in some cases fluoresence, changes resulting from calcium binding and are thus Ca^{2+} indicators. These chelators have eight donor sites ranged in almost the same geometry as in the well known calcium-selective chelator EGTA. Fig. 3 shows the structure of a fluorescent member of this series of compounds, quin2. Fluoresence offers a number of advantages for work in living cells including a much greater sensitivity for detection, and reduced interference from light scattering. Most of the work so far done in cells with these indicators has used quin2 (e.g. Refs. 37-37). Fig. 4 shows the fluoresence properties of quin2 that make it suitable as an indicator for $[Ca^{2+}]_i$. Against a background of cations designed to roughly mimic those in cytoplasm of the cells in which the dye has been most used, the fluoresence intensity increases six fold going on the Ca-free to the Ca-saturated form, without a shift in wavelength. The apparent Kd is 115 nM and the dye is thus

for measuring $[Ca^{2+}]_i$ around the resting level and over a range of approximately 10-1000nM. Equally, there is not much resolution above 1 μM just as a pH indicator is not much use more than one pH unit from its pK_a. This limitation can in principle be overcome by making a related dye with a lower affinity for use in the higher range of $[Ca^{2+}]_i$ (Ref. 4). The speed of response of these dyes has not been examined in detail but from the properties of the parent compound it is likely that the time constants for response to increasing $[Ca^{2+}]$ are faster than those for aequorin and bis-azo dyes.

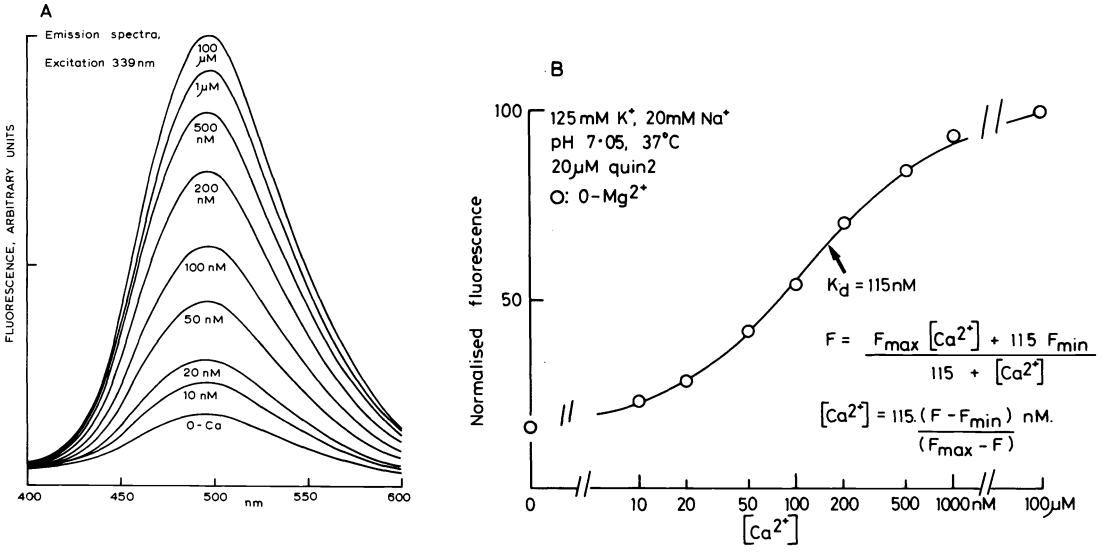


Fig. 4. Fluorescence response of quin2 to $[Ca^{2+}]_i$. A, shows emission spectra from 20 μM quin2, in a background at 125 mM K^+ and 1 mM free (Mg^{2+}) and pH 7.05. The procedure for altering (Ca^{2+}) is given in Ref. 27. B plots the signal at 492 nm against $[Ca^{2+}]$. The data fit well to a simple 1:1 binding relation shown in the Figure. The solid line is the theoretical curve for an apparent K_d = 115 nM.

Loading into intact cells

These tetra-anion indicators are highly impermeant through cell membranes. They can however be incorporated into populations of cells by use of a hydrophobic "pro-indicator". The carboxylates are esterified with acetoxymethyl groups to give the non-polar tetra-acetoxymethyl ester. The ester readily diffuses across the cell membrane and enters the cytoplasm. Cytoplasmic esterases hydrolyse the ester, regenerating the impermeant tetra-anion which is now trapped, just where it is wanted, in the cytoplasmic compartment. The cells are washed free of extraneous dye and resuspended. The fluoresence signal from the cells now reports $[Ca^{2+}]_i$.

This technique is most readily applicable to, and has so far been mostly used with, small cells in a suspension such lymphocytes and blood platelets. These of course are just the sort of cells for which the other methods are most inapplicable, and so this new technique is complimentary to those outlined above, and has greatly extended the range of cell types in which $[Ca^{2+}]_i$ can be monitored.

Using quin2

Fig. 5 shows records from human blood platelets that have been loaded with quin2 by incubating them with the acetoxymethylester for thirty minutes and then resuspending them in physiological saline. The signal was recorded

and then a calcium ionophore, ionomycin, added. Ionomycin greatly increases the rate at which Ca^{2+} can cross the cell membrane and so produces an increase in $[Ca^{2+}]_i$ which is reported by the increase in quin2 fluoresence. At this stage however the signals froms the cell are uncalibrated. Calibration can be done by subsequently releasing all the dye from the cells into the external medium by destroying the cell membrane with a detergent, TritonX 100. The same amount of dye is present in the same cuvette with the same perimeter settings as previously, but now we can set the free calcium concentration. In this case it is initially 1 mM so we determine the Ca-saturated fluorescence of the dye, F_{max}. Then the $[Ca^{2+}]$ is made exceedingly low by adding excess EGTA and raising the pH to increase the Ca affinity of EGTA. The Ca-free fluorescence, F_{min}, is then recorded. The $[Ca^{2+}]_i$ corresponding to any observed fluorescence, F, from quin2 in the intact cells is then calculated according to the equations as shown resting $[Ca^{2+}]_i$ was here calculated as 79 nM and the value after addition of the calcium ionophore was 2.3 μM.

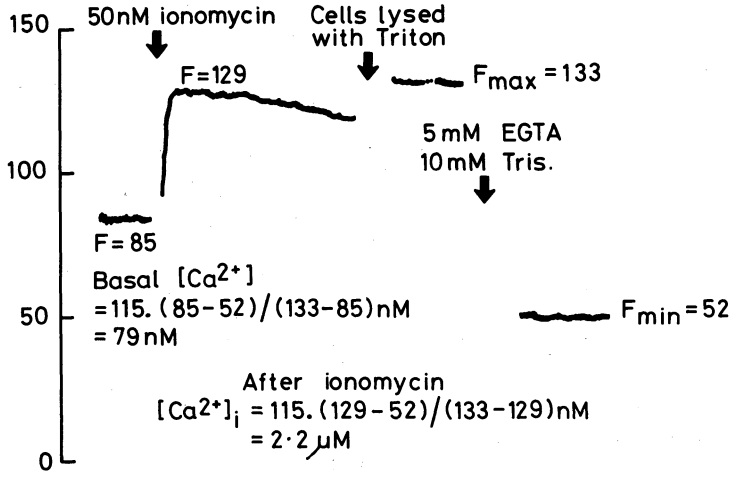


Fig. 5. Using quin2 to measure $[Ca^{2+}]_i$ in human blood platelets. The fluorescence signal was recorded for cells resuspended in physiological saline. Ca ionophore, ionomycin was then added and the fluorescence measured as Ca entered the cells and $[Ca^{2+}]_i$ increased. To calibrate the observed fluorescence, F, the quin2 was released from the cells into the medium which had 1 mM Ca^{2+} and F_{max} measured. $[Ca^{2+}]$ was then reduced to below 1 nM and F_{min} measured. The resting and stimulated $[Ca^{2+}]_i$ were then calculated from the relation shown in Fig. 4 as indicated in this figure.

For cells in suspension the method is relative straightforward and readily allows comparison of changes in $[Ca^{2+}]_i$ produced by different stimuli with the altered functions of the cells. The use of quin2 has already provided valuable data for cells in which $[Ca^{2+}]_i$ was known from indirect evidence to be an important regulator but had never actually been measured (Refs 37-37). There are however a number of limitations and problems that need to be considered.

Intracellular quin2 concentrations of 0.5 to 1 mM are needed to get a signal conveniently above the background cell fluorescence. This is because the excitation and emission wavelengths for quin2 come close to those for various fluorescent endogenous compounds including NADPH, and because quin2 has a rather poor extinction coefficient so that the signal is weak. It is expected that related indicators can be designed and synthesised to give a larger fluorescence at longer wavelengths so that cell autofluorescence becomes a negligible part of the signal (Ref. 4). Indicators with a higher K_d will also be useful to better quantify $[Ca^{2+}]_i$ in the range 1 to 10 μM expected for some activated cells. Calibration could be greatly facilitated by having a dye that showed a wavelength shift rather altered emission intensity on binding Ca^{2+} (Refs. 3 & 4).

As with any intracellular probe one has to ask whether the cell effects its behaviour and whether the probe perturbs the cell. The available evidence, detailed elsewhere (Refs. 32 & 33) indicates that the properties of quin 2 are much the same in cytoplasm as in calibration solutions. One point which needs watching is quenching of quin2 fluorescence by heavy metals such as Mn, Cu and Zn. The presence of trace amounts in the physiological saline can interfere with the calibration procedure illustrated in Fig. 5 (Refs. 3 & 33). It is also possible that intracellular quin2 will chelate heavy metals, which could have two adverse effects. 1) It might interfere with biological activity dependent on these metals, though the normal behaviour of most cells when loaded with quin2 argues that this effect is not serious. 2) Quenching of the fluorescence signal could lead to under-estimates of $[Ca^{2+}]_i$. The available data suggest this is not usually a major problem at quin2 contents around 1 mM, probably because the amount of quin2 bound by heavy metals is then a negligible proportion of the total.

In most of the cells tested there has been little evidence of toxicity, though certain adverse effects of quin2 loading into lymphocytes are reported in Ref. 37. A quin2 content in the millimolar range makes a significant addition to the natural Ca buffering of the cytoplasm which has to be considered in interpretation of the data (Refs. 3, 4, 33 & 34). This extra buffering can be relatively unimportant, a nuisance, or a useful experimental tool, depending on circumstances (Refs. 3, 4, & 34). So far quin2 has been used in cell suspensions in which the observed signal is the sum of the fluorescence of many thousands of cells. It is thus highly desirable that a homogenous population is used so that the summed signal represents that of individual cells. The use of suspensions precludes measurement of transient changes in $[Ca^{2+}]_i$ if they are not synchronous, or of spatial gradients. It may be possible to measure quin2 signals from single cells though it will be technically difficult, and much better results can be expected with improved indicators.

The introduction of quin2 and a method for loading it into intact cells has significantly extended the range of methods for measuring $[Ca^{2+}]_i$. But, there are still significant limitations to the technique some of which can be overcome by molecular modification to produce a better tetracarboxylate fluorescence indicator (Ref. 4) and some will need a further radical change in methodology.

NMR probes

Very recently G.A. Smith (Ref. 12) has synthesised a series of fluorinated derivatives of the 'parent' chelator of the Tsien series, BAPTA (Ref. 30). These fluorinated compounds retain the high affinity for Ca^{2+} and selectively over Mg^{2+}. They show large chemical shifts on binding Ca^{2+} and several other divalent metals. For some of the compounds the Ca bound and Ca free forms as in the slow exchange and for others they are in fast exchange. In either case it is im principle feasible to measure the proportion of free and bound forms and calculate the free $[Ca^{2+}]_i$ from the dissociation constant. Since other polyvalent metals produce different shifts it may be possible to detect, identify and even quantify the binding of such 'interfering' metals to the probes. This is potentially a worthwhile advantage over the fluorescent tetracarboxylate indicators. The probes are incorporated into cells in the manner described above for quin2 - i.e. by permeation of the acetoxymethyl ester and cytoplasmic hydrolysis to regenerate the tetracarboxylate 'NMR probe'. These probes presently share the problem of quin2, that relatively high contents are needed to get direct signals. Significant disadvantages compared with quin2 include the need for very expensive and complex equipment currently available in only a few centres and the requirement for many more cells maintained in denser

suspensions. The biological data reported so far is confined to an approximate confirmation of the value for resting $[Ca^{2+}]_i$ found in thymic lymphocytes with quin2 (Ref. 32) and an increased (Ca^{2+}) after exposure to a derivative of concavalin A (Ref. 12). Further data from this up-to-the minute variant of a new technique are awaited with interest.

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