REVIEW ARTICLE

Fluorescence and bioluminescence measurement of cytoplasmic free calcium

Peter H. COBBOLD* and Timothy J. RINK†‡

*Department of Human Anatomy and Cell Biology, University of Liverpool, P. O. Box 147, Liverpool L69 3BX, and †Smith Kline & French Research Ltd., The Frythe, Welwyn, Hertfordshire AL6 9AR, U.K.

1. INTRODUCTION

The first measurements of cytosolic free Ca^{2+} , $[\text{Ca}^{2+}]_i$, in living cells were made with aequorin in giant muscle cells (Ridgway & Ashley, 1967) in the late 1960s. During the 1970s two further methods were introduced, bis-azo absorbance dyes (mainly arsenazo III) and calcium-selective microelectrodes (Ashley & Campbell, 1979). However, even as late as 1981, $[\text{Ca}^{2+}]_i$ had been reliably measured in very few cell types other than giant cells of invertebrates and the techniques were largely confined to the laboratories of those specialized in excitable cell physiology and familiar with microelectrodes or micro-injection and advanced optical or electronic technologies. In 1982 a new generation of fluorescent dyes was introduced, with quin2 (Tsien *et al.*, 1982a) and a chemical trick for loading it non-disruptively into populations of cells of any size (Tsien, 1981). This technique in its basic form is simple enough, and needs only basic laboratory instrumentation, so that it is now routinely used in hundreds of laboratories. The technique has been rapidly developed with the invention of the superior dyes, fura-2 and indo-1, and technologies for monitoring $[\text{Ca}^{2+}]_i$ in single cells, whole fields of identified cells, and even localized areas within cells, and also improvements in time resolution down to the millisecond range. An outline of these developments forms part of this review. Clearly, with hundreds of papers already published we can only point out the major features, advantageous and problematic, of the technique and hope to provide a critical guide to the literature that details the important points.

Another development has been the refinement of the use of the calcium sensitive photoproteins aequorin and obelin. Important novel data on microinjected small single cells are now coming from specialist laboratories and the techniques are not as fearsomely difficult as commonly thought. Part of this review is therefore devoted to an explanation of this method, its advantages and pitfalls, and its successes. We will not consider bis-azo dyes or calcium-selective microelectrodes. There are several detailed accounts of these techniques and their place in relation to other approaches (e.g. Thomas, 1982; Blinks *et al.*, 1982; Rink, 1983; Tsien & Rink, 1983). Nor will we review the fluorine derivatives of the fluorescent dyes that have provided n.m.r. probes (Metcalf *et al.*, 1985). This review covers the technical considerations of using fluorescent and bioluminescent probes for measuring $[\text{Ca}^{2+}]_i$; we make no attempt to cover the

extensive and proliferating data obtained from such measurements.

2. QUIN2, FURA-2 AND INDO-1

These fluorescent Ca^{2+} chelators were developed by Tsien and his colleagues (Tsien *et al.*, 1982a; Grynkiewicz *et al.*, 1985) from the novel Ca^{2+} chelator BAPTA (Tsien, 1980), which is the double aromatic analogue of EGTA. BAPTA has significant advantages over EGTA as a calcium chelator, including: approx. 10-fold better calcium:magnesium selectivity, negligible pH sensitivity of calcium binding above pH 7, more rapid binding and dissociation, a large shift in u.v. absorption spectrum on binding Ca^{2+} , and even a sizeable fluorescence signal. BAPTA is actually a good optical indicator in the $[\text{Ca}^{2+}]$ range required for the cytosol. However, its spectrum deep in the u.v. is unsuitable for biological use due to possible u.v.-induced cell damage and to severe interference from cell proteins and nucleotides. Quin2, fura-2 and indo-1 have added fluorophores, and were designed to retain the excellent Ca^{2+} selectivity and sensitivity of BAPTA while providing excitation and emission maxima in the useable spectrum. The structures and key properties of quin2, fura-2 and indo-1 are shown in Table 1 and Fig. 1.

2.1. Quin2

Quin2 provided the first measurements of resting and activated $[\text{Ca}^{2+}]_i$ in many important cell types, and is still the best tool for some purposes. Quin2 binds Ca^{2+} with 1:1 stoichiometry and an apparent K_d of 114 nM in solutions designed to have the cationic composition of mammalian cell cytosol. Its K_d and fluorescence are not importantly affected by the nature of the monovalent ions usually encountered in cells or experimental solutions, although the K_d is increased with increasing ionic strength (Poenie *et al.*, 1985). The K_d is somewhat temperature-sensitive, increasing about 2-fold from room temperature to 37 °C. The selectivity against Mg^{2+} is approx. 10^4 -fold, which is not as good as that of BAPTA or the other two dyes owing to the additional nitrogen in the chelation claw, but $[\text{Mg}^{2+}]_i$ seems to be well clamped at near 1 mM in most cells for most of the time and does not constitute a problem in practice. Many other divalent cations, e.g. Mn^{2+} , Fe^{2+} , Zn^{2+} , Co^{2+} and Ni^{2+} , will bind to quin2 and the other dyes more strongly than does Ca^{2+} , and this can create problems or opportunities for the experimenter. These cations quench

Abbreviations used: AM, tetra-acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} ; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid.

‡ To whom correspondence and reprint requests should be addressed.

Table 1. Physical constants for fluorescent calcium chelators

These physical constants for the dyes have been extracted from Tsien (1980), Tsien *et al.* (1982) and Grynkiewicz *et al.* (1985). The K_d values were measured at 37 °C in medium containing approx. 120 mM-KCl and with pH 7.05. The $\lambda_{\max}$ values for fura-2 excitation are fully corrected; the other values are those obtained on a Perkin-Elmer MPF fluorimeter uncorrected for lamp and photomultiplier characteristics.

Dye	K_d (nM)	Absorbance $\lambda_{\max}$ (nm)		Maximum absorption coefficient ($M^{-1} \cdot cm^{-1}$)	Emission $\lambda_{\max}$ (nm)		Quantum efficiency	
		Anion	Calcium ligand		Anion	Calcium ligand	Anion	Calcium ligand
Quin2	114	339	365	6	492	492	0.14	0.03
Fura-2	224	362	335	33	512	505	0.23	0.49
Indo-1	250	349	331	34	485	410	0.38	0.56

Fig. 1. Structures and fluorescence spectra for quin2, fura-2 and indo-1

The solid lines show the spectra for the calcium-saturated form of the dye and the broken lines show the spectra for the free tetra-anion, all in medium containing approx. 120 mM-KCl with pH 7.05, at 37 °C. The data are extracted from Grynkiewicz *et al.* (1985). The excitation spectra for fura-2 are corrected for instrumental anomalies; the others are not and the 'glitch' in the 'no-calcium' indo-1 line is an artefact due to the xenon lamp characteristics. Note that the vertical scale is marked in *relative* intensity; on an absolute scale, equivalent concentrations of fura-2 and indo-1 give about 30 × more signal than does quin2.

fluorescence. There is no evidence that quin2 binds significantly to cellular macromolecules.

The key feature which allows quin2 to report $[Ca^{2+}]_i$ is the 6-fold increase in fluorescence signal produced as calcium saturates the ligand, when the excitation wavelength is near 339 nm. There is an isosbestic point, and with excitation around 370 nm increasing $[Ca^{2+}]$ actually decreases the quin2 signal. In principle, therefore, a 'ratio' dual-wavelength excitation technique could be used for quin2 (for further explanation see section 2.2 below). However, the very weak fluorescence at 370 nm makes the signals very susceptible to background

variation and other artifacts. Few investigators have used quin2 in ratio mode. Even with excitation at near 339 nm the quantum yield is not good owing to the relatively small absorption coefficient (i.e. relatively few of the incident photons are absorbed) and small quantum yield (relatively few of the absorbed photons excite a fluorescence emission). This means that quin2 usually has to be loaded to an intracellular concentration of greater than 0.5 mM for signals well above autofluorescence, with consequent addition to cytosolic calcium buffering and potential side-effects of the dye-loading procedure.

2.2. Fura-2

Fura-2 has several properties that are advantageous compared with those of quin2 (Grynkiewicz *et al.*, 1985; Tsien *et al.*, 1985). Its better absorption coefficient and quantum yield make it about 30 times brighter, so that loadings of 20–30 μM can give signals well above autofluorescence. To give an idea of the brightness, lymphocytes loaded with approx. 100 μM -fura-2 have a bluish-green glow easily visible when looked at under standard epifluorescence microscopy, and a single cell gives up to 10^5 counts/second from a moderate performance photomultiplier. With fluorescence as opposed to luminescence, the energy comes from the incident light and each dye molecule can be reused, until it becomes bleached. Platelets only 1 μm thick are visible under epifluorescence microscopy when loaded with approx. 100 μM -fura-2, and provide an adequate light flux for digital imaging with a SIT video camera (Hallam *et al.*, 1986).

The excitation spectrum of fura-2 is favourable in two ways. It is slightly red-shifted compared with that of quin2, just enough to let one use glass rather than quartz optics, and there is a very marked shift in the excitation spectrum: while the signal with 340 nm excitation increases 3-fold with Ca^{2+} saturation, the 380 nm signal decreases 10-fold. [The extent of these changes in signal depends on the wavelengths and band-pass of excitation and on the characteristics of the particular apparatus. These values are roughly those given by a Spex Fluorolog set up as detailed by Grynkiewicz *et al.* (1985).] This provides an excellent basis for ratio work and a rather large dynamic range of ratio signals, i.e. one may have useable ratios from less than 1 to greater than 15. The K_d of fura-2, 224 nm, is also rather better than that of quin2. This means that the usual resting level of $[\text{Ca}^{2+}]_i$ is well below half-saturation for the dye and allows reasonable accuracy of measurements in the range 1–10 μM . Fura-2 has better calcium:magnesium selectivity than quin2, the K_d for Mg^{2+} being 6–10 mM, making Mg^{2+} interference unimportant. The transition metals bind rather more weakly to fura-2 than to quin2 but still more strongly than does Ca^{2+} . Mn^{2+} quenches the fluorescence of fura-2 but Zn^{2+} behaves rather differently, looking somewhat like Ca^{2+} in its effects on the fluorescence spectrum (Grynkiewicz *et al.*, 1985). The fura-2 association and dissociation rate constants for Ca^{2+} were $5.95 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, and 97 s^{-1} measured by temperature jump at 20 °C (J. Kao & R. Y. Tsien, personal communication). The composite relaxation time for an indicator responding to changes in $[\text{Ca}^{2+}]$ is $K_a([\text{Ca}^{2+}] + K_d)$, so that fura-2 (at 20 °C) has about a 5 ms time constant near the K_d and about 1.5 ms at 1 μM - $[\text{Ca}^{2+}]$.

2.3. Indo-1

Indo-1 shares the brighter fluorescence and higher K_d , at 250 nm, of fura-2. It has not only an excitation shift (though at wavelengths rather too short for convenience) but also an emission shift which suits it to applications where a single-wavelength excitation and dual-wavelength detection are preferred. The kinetics have not yet been directly measured, though they are expected to be similar to those of fura-2, and the effects of transition metals have not been quantified.

2.4. Bleaching (photolability)

Ideally, dyes for measuring $[\text{Ca}^{2+}]_i$, or anything else, should not bleach. It turns out that quin2 is surprisingly photolabile, each quin2 molecule being bleached, on average, after absorbing only three or four photons (R. Y. Tsien, personal communication). You can see signs of this very readily if you measure the signal from an unstirred quin2 solution illuminated in a standard fluorimeter. The signal typically falls at several %/min. Stirring almost halts the decline as new dye is continually brought into the beam, which illuminates only a small proportion of the cuvette volume. For this reason quin2 is fine for use in cell suspensions, but this is almost its only sensible remaining application.

Fura-2 bleaches much less rapidly than quin2, which is just as well since the dye is often continuously illuminated in the cells being measured in monolayers, or in single cell work. There are no reports of bleaching causing serious problems with fura-2, and the effect on measured $[\text{Ca}^{2+}]_i$ of minor loss of photoresponsive molecules is taken care of by the ratio method. Indo-1 bleaches at a rate between that of quin2 and fura-2, and we noticed a significant loss of signal over tens of seconds of measurement indo-1-loaded endothelial cells (R. N. McBurney, T. J. Rink & T. J. Hallam, unpublished work), albeit at somewhat brighter excitation than strictly needed. We do not know if bleaching might lead to photodamage via free radicals.

2.5. Loading

Inventing BAPTA, quin2, fura-2 and indo-1 was a major advance. Finding a way of getting these hydrophilic tetra- or penta-anions non-disruptively into the cytosol of living cells was another. The trick was to make acetoxymethyl (AM) esters that turn the dyes into lipophilic membrane-permeant derivatives which are cleaved by cytosolic esterases to generate the free dye, now trapped in the cytosol (Tsien, 1981). This technique can make dye loading trivially simple; one merely incubates the cells with the acetoxymethyl ester of the chosen dye, waits some tens of minutes, and washes them. In theory all the available dye should progressively accumulate in the cytosol so that 1 μM -AM ester incubated with a 0.1 % cytocrit would give 1 mM final loading. In fact loading efficiency is typically in the range of 10–30 %. Most mammalian cells that have been tested will load quite readily with quin2 at 37 °C though we have found occasional recalcitrant tissues, e.g. guinea pig ileum. There have been few successes with invertebrate cells, partly because of the lower temperatures and larger surface-to-volume ratio of large cells.

There are even reports of successes with plant cells, and some failures (Reiss & Nobiling, 1986; Cork, 1986; Brownlee & Wood, 1986). The AM esters are not water-soluble and are usually introduced into the medium from dimethyl sulphoxide solutions. The presence of serum albumin can aid loading, perhaps by providing a 'carrier function'. We routinely load blood platelets with quin2-AM or fura-2-AM in citrated plasma.

2.5.1. Quin2-AM. In most cells quin2 seems to load very largely into the cytosol, with little incorporation into organelles. There are probably two main reasons for this: (1) the relevant esterases are mainly cytosolic so that once one acetoxymethyl group is cleaved you have a charged anion which is not very membrane-permeant

and so stays in the cytosol rather than passing into an organelle; (2) the organelles lack the requisite esterase. We made fairly careful tests to exclude significant non-cytosolic locations for ester-loaded quin2 in lymphocytes (Tsien *et al.*, 1982a). However, some workers have described the compartmentalization of quin2 (Goligorsky *et al.*, 1986) and one needs to be aware of the problem and of ways to check it in any particular experimental preparation. Another problem of ester loading is incomplete hydrolysis, which leaves some fluorescence that is not responsive to $[Ca^{2+}]_i$, or has a high K_d . One can follow the formation of the anion from the ester by observing the spectral shift but this method may not rule out small but significant proportions of partially hydrolysed dye. A good but tedious test is to lyse the cells following the loading procedure and to check the fluorescence response of the liberated dye to show that it has a 6-fold increase in fluorescence going from the calcium-free to the calcium-saturated form, and preferably show that the K_d is where it should be (Tsien *et al.*, 1982a).

2.5.2. Fura-2-AM. Ester loading has been more of a problem with fura-2, perhaps because of the greater hydrophobicity of fura-2-AM. Some cells just don't seem to want to load, hydrolysis seems to be slower, and compartmentation leakage more of a problem. Loading may be aided by providing some form of 'carrier' for the fura-2-AM. Plasma protein may serve this function, as we have found no difficulty loading fura-2 into human platelets in plasma anticoagulated with acid citrate dextrose to give a final pH of 6.6. Poenie *et al.* (1986) used 'Pluronic' as a dispersing agent.

However, the major problem with ester-loaded fura-2 has been significant accumulation into intracellular organelles (e.g. Almers & Neher, 1985; Neher & Almers, 1986; Poenie *et al.*, 1986; Williams *et al.*, 1985; Goligorsky *et al.*, 1986; Defeo & Morgan, 1986). This compartmentation is most readily noticed when observing ester-loaded cells by fluorescence microscopy. There may be 'hot-spots' with excitation at 340 nm indicating preferential accumulation of dye, and usually the ratio shows a high value, suggestive of a high- $[Ca^{2+}]$ environment. In some cases the ratio may be low (e.g. in cardiac myocytes; R. Jacob, personal communication) pointing to either very low $[Ca^{2+}]$ or unhydrolysed ester which has an excitation pattern more like that of calcium-free than calcium-saturated dye. Another indication of compartmentation is difficulty in obtaining sensible calibrations or the expected responses to stimulation. The available evidence suggest that fura-2 or its AM ester can accumulate in secretory granules, sarcoplasmic reticulum, mitochondria and other organelles, depending on the cell and the conditions. The problem of compartmentation has seemed considerably worse for cultured cells in monolayers than for cells in suspension and a recent thorough study by Malgaroli *et al.* (1987) has provided some important clues as to some of the mechanisms. They suggest that perinuclear fluorescence spots in cultured fibroblasts resulted from endocytosis of micelles of fura-2-AM which were then incorporated and hydrolysed in lysosomal compartments; the fura-2 spots co-localized with Acridine Orange fluorescence. The problem was largely obviated by loading at 15 °C which seemed to inhibit endocytosis but not permeation and cytosolic hydrolysis. Accumulation from the cytosol

does not seem to be the problem. Almers & Neher (1985) found uniform fluorescence in mast cells with fura-2 incorporated via a whole-cell patch pipette, and we (A. Harootunian, R. Y. Tsien & T. J. Rink, unpublished work) found that fura-2 microinjected into the cytoplasm of BC3 H1 cells showed none of the perinuclear compartmentation in these cells loaded with fura-2-AM, even after many tens of minutes at 37 °C.

This perinuclear positioning of dye accumulated in regions when it shows a high ratio, because of high $[Ca^{2+}]_i$ or low pH, can give a false impression of a low $[Ca^{2+}]_i$ in the nucleus. However, the nucleus is uniform with the cytoplasm in microinjected cells. A dramatic demonstration of this compartmentation can be seen by dropping digitonin on to the cells, whereupon the cytoplasmic fura-2 immediately disperses, leaving only the 'hot-spots' behind. One should be able to test for compartmentation in fura-2-loaded cell suspensions by observing the instantaneous drop in signal when digitonin is applied in the presence of external Mn^{2+} , compared with the drop caused by subsequently added Triton. Presumably cells in suspension are less susceptible to intraorganelle accumulation of fura-2 because they do not lie at the bottom of the dish with micelles mining down on them, and tend to endocytose less actively.

Fura-2 also seems to leak out of some cells, e.g. lymphocytes, macrophages and PC12 cells, faster than does quin2. This is somewhat variable from cell type to cell type and preparation to preparation, but does not seem merely to reflect cell death or breakdown of permeability barriers. For instance, fura-2 leakage from PC12 cells was not associated with loss of lactate dehydrogenase or increase in nuclear staining by ethidium bromide (Malgaroli *et al.*, 1987). It seems unlikely that penta-anionic fura-2 would be more intrinsically membrane permeant than tetra-anionic quin2, and there is a suspicion that fura-2 is somehow taking a ride on anion transport mechanisms across cell membranes. Leakage has been noted to be quite temperature-dependent (R. Jacob, personal communication; Malgaroli *et al.*, 1987) and in macrophages can be reduced by probenecid (F. Di Virgilio, personal communication). We should note that leakage is not a problem with all cells; we have little problem with human platelets, neutrophils or endothelial cells.

Less is known about indo-1 in terms of loading but there is one report of difficulty of hydrolysis and possible compartmentation (Luckhoff, 1986). Anecdotal evidence suggests that indo-1 may have less of a leakage problem than fura-2.

2.5.3. Other loading methods. Clearly the methods that have been used to get aequorin into cells can be applied to the potassium salts of the fluorescent dyes. Micro-injection has been mentioned above, and another approach is to introduce the dye into a single cell via a 'patch-clamp' micropipette (Almers & Neher, 1985). In one recent study of mitosis in PGK2 cultured cells the authors reported that ester loading gave unsatisfactory results with fura-2 and they turned to the low- Ca^{2+} , low- Mg^{2+} , ATP^{4-} method of 'reversible lysis' (Ratan *et al.*, 1986). Mostly though, the (deceptive) ease of ester loading will make people concentrate on that method.

2.6. Adverse effects and effects of Ca^{2+} buffering

Obviously one has to know whether loading a cell with

one of these dyes has had adverse effects, either due to the predictable effects of the buffering properties of the dye itself or to side effects of hydrolysis byproducts. The effects of added Ca^{2+} buffering have been discussed in detail elsewhere (Tsien *et al.*, 1982a, 1984). The important point is that there is no effect on steady state Ca^{2+} in normal conditions where $[\text{Ca}^{2+}]_i$ is set by the balance of pump/leak at the plasma membrane.

If large amounts of dye are loaded into the cytosol in the absence of external Ca^{2+} , then the dye will strip Ca^{2+} from intracellular pools and very low $[\text{Ca}^{2+}]_i$ can be achieved (Tsien *et al.*, 1982a; Pozzan *et al.*, 1982; Di Virgilio *et al.*, 1984). This is in fact a very useful experimental set-up for seeing what cells can do when $[\text{Ca}^{2+}]_i$ is held 5–10 times below the normal level. Increasing the Ca^{2+} buffer capacity of cytosol naturally serves to blunt Ca^{2+} transients due to brief inputs of Ca^{2+} into the cytosol; a good example of this is shown in a recent comparison of parathyroid cells heavily loaded with quin2 and lightly loaded with fura-2 (Nemeth & Scarpa, 1986). The extent of the $[\text{Ca}^{2+}]_i$ rise caused by discharge of finite internal stores is of course greatly reduced by increasing Ca^{2+} buffering (see e.g. Pollock & Rink, 1986), and the rate of approach to a new steady state following a step increase in Ca^{2+} entry is slowed by increasing the quin2 content of cells (Tsien *et al.*, 1982a). To observe events closest to those in unperturbed cells the lowest practicable loading of fura-2 or indo-1 should be used. Estimating the amount loaded normally involves measuring cell volume, cytocrit and F_{max} (fluorescence of calcium-saturated dye), and comparing the latter with a standard solution of the potassium salt of the dye.

Each acetoxymethyl group hydrolysed from an AM ester generates two protons, one acetate and one formaldehyde. It is remarkable how little toxicity is produced by loading with millimolar amounts of quin2. However, there are instances of side effects of quin2 loading (see e.g. Tsien *et al.*, 1982a; Hesketh *et al.*, 1983; Rink & Pozzan, 1985; Pollock *et al.*, 1986), and each investigator should check this out for him/herself.

So far no specific problems are reported with unexpected side effects from fura-2 or indo-1 loading.

2.7. Availability

Quin2, fura-2 and indo-1 are commercially available as the potassium salts or acetoxymethyl esters from several suppliers, including Molecular Probes, Eugene, OR, U.S.A. and from Calbiochem. Detailed stability measurements have not been made, but it is a sensible precaution to keep stocks of solid desiccated in the deep-freeze. The AM esters are subject to hydrolysis, so we prefer to divide stock solutions into dimethylsulphoxide and keep them carefully desiccated and frozen.

2.8. Instrumentation and types of measurement

2.8.1. Cell suspensions. Measurements with cell suspensions can be very simple, using a moderately good fluorimeter capable of exciting and recording the appropriate wavelengths and preferably having a thermostatted stirred cuvette. The procedures for getting satisfactory results and avoiding many of the pitfalls have been discussed in the original descriptions of quin2 (Tsien *et al.*, 1982a,b) and in subsequent reviews (Rink & Pozzan, 1985). Clearly the quality of the results will depend on the quality of the cell suspension and it is

important to show viability and proper function of the dye-loaded preparation in the cuvette. The signal recorded will be the summed signal of all the cells in the light path. This may well conceal: heterogeneity in the cell population; intracellular localization of $[\text{Ca}^{2+}]_i$ changes; or temporal dispersion of the signal in different cells. For example, if half the cells are stimulated to several micromolar $[\text{Ca}^{2+}]_i$ while half remain at the basal level near 100 nM, the measured average $[\text{Ca}^{2+}]_i$ will be around 350 nM. If $[\text{Ca}^{2+}]_i$ oscillates between 100 nM and 1 μM for short bursts out of synchrony, a much smaller average rise in total signal will be recorded and interpreted as a small rise in $[\text{Ca}^{2+}]_i$. If, however, the fluorescence signal rises to the point where almost all the dye molecules are in the calcium-bound form one knows that nearly all the cells must have had a large elevation of $[\text{Ca}^{2+}]_i$. Another common difficulty comes from leakage of the dye into the medium. If the external Ca^{2+} is in the millimolar range, leakage will be interpreted as a rise in $[\text{Ca}^{2+}]_i$; if the external Ca^{2+} is made very low with EGTA, leakage could look like a fall in $[\text{Ca}^{2+}]_i$. These errors in suspension measurements cannot be overcome by using dual wavelength measurements; they can be checked for by manipulation of external $[\text{Ca}^{2+}]$, or by using quenching transition metals, or by measurement of dye in the suspension supernatant (Rink & Pozzan, 1985).

2.8.2. Ratio methods. Dual wavelength excitation or emission, and the ratio method for obtaining $[\text{Ca}^{2+}]_i$, can be used with cell suspensions, given the means of alternating the exciting wavelength, or recording the emission at two wavelengths (Tsien *et al.*, 1985). For a ligand which exists in only two forms, free and calcium-bound, the signal from any two wavelength pairs is uniquely determined by the ratio of free and bound dye and therefore by the free $[\text{Ca}^{2+}]$. Provided that the non-dye fluorescence is either a small part of the signal, or is known and stable, the ratio method eliminates most of the variation due to instrumental fluctuations and changes in the dye content of the cells, e.g. due to bleaching or leakage. These advantages are most important when working with cell monolayers or single cells but can be useful in suspension. For instance, blood platelets aggregate in response to many agonists and this produces a loss of the fluorescence signal from trapped dye and looks, at a single wavelength, like a fall in $[\text{Ca}^{2+}]_i$. The loss of signal however is proportionately the same at both wavelengths in dual wavelength recording so that the ratio of the two signals can report $[\text{Ca}^{2+}]_i$ even during the formation of aggregates (Tsien *et al.*, 1985). A discussion of the technology for alternating two excitation wavelengths or for splitting emission beams is beyond our scope here. Several groups (including T.J.R.'s) use a modified Spex Fluorolog which has a rotating 'butterfly' mirror to provide alternating excitation from two monochromators set at the desired wavelengths, and a digital data acquisition system to record each of the two multiplexed signals, together with the necessary software for the requisite manipulation to generate $[\text{Ca}^{2+}]_i$ from the raw data (for description see Tsien *et al.*, 1985). Another approach is to use a rocking plate or spinning wheel, containing interference filters of the appropriate band pass, in front of the excitation light source. Again a purpose-built data capture and analysis system is needed.

2.8.3 Stopped-flow fluorescence. In many cells, $[Ca^{2+}]_i$ can increase within 1 s of addition of agonist and it is difficult to get decent time resolution in this period with stirred cuvettes and standard fluorimeters due to uncertainties in the time of addition of the agonist and also of the mixing time. We have recently shown that stopped-flow fluorescence measurements can reveal sub-second kinetics of $[Ca^{2+}]_i$ in fura-2-loaded platelets and disaggregated parotid cells (Sage & Rink, 1986a,b; Merritt & Rink, 1987). Here the timing of agonist cell interactions is known to within a few milliseconds and the time course of the fluorescence change can be measured with a time resolution of tens of milliseconds.

2.8.4. Monolayers, tissues and organs. It is possible to measure $[Ca^{2+}]_i$ in monolayers with quin2 but the signal is often perilously close to the background and may be hard to calibrate (Conrad & Rink, 1986). The simplest approach is to wedge the cover-slip diagonally between the corners of a square or oblong cuvette in the fluorimeter and experiment with the position of the monolayer relative to the excitation beam to get the best available signal. The use of fura-2 is a great advantage in these conditions, giving a much brighter signal and allowing the use of dual-wavelength ratio measurements (e.g. Hallam & Pearson, 1986).

One can take a similar approach with thin sheets of tissue held across the excitation beam. We obtained some preliminary signals from frog sartorius muscles loaded with quin2 but it was hard to get enough quin2 into the tissue to obtain signals usefully above autofluorescence (S. W. Smith, R. Y. Tsien & T. J. Rink, unpublished work). More recently, very good results have been obtained with fura-2-loaded sheets of smooth muscle from guinea-pig ileum providing simultaneous $[Ca^{2+}]_i$ and tension measurements (Himpens & Somlyo, 1987). For the best results one wants very thin sheets, to allow some chance of even dye loading, and as homogeneous a cell population as possible. Another approach has been to load whole organs with fura-2 via perfusion of the blood supply and to use front-face dual-wavelength fluorescence to obtain a measure of $[Ca^{2+}]_i$ in surface cells. Preliminary data have been obtained in this way in beating rat hearts (Lattanzio & Pressman, 1986).

2.8.5. Flow cytometry. Indo-1 has the properties best suited for flow cytometry where excitation with a single wavelength and recording the emission of two or more wavelengths is routine. The ratio method allows $[Ca^{2+}]_i$ to be calculated from the data captured in the fraction of a second that the single cell is irradiated by the laser beam. With this technique $[Ca^{2+}]_i$ can be correlated with other variables measurable by flow cytometry, e.g. cell size and specific surface antigens (Lazzari *et al.*, 1986; Rabinovitch *et al.*, 1986). The relative size of sub-populations that have different $[Ca^{2+}]_i$ can be sorted out. Time resolution is usually not good in that it takes tens of seconds between adding a stimulus and getting the cells into the measuring chamber, but reducing this time delay to a few seconds should be technically feasible. This approach has definite attractions for certain applications, especially in cell types where other variables have been extensively studied in this way, for instance lymphocytes. The drawback is the expense of the equipment and the need for highly trained operators to achieve good results. This will most likely remain a

technology for specialist centres and not one that is widely distributed in general cell biology laboratories.

2.8.6. Epifluorescence microscopy. An increasingly used technique is quantitative epifluorescence microscopy. There are several variants in use with each investigator devising his or her own equipment. Probably most people have used Zeiss IM35 microscopes, but Nikon Diaphots are fine and some workers have chosen (or had already available) Leitz Diavert apparatus, or the Zeiss Axiomat System. The wavelength pair usually chosen for fura-2 measurement in a scanning fluorimeter are 340 nm and 380 nm. Unless one has a microscope with quartz optics, and most groups do not, 340 nm is too far into the u.v. to get adequate transmission of the excitation beam through the optical components. A shift of 10 or 15 nm to 350 or 355 will often make a substantial difference and still provide some increase in signal with increasing $[Ca^{2+}]_i$. One may prefer to move the lower wavelength to approx. 365 nm, the isosbestic point, and rely on the longer excitation wavelength to provide all the $[Ca^{2+}]_i$ -dependent shift. Even to work at 350–365 nm one has to select objective lenses capable of passing near-u.v. One excellent lens for this purpose is the glycerol immersion Nikon UV-F 40.

The two main ways used for recording the fluorescence are: (1) dual wavelength measurement of single cells or small clumps of cells picked out by pin-holes judiciously placed in the emission beam (e.g. Poenie *et al.*, 1985); (2) digital imaging fluorescence microscopy to examine $[Ca^{2+}]_i$ in whole fields of cells and different locations within single cells (e.g. Tsien & Poenie, 1986). In principle the digital imaging technique should be able to provide everything one can see with the single cell method and much else besides. However, the imaging requires expensive and complex apparatus and presently does not have the time resolution available by simply monitoring the counts on a photomultiplier from single cells. A photomultiplier can give a continuous record for each excitation wavelength and, with suitable data processing, a continuous readout of the ratio signal and thus of $[Ca^{2+}]_i$. Time resolution will be limited by the speed of alternating the two excitation beams, where fura-2 is used, and by the processing power of the data acquisition system. For single cell work indo-1 and dual wavelength *emission* may have advantages. One can illuminate with a single continuous beam, and take the emitted light through appropriate filters and beam-splitters into two photomultipliers. Now the time resolution need be limited only by the signal-to-noise ratio and kinetics of indo-1. For events which can be repeated at accurately timed intervals one can of course enhance the signal-to-noise ratio by signal averaging. Working with single cells one clearly avoids the problems of population heterogeneity and can pick out the temporal pattern of the signal in individual cells. Given the right equipment and expertise it is also possible to monitor the cell electrophysiologically by impalement or patch-clamp so that one can get a real time functional correlation with $[Ca^{2+}]_i$. Another (slow) way to monitor the functional response of the observed cell is manually to alternate fluorescence recording with Nomarski or phase contrast observation of morphology to observe cytokinesis, cell shape, dispersion of organelles, etc. Much better, but requiring customization of the instrument, is to record an i.r. image in parallel with the fluorescence. However,

a major difficulty with working on single cells is that many of the biochemical and functional correlations one wants to look for (and are quite readily measured in cell suspensions), e.g. phosphorylation, measurement of cyclic nucleotides, glucose production, catecholamine secretion, cannot yet be monitored in single cells. The challenge now is to develop biochemical analysis at the single cell level. For instance, suitable fluorescent probes for cyclic AMP and cyclic GMP will be very welcome.

Digital imaging microscopy with a pixel-by-pixel quantitative analysis can show $[Ca^{2+}]_i$ not only in different cells in a whole field but also within localized regions of one cell (Poenie *et al.*, 1986). This has been a very exciting recent development and is well summarized in a recent review (Tsien & Poenie, 1986). At present, the time resolution of this technique is limited to about 0.5 Hz, but this is good enough for many purposes. The heterogeneity of cell response between different cells in the population is startlingly revealed by this technique which is also ideally suited for studying the effects of interaction between different cell types, e.g. killer cells and their targets. As currently used this technique requires dual wavelength excitation in a good epifluorescence microscope, an image capture system (e.g. video camera) and an image processing computer capable of digitizing and manipulating the images at approx. 1 Hz. The necessary hardware and software is still expensive and needs careful tending by a skilled operator. Advance in this field is, however, rapid and year by year the technology is expected to become cheaper, and more user-friendly and also capable of improved time resolution. Important imminent developments in video cameras and position-sensitive photomultipliers should improve the quality and rate of image formation, and cheaper image processing packages will be capable of being interfaced with relatively cheap microcomputers.

2.9. Calibration of signals

We do not propose to detail here the theory and practice of calibration of the fluorescent indicators. Several previous publications have covered this ground with some detail (Tsien *et al.*, 1982a; Rink *et al.*, 1983; Rink & Pozzan, 1985). Rather we shall outline principles and some of the problems and their possible solutions. Each of the dyes bind Ca^{2+} with simple 1:1 stoichiometry, so that in simple solutions the proportion of free and bound dye depends on the K_d for those conditions and the free $[Ca^{2+}]$. Or, if one knows the proportions of calcium-bound dye and free dye one can readily obtain the $[Ca^{2+}]$ from $K_d \cdot (\text{calcium-bound dye/free dye})$. Because of this simple 1:1 stoichiometry the relationship between fluorescence and $[Ca^{2+}]$ is approximately linear in the region of the K_d . However, the amount of fluorescence change for a given change in calcium rapidly falls off as one moves an order of magnitude above or below the K_d . This means that the resolution and accuracy of these dyes, even used in a dual wavelength mode, is not satisfactory above about 2–3 μM for quin2 or above 5–10 μM for fura-2 and indo-1. On the other hand, these dyes do offer accurate measurement for $[Ca^{2+}]_i$ in the range below the normal resting value, 10–100 nM. Idealized calibration curves for quin2 and fura-2 are shown in Fig. 2.

2.9.1. Quin2. In principle, if one knows the background fluorescence and the cytosolic concentration of

Fig. 2. Idealized calibration curves for quin2 (a) and fura-2 (b)

(a) shows the normalized fluorescence, 339 nm excitation and 500 nm emission, as a function of $[Ca^{2+}]$ over the range 10^{-8} – 10^{-5} M. Note the near-saturation of the response above 1 μM . (b) shows the ratio of the fluorescence signals with 340 nm and 380 nm excitation and 500 nm emission for fura-2 as a function of $[Ca^{2+}]$. Note the apparently poorer resolution below 10^{-7} M and the apparently good resolution in the range 10^{-6} – 10^{-5} M. This latter partly reflects the higher K_d of the dye (see text and Table 1). However the increasing ratios above 10^{-8} M depend very heavily on small (in absolute terms) falls in the 380 nm values, near to background fluorescence and hence potentially subject to considerable experimental variation. The limiting values of the fluorescence ratios, here approx. 1 and 35, depend on the wavelengths chosen, the band pass and the machine characteristics. This curve was derived from values reported by Grynkiewicz *et al.* (1985) using the equation given in the text. With many applications, especially with fluorescence microscopy where the shorter wavelength for excitation is often set at 350 nm or even higher, a much smaller range of ratio values is obtained.

the dye, and the quantitative relation between dye concentration and fluorescence, any given measured fluorescence level, even at one excitation wavelength, would define the proportions of calcium-bound and free dye and allow one to calculate $[Ca^{2+}]_i$. In practice these conditions are not fulfilled and with measurement of only one wavelength pair a post-experiment calibration is needed, preferably for each experimental run, but always for each preparation. One aims to define F_{max} , the fluorescence of calcium-saturated dye, F_{min} , the fluorescence of free dye, and/or F_{Mn} , the fluorescence signal after the dye is quenched with Mn^{2+} , i.e. the autofluorescence. In many cells F_{max} can be obtained within the cell by applying the calcium ionophore ionomycin in the presence of 1 mM-external Ca^{2+} . In some cells

ionomycin is surprisingly reluctant to translocate calcium and lysis is then needed to expose the dye to external Ca^{2+} and obtain F_{max} . F_{min} can then be obtained by removal of Ca^{2+} with EGTA. F_{Mn} can also readily be obtained after lysis, and can also be obtained in suitable cells following treatment with ionophore since ionomycin readily translocates Mn^{2+} into the cytosol. For any given F recorded during the experiment:

$$[\text{Ca}^{2+}]_i = K_d[(F - F_{\text{min}})/(F_{\text{max}} - F)]$$

and

$$F_{\text{min}} = F_{\text{Mn}} + 1/6(F_{\text{max}} - F_{\text{Mn}})$$

It is important to note that any changes in autofluorescence during the experimental procedures will invalidate this procedure and this point must be checked. Often the autofluorescence (much of which comes from NADP^+ and NADPH) is surprisingly and helpfully stable. But many compounds of interest, e.g. FCCP, quinine, trifluoperazine and A23187, are themselves quite fluorescent at quin2 (and fura-2) wavelengths.

2.9.2. Fura-2. Dual-wavelength measurements can allow instant calculation of $[\text{Ca}^{2+}]_i$ without the need for post-calibration. With two measurements the concentration of dye should be immaterial as the ratio of the signals, unlike the signal at a single wavelength pair, is not dependent on dye concentration. If one is confident of the autofluorescent background, and knows the limiting values for ratios of the fluorescences of the two wavelengths for fura-2 and calcium-fura-2 in the particular experimental conditions, calibration is not necessary for each preparation. $[\text{Ca}^{2+}]_i$ values are calculated from fura-2 ratios (R) by the equation:

$$K(R - R_{\text{min}})/(R_{\text{max}} - R) = [\text{Ca}^{2+}]$$

where R_{min} and R_{max} are the ratios (e.g. 340 nm/380 nm) obtained in zero or saturating calcium concentrations, K is the product $K_d(F_0/F_s)$, where K_d is the effective dissociation constant, F_0 is the 380 nm excitation signal in the absence of calcium and F_s is the 380 nm excitation signal at saturating calcium concentrations (Poenie *et al.*, 1985). In practice one normally feels much more confident of the numbers if $[\text{Ca}^{2+}]_i$ can be raised to saturating values with ionomycin at the end of the experiment and if the autofluorescence is then obtained by adding Mn^{2+} . Note that in monolayers and in any single-cell work we cannot use the lysis method to obtain F_{max} as the dye leaks from the area of excitation and emission. (For an attempt to subvert this restriction in cells that refuse to respond properly to ionomycin, see Conrad & Rink, 1986.)

The validity of the calibration procedures outlined above depends, as with any other probe, on the calibration *in vitro* being applicable to the cytosol and on the dye being confined to the cytosol. For quin2, so far as we now know, these conditions often hold. An interesting exception was found in Ehrlich+Yoshida associate cells (Arslan *et al.*, 1985). An unusually high Zn^{2+} content caused quenching of the quin2 signal and an underestimate of $[\text{Ca}^{2+}]_i$ that varied with the amount of quin2 loaded. The more the quin2 the smaller was the proportion of quenching by the fixed amount of Zn^{2+} and the less the error. As pointed out above, fura-2 seems to suffer from a greater tendency to compartmentation and incomplete hydrolysis and naturally this will frustrate

efforts at rigorous quantification. Fluorescence polarization measurements can give useful information on the local environment of dye molecules, showing changes in binding of macromolecules or when the viscosity of the microenvironment of the dye changes. Preliminary studies with quin2 showed no measurable difference in polarization of the dye in saline or when trapped in the cytosol of lymphocytes (R. Y. Tsien & T. J. Rink, unpublished work), so we concluded that quin2 was not importantly perturbed by binding or cellular microviscosity. However, fura-2 in the cytosol can show polarization changes that resemble those seen in highly viscous solutions. Depending on the polarization characteristics of a particular apparatus used, this may have a significant effect on the excitation spectrum and seriously disturb the calibration (Grynkiewicz *et al.*, 1985; Poenie *et al.*, 1986). We think that absolute levels of Ca^{2+} as reported by quin2 may be more reliable than those reported by fura-2 if there is a discrepancy. In one cell in which we have substantial experience of both techniques, the human blood platelet, measured $[\text{Ca}^{2+}]_i$ both at rest and during activation turns out to be closely similar in quin2-loaded and fura-2-loaded cells (e.g. Pollock *et al.*, 1986; Pollock & Rink, 1986).

2.10. Future prospects

Improvements can be anticipated in the dyes, the loading and in instrumentation. Dyes with lower Ca^{2+} affinity would be helpful to quantify stimulated levels of $[\text{Ca}^{2+}]_i$. Longer wavelengths would obviate the concern over absorbance by lenses and would move away from significant autofluorescence. Derivatives of BAPTA with fluorescein or rhodamine fluorophores remain an attractive prospect, and for some applications one might be prepared to forego the advantages of ratio methods for a brighter signal, much enhanced signal-to-noise-ratio and a larger dynamic range e.g. 20-fold increase in photon emission going from calcium-free to calcium-saturated dye. With such advances one might be able to reduce the Ca^{2+} buffering introduced into the cytosol to approx. $2 \mu\text{M}$, comparable with that achievable with microinjected aequorin. Variants of the ester-loading method that avoid serious compartmentation are expected. Probably these will be empirically determined procedures, varying from preparation to preparation, but an esterifying group superior to acetoxymethyl remains an objective. We also anticipate increasing use of microinjection as a way of incorporating both reporter and modulator hydrophilic compounds into cells, and the biochemistry and molecular biology of the single cell is increasingly an object of study. Improvements in instrumentation will include the development and refinement of the imaging technology with standard epifluorescence microscopes, including better time resolution. More dramatic will be the introduction of confocal systems which should provide real-time serial optical sections approx. $0.5 \mu\text{m}$ thick of dual wavelength emission ratio images and permit far better perception of three-dimensional $[\text{Ca}^{2+}]_i$ transients within cells and also patterns of $[\text{Ca}^{2+}]_i$ through whole tissues and organs (White & Amos, 1987).

3. PHOTOPROTEINS

Aequorin is a luminescent 21 kDa protein present in photocytes in the periphery of the umbrella of the

jellyfish *Aequorea forskalea*. A similar photoprotein, obelin, is found in hydroids of *Obelia* spp. (Campbell, 1974). Following the discovery of its calcium-triggered light emission (Shimomura *et al.*, 1962), aequorin was first used to monitor $[Ca^{2+}]_i$ in a giant muscle cell (Ridgway & Ashley, 1967). Aequorin has since been used to measure $[Ca^{2+}]_i$ in progressively smaller single cells (reviewed by Blinks, 1978; Blinks *et al.*, 1976, 1982). Over the past few years detailed improvements to the technique have led to aequorin measurements in single mammalian cells, including oocytes (Cuthbertson *et al.*, 1981, 1985), heart myocytes (Cobbold & Bourne, 1984), hepatocytes (Woods *et al.*, 1986), neurones (Neering & McBurney, 1984) and, the smallest to date, adrenal chromaffin cells approx. 12–14 μ m in diameter (Cobbold *et al.*, 1987). Aequorin measurements on populations of cells have recently become feasible through the development of procedures for reversible cell permeabilization, to allow extracellular photoprotein access to the cytoplasm. These procedures include liposome or erythrocyte fusion (Campbell & Dormer, 1975, 1978; Campbell *et al.*, 1981, 1985), hypo-osmotic shock treatment (Borle & Snowdowne, 1982; Snowdowne & Borle, 1984a,b; Tucker *et al.*, 1986), scrape-loading (McNeil & Taylor, 1985; McNeil *et al.*, 1985), ATP or EDTA permeabilization (Morgan & Morgan, 1982, 1984; Kojima & Ogata, 1986) and spin-loading (Borle *et al.*, 1986; James-Kracke, 1986). As a result of these recent advances there can be few cells in which photoproteins cannot be used for measuring $[Ca^{2+}]_i$, though clearly some of these techniques for populations of cells are significantly disruptive.

3.1. Properties of aequorin

The features of aequorin that make it suitable for intracellular $[Ca^{2+}]_i$ measurements, or which impose constraints in its use, are listed here, citing only those references that have appeared since earlier comprehensive reviews (Blinks, 1978; Blinks *et al.*, 1976, 1982).

(a) Molecular mass 21 kDa. Aequorin must be micro-injected (section 3.2.1.) or introduced by temporary permeabilization of cells (section 3.2.2). Once in the cytosol, aequorin, a highly negatively charged protein, will remain in the cell for many hours, presumably lacking the correct signal sequences for export, or sites for ubiquitination; in the jellyfish it resides in the cytosolic compartment. Spurious signals from aequorin entering calcium-rich intracellular compartments are unlikely to occur.

(b) Heterogeneity. Native aequorin is a mixture of eight iso-aequorins, which can be separated in milligram quantities by h.p.l.c. and whose sensitivities to calcium differ (Shimomura, 1986).

(c) Quantum yield is approx. 0.15, luminescent emission λ_{max} 470 nm. A good low-noise bi-alkali photomultiplier (section 3.3) will be capable of detecting one photoelectron count for every (about) seven photons of 470 nm light falling on the photocathode. Together with the high quantum yield of aequorin, this will allow about one molecule in every 50 molecules of aequorin to be detected as a single count with relatively simple instrumentation.

(d) The luminescent reaction. The protein contains a tightly bound luminophore, coelenterazine (Shimomura & Johnson, 1978). The luminescent reaction involves oxidation of coelenterazine with oxygen carried

by the protein; molecular oxygen is not required, nor is ATP. The rate of the reaction is strongly dependent on calcium (see below). A slow rate (approx. $10^{-6} s^{-1}$) is found in the absence of calcium, the 'calcium-independent light'; this imposes a lower limit of approx. $10^{-7} M$ on $[Ca^{2+}]_i$ measurements (Allen *et al.*, 1977). The luminescent reaction is 'once-only' in nature, although spent aequorin can be recharged with added synthesized coelenterazine in the presence of molecular oxygen and mercaptoethanol.

(e) Sensitivity to calcium. The rate of the luminescent reaction rises approx. 10^5 -fold as $[Ca^{2+}]_i$ is raised from $10^{-7} M$ to $10^{-5} M$ (see section 3.4). At $10^{-5} M$ - Ca^{2+} the rate of consumption of the protein is approx. $0.1 s^{-1}$, restricting the duration of $[Ca^{2+}]_i$ measurements at this concentration. Roughly speaking, at $10^{-6} M$ - Ca^{2+} approx. 10^{-4} of the protein is consumed per second. So, for brief excursions in $[Ca^{2+}]_i$ to 10^{-6} or $10^{-5} M$ the dramatic increase in reaction rate greatly facilitates precise detection of the signal with relatively simple instrumentation. The steep relationship between reaction rate and $[Ca^{2+}]_i$ is due to the presence of three calcium-binding sites on the protein, predicted from luminescence dose-response curves (Moiescu *et al.*, 1975) but confirmed from sequence analysis of the cloned gene (Inouye *et al.*, 1985; Tsuji *et al.*, 1986; Prasher *et al.*, 1985).

(f) The kinetics of the luminescent reaction are sufficiently fast to detect the $[Ca^{2+}]_i$ transient in heart muscle contraction, rate constant approx. $100 s^{-1}$.

(g) Mg^{2+} depresses the sensitivity to Ca^{2+} . For as accurate calibration as possible intracellular $[Mg^{2+}]$ needs to be known (see section 3.4).

(h) Ionic strength influences sensitivity to calcium and calibration procedures should use an ionic strength as close as possible to that in the cells in question (see section 3.4). At low ionic strength, EDTA and EGTA depress sensitivity to calcium independently of their ability to chelate Ca^{2+} (Shimomura & Shimomura, 1984), but this will not be a problem at ionic strengths pertaining in mammalian cells.

(i) Over the physiological range (6.6–7.4) pH has no important effect on calcium-sensitivity.

(j) Strontium, lanthanum and other lanthanides will induce luminescence, at concentrations comparable with calcium.

(k) Enhanced sensitivity to calcium is given by acetylation of aequorin (Shimomura & Shimomura, 1982, 1985), although losses of activity during this process may not necessarily improve signals from cells.

(l) Aequorin is inactivated by trace levels of silver and mercury; solutions that contact aequorin should not have reference electrodes that might leak those ions immersed in them.

(m) Stability, handling and availability. Freeze-dried aequorin is stable 'indefinitely' at $-70^\circ C$, and several days storage at room temperature induces little loss. In solution at $-70^\circ C$ it is stable for months and tolerates many freeze-thaw cycles without serious loss. At high concentration (approx. 10–150 mg/ml) it can be dialysed on a micro-scale (approx. 0.2 μ l aliquot) in a few hours (Cobbold *et al.*, 1983). Whenever low aequorin concentrations are acceptable the freeze-dried powder is simply dissolved in a suitable calcium-free buffer.

Aequorin is available from Dr. J. R. Blinks, Department of Pharmacology, Mayo Foundation, Rochester, MN, U.S.A.

3.2. Introduction of photoproteins into cells

3.2.1. Microinjection. The conventional approach uses borosilicate micropipettes, usually of the filament-lined variety, which are washed, pulled and loaded with a few microlitres of filtered aequorin solution (approx. 10 mg/ml) via the butt end. High gas pressure (approx. 10 atm.) is used to force aequorin out of the tip, and a carefully machined pipette holder is needed to minimize pipette movement under the high pressure (Blinks *et al.*, 1978). Electrical recording through a platinum (not silver) wire is used to monitor impalement of the cell. An alternative procedure developed in P.C.'s laboratory for use in amoebae, mouse oocytes, rat heart and liver cells and bovine chromaffin cells involves filling only the tip of the pipette with concentrated aequorin solution (up to 150 mg/ml). Originally we forced aequorin into the pipette through the tip with gas (Cobbold *et al.*, 1983) and, later, hydraulic pressure (P. Cobbold, unpublished work). These approaches have been superseded by a very simple procedure (outlined in Woods *et al.*, 1986) in which the tip of a freshly-pulled pipette is dipped for a few seconds in light liquid paraffin (BDH) so that a short (approx. 20 μ m) column of oil enters. The tip is then immersed in a microdroplet of concentrated (approx. 150 mg/ml) dialysed aequorin solution, held under the oil on the surface of a plastic culture dish. Aequorin enters spontaneously to form a column approx. 50 μ m long. The pipette is then mounted in a rigid holder for microinjection as previously described (Cobbold *et al.*, 1983). Cells are injected with a measured volume of aequorin, using the tip-to-meniscus distance to estimate the volume, to approx. 0.5 % of their volume, with a high success rate. Previously published low success rates (Cobbold *et al.*, 1983) were subsequently found to be caused by insufficient pH buffer in the aequorin solution; aequorin is now dialysed against (mM): KCl (AristaR), 150; Pipes, 1; EGTA, 0.025; EDTA, 0.1; dithiothreitol, 1; KOH (AristaR), to pH 7.3 dissolved in double glass distilled water. Dialysis of approx. 0.2 μ l of aequorin solution (approx. 150 mg/ml) is carried out for 4–6 h at room temperature across a 5 mm length of Bio-Rad Biofiber 50A tubule (unfortunately no longer available). These procedures allow single cells to be loaded with quite high concentrations of aequorin (approx. 0.5–1 mg/ml) with minimal injection volume (approx. 0.5 % cell vol.), and are simple, quick and reliable in cells down to hepatocytes in size 25–30 μ m diameter (Woods *et al.*, 1986), and feasible in cells of approx. 12 μ m diameter, e.g. chromaffin cells (Cobbold *et al.*, 1987).

Cells that prove difficult to impale with sharp pipettes may lend themselves to being loaded with aequorin through a patch pipette, as used in salamander rods. Aequorin solution added to the patch pipette after the plasmalemma has been disrupted is allowed to diffuse into the cell for several minutes (McNaughton *et al.*, 1986).

3.2.2. Reversible permeabilization procedures. (a) Cell-cell fusion or liposome fusion. Obelin has been loaded into either resealable ghosts of pigeon erythrocytes or into liposomes, which were subsequently fused to target cells (Campbell & Dormer, 1975; Hallett & Campbell, 1982). Interpretation of the signals was recognized as being complicated by signals from unfused or phagocytosed erythrocytes or liposomes, and the possible deleterious effects on the physiological status of the target cells of the fusion treatment and of the presence of

foreign membrane in the plasmalemma (Campbell *et al.*, 1985).

(b) Hypo-osmotic shock treatment. Brief exposure of hepatocytes or cultured kidney cells to a cold hypo-osmotic medium (3 mM-ATP, 3 mM-Hepes) containing aequorin (5–10 mg/ml) followed by slow readdition of KCl to iso-osmoticity allowed aequorin to be incorporated into populations of cells. About 7 % of the original cell protein content was lost to the medium, but cell recovery as judged by growth, mitogenesis and intracellular enzyme activity suggested good short-term (20 min) and long-term (6 h) viability. Unstimulated kidney cells gave a detectable resting signal which was equivalent to approx. 5×10^{-8} M $[Ca^{2+}]_i$ (Borle & Snowdowne, 1982; Snowdowne & Borle 1984a,b; Tucker *et al.*, 1986).

(c) Permeabilization by EGTA or ATP. Aorta strips have been reversibly permeabilized in an iso-osmotic medium which included 3 mM-ATP, 0.1 mM-EGTA and 200 μ g of aequorin/ml; resealing was induced by adding 10 mM-Mg²⁺. Contractile responses to phenylephrine and angiotensin II decreased by only 0.3 % following loading, and the aequorin signals were qualitatively similar, but of greater amplitude because of greater incorporation of aequorin, to signals from microinjected preparations (Morgan & Morgan, 1982, 1984; Neering & Morgan, 1980). A similar approach has been used over prolonged periods for skeletal myoblasts (James-Kracke, 1986), for adrenal glomerulosa cells (Kojima & Ogata, 1986), and for platelets (Ware *et al.*, 1985).

(d) Scrape-loading. Cultured cells that attach to dishes were loaded with aequorin by scraping cells, previously washed free of extracellular calcium, into a film of iso-osmotic medium containing aequorin (1 mg/ml). Shortly after scrape-loading, normal medium was added back to the cells which were left to recover for 1–24 h before making aequorin measurements. About 10–12 h was required for recovery of normal morphology of fibroblasts (McNeil & Taylor, 1985; McNeil *et al.*, 1985). About 50 % of cells did not survive the scraping procedure, but the remaining 50 % showed good viability 24 h later, and had a normal growth rate. A measurable resting signal was detected from approx. 10^4 cells scraped in 20 μ g of aequorin.

(e) Spin-loading. EGTA-washed cultured kidney cells suspended at high cytocrit in cold iso-osmotic aequorin-containing calcium-free medium were centrifuged at 300 g for 30 s, then resuspended in normal Krebs-Henseleit buffer at 37 °C (Borle *et al.*, 1986). It is claimed that this procedure is as effective as scrape-loading, but only 30 % as effective as hypo-osmotic shock treatment, in loading the cells with aequorin. The intracellular aequorin content was approx. $(1-5) \times 10^{-9}$ M. A resting glow was detectable. The technique can also be used on rat liver and heart muscle cells. This loading protocol differs little from normal handling procedures used for isolated cells.

3.2.3. Single cell or population measurements? The choice of loading technique, microinjection or reversible permeabilization of cell populations, will have a profound influence on the ultimate interpretation of the experiments, so this decision is probably the most crucial for using photoprotein. It is very likely that signals from cell populations will be open to misinterpretation on several counts. Firstly, large signals will arise from a dying cell;

an unhealthy cell with approx. 5×10^{-6} M $[Ca^{2+}]_i$ will generate as much light as about 10^4 resting cells (see section 3.4.4), and hence cell viability must be excellent if pathological $[Ca^{2+}]_i$ changes are not to mask physiological responses. Secondly, studies on the role of $[Ca^{2+}]_i$ in pathogenesis will be clouded by the likelihood that reversible permeabilization has generated hidden stresses (e.g. loss of substrates and cofactors) that will impair cell responses to injurious conditions. Thirdly, the lack of synchrony between cells in a population, in both pathological events and in transient physiological responses, will be a particularly serious drawback in population studies. There is emerging evidence that physiological $[Ca^{2+}]_i$ responses to extracellular stimuli may not be sustained, but can be transient and often repetitive, as for example in oocytes at fertilization (Cuthbertson *et al.*, 1981; Cuthbertson & Cobbold, 1985; Poenie *et al.* 1985), secretory cells (Rapp & Berridge 1981; Neher & Almers, 1986; Cobbold *et al.*, 1987), and in liver cells responding to calcium-mobilizing hormones (Woods *et al.*, 1986, 1987). Clearly population measurements will not resolve these transients and will lack the necessary information to answer questions concerning the detailed second-by-second control of $[Ca^{2+}]_i$. Likewise, the ability to correlate $[Ca^{2+}]_i$ with the microscopic appearance of single cells has been shown to be valuable in studies on cellular pathogenesis in heart (Cobbold & Bourne, 1984; Allshire *et al.*, 1987) and liver cells (Lemasters *et al.*, 1987).

Unfortunately, it seems that the simple reversible permeabilization procedures do not load sufficient aequorin to allow signals to be detected from a single cell. Thus at 5×10^{-9} M intracellular aequorin (Borle *et al.*, 1986), a spin-loaded kidney cell of 5 pl volume will contain only 1.5×10^4 aequorin molecules [and only 300 photoelectrons will be detected; see section 3.1(c)]. By contrast, microinjection can introduce about 1.5×10^7 molecules of aequorin into a hepatocyte, at an intracellular concentration of about 2 μ M (Woods *et al.*, 1986, 1987).

In the future it should prove feasible to make measurements on single cells by transfecting a population of cells with the aequorin gene (Inouye *et al.*, 1985; Prasher *et al.*, 1985) incorporated into a suitable expression vector. The intracellularly-synthesized apo-protein could then be charged by adding to the medium coelenterazine, which can be synthesized chemically (Shimomura & Johnson, 1978; Inoue *et al.*, 1977) and which is likely to be sufficiently permeant to the plasmalemma. This procedure should avoid the intricacies of microinjection, be applicable to cells not amenable to microinjection, avoid the possible pathological stresses of reversible permeabilization, eliminate the complications of aequorin heterogeneity, while at the same time satisfying the ideal of measuring $[Ca^{2+}]_i$ in single cells.

3.3. Instrumentation

3.3.1. Signal detection. A photomultiplier is an expensive but extremely effective quantum counter; a tube with a bialkali photocathode will detect approx. 15% of 470 nm photons incident upon it, i.e. its quantum efficiency at 470 nm is approx. 0.15. Since about 15% of aequorin molecules will release a photon [section 3.1(c)], it is possible to detect one photoelectron 'count' for about every 50 molecules of aequorin reacting with calcium. To achieve this, the counting geometry, photo-

multiplier dark count and background sources of luminescence must be optimized.

(a) Counting geometry. The light lost between its source in the cell and the photocathode must be minimized. We have published an arrangement (Cobbold *et al.*, 1983) in which a single aequorin-injected cell is held in a warmed, perfusable chamber of about 100 μ l capacity. The chamber is machined from 316 stainless steel and polished with jewellers' rouge, and positioned with an approx. 3 mm air gap directly in front of the photomultiplier. The air gap, aided by a steady air flow, maintains both thermal isolation from the cooled (5 $^{\circ}$ C) photocathode, and electrical isolation. Other approaches use light guides (Cannell & Allen, 1983), parabolic reflectors (Blinks *et al.*, 1982) or light collection through a microscope objective, which will at best collect only 5–10% of the isotropically radiated light, depending on the numerical aperture of the lens (McNaughton *et al.*, 1986).

(b) Background count. The lower the dark count or background, the lower the signals that can be detected within a reasonable recording duration. A bialkali photocathode minimizes the dark count rate, which is mainly generated by electrons released thermally from the photocathode. The smaller the area of the photocathode, the fewer thermal electrons released; hence we use a tube with a 1 cm diameter photocathode (E.M.I. 9789A). Tubes are available with still smaller photocathodes (e.g. 3 mm or 1 mm diameter), but these introduce problems concerning losses due to poor counting geometry. Exposure of a tube to room lighting or brighter should always be avoided, even if no voltage is applied; this is because glass becomes phosphorescent in response to u.v. light, and this phosphorescence requires a long time to decay. Cooling of bialkali tubes to about 0 $^{\circ}$ C, but not much lower, is desirable.

Unexpected sources of luminescence may be revealed, e.g. brass, epoxy resin, bovine serum albumin, and background luminescence from populations of cells may been to be assessed, especially in systems in which reactive oxygen metabolites are produced (Cadenas & Sies, 1984).

3.3.2. Photon counting or D.C. measurement? The anode current from a photomultiplier consists of a D.C. leakage current on which are superimposed brief current pulses, each a few nanoseconds in duration and consisting of about 10^7 electrons, that are generated from each photoelectron emitted from the photocathode. Although D.C. measurements using a simple nanoammeter can give a useful measure of incident light, there are several advantages in distinguishing the current pulses from the D.C. component, i.e. photon counting. Firstly, the D.C. current leakage, which can vary from tube to tube and with time and which is unaffected by cooling, is rejected. Secondly, integrated D.C. measurements record all the current generated by a cosmic ray (several tens of photons incident on the tube), while a photon counter will record just one count, being unable to separate the time of arrival of the Čerenkov counts. Thirdly, the gain per photoelectron varies and this will be reflected in D.C. measurements, (as 'shot noise') but effectively standardized by the discriminator circuit of a photon counting system. Fourthly, the quantal nature of light is preserved in a photon counting system, and the digital output of a photon counter is ideal for interfacing to micro-

computers, which greatly facilitates data acquisition and analysis.

Photon-counting instrumentation can be assembled from modular components of radionuclide measuring systems (listed in Cobbold *et al.*, 1983) and can have a high band width (up to 150 MHz).

We (P.C. and R. Cuthbertson) have developed a less expensive, microcomputer-based system, whose cost is approximately one-tenth of the cost of a dual wavelength excitation system for fluorimetric measurements. This system has proved invaluable for calibrating aequorin signals, by computing (off-line) the fractional rate of aequorin consumption throughout the experiments (see section 3.4). (Software for data acquisition and analysis, running on MS-DOS, is available from Dr. Roy Cuthbertson of P.C.'s laboratory.)

3.3.3. Imaging. Imaging requires that a microscope lens is used to form an image of the cell, introducing losses of about 95% of the aequorin luminescence. Hence sustained signals from relatively large cells, or iterative signals, are needed in order to form distinguishable images of $[Ca^{2+}]_i$ in cells. Early vacuum-tube imaging technology (Reynolds, 1972) has been used in giant salivary gland cells (Rose & Loewenstein, 1975, 1976), oocytes (Ridgeway *et al.*, 1977; Gilkey *et al.*, 1978; Eisen *et al.*, 1984; Eisen & Reynolds, 1984) and amoebae (Cobbold, 1979, 1980). Recent technology, based on cooled photocathodes and microchannel plates coupled to position-sensitive anodes that generate computer-compatible x, y co-ordinates for each photoelectron, is now available (e.g. Instrumentation Technology, St. Leonards on Sea, U.K.) and is being applied to aequorin signals. Images of aequorin luminescence will be markedly disproportionately brighter from zones of raised $[Ca^{2+}]_i$, because of the 'cube-law' relationship between $[Ca^{2+}]_i$ and the rate of aequorin discharge (see section 3.4). This property of aequorin effectively enhances image contrast, making zone of elevated $[Ca^{2+}]_i$ easier to detect. However, the amount of information contained in aequorin images of cells, especially small cells, severely restricts the detection of such zones of elevated $[Ca^{2+}]_i$. To take an example, a rat liver cell, if imaged, would yield a total of about 1.5×10^4 detected counts (calculated from the data in section 3.2.3 and assuming the microscope objective collected only 5% of the isotropically-radiated light). Thus, if the whole of the cell rose to a $[Ca^{2+}]_i$ of 10^{-6} M, this will give an image containing only 1.5 counts/s (see section 3.4.3), which would require tens of seconds exposure for the image, say 1 cm diameter, to be detectable above the detector's dark count of 1–2 photoelectrons $\cdot s^{-1} \cdot cm^{-2}$. At unphysiological levels of 10^{-5} M $[Ca^{2+}]_i$, the signal is brighter, and easily detectable (about 150 counts/s), and could be detected in a few seconds even if it occurred in only a small percentage of a cell's volume. These calculations indicate that the imaging of physiological levels of $[Ca^{2+}]_i$ using aequorin is of borderline feasibility in mammalian cells. The ability of fluorescent probes such as fura-2 to image micromolar $[Ca^{2+}]_i$, in exposures of a second or so, in small (about 2–3 μm) zones of mammalian cells (Connor, 1986) cannot be matched by photoproteins.

3.4. Calibration

The 'once only' nature of the luminescent reaction of

aequorin means that, at a constant calcium concentration, a constant fraction of the remaining aequorin molecules should discharge per second. In fact, the predicted purely exponential decay of luminescence is not found. This can be attributed to the heterogeneity of native aequorin [section 3.1(b)], or possibly to post-translational modifications such as acetylation [section 3.1(k)], but calcium-response curves for aequorin prepared from the cloned gene are not yet available. However, this uncertainty is not important unless a significant fraction of each aequorin sample is used during an experiment. Calibration procedures rely upon determining either the aequorin luminescence intensity, or the rate of aequorin consumption, which is then normalized as a fraction of the maximum intensity or rate of aequorin consumption (section 3.4.1). These values can then be compared with values obtained in a series of calcium buffers *in vitro* (section 3.4.2).

3.4.1. Normalization of signals from cells. Calibration of the signal from cells requires a knowledge of the total aequorin luminescence contained in the preparation. Discharge of aequorin remaining in a preparation is usually induced at the end of an experiment by either a detergent such as Triton-X or, for single mammalian cells, by lysis in distilled water. Thus it is not necessary, or indeed feasible, to microinject known volumes of aequorin of known activity into cells. Knowledge of the total aequorin luminescence allows retrospective calibration of the aequorin signals recorded from the cells prior to lysis. Two procedures are used to normalize the luminescence from the cell as a function of the total aequorin it contains.

(a) Fractional luminous intensity, L/L_{max} , where L represents the light intensity at unknown $[Ca^{2+}]$ and L_{max} the light intensity that the same quantity of aequorin would generate in saturating $[Ca^{2+}]$. L_{max} has to be calculated from the total light yield, usually represented as nA $\cdot$ s (being the area under the anode current trace) when the aequorin-loaded preparation is lysed with Triton-X (Allen & Blinks, 1978, 1979). This quantity of light is then normalized into a peak light intensity assuming a time-constant for the decline in intensity in saturation $[Ca^{2+}]$ of $0.8 s^{-1}$. Calibration curves *in vitro* of L/L_{max} at known $[Ca^{2+}]$ values, and using ionic conditions and temperature that match the intracellular milieu (see section 3.4.2), are then used to calibrate the value *in vivo*.

(b) Rate of aequorin consumption. This procedure normalizes the signals by expressing them as a fractional rate of aequorin consumption, i.e. counts/s divided by the total number of counts remaining, after subtraction of background (Cobbold, 1980; Campbell *et al.*, 1981). It is used by our group in conjunction with photon counting and a microcomputer (section 3.3.2). This method, developed by Dr. K. S. R. Cuthbertson, has been used to analyse aequorin signals from single oocytes, heart, liver, and chromaffin cells (Cuthbertson & Cobbold, 1985; Cobbold & Bourne, 1984; Woods *et al.*, 1986, 1987; Cobbold *et al.*, 1987). Consumption of aequorin during the experiment is automatically accounted for.

3.4.2. Calibration *in vitro*. Normalized signals *in vivo*, as either L/L_{max} or counts/s per total remaining counts, are calibrated in terms of $[Ca^{2+}]_i$ by measurements made on aliquots of aequorin in calcium buffers *in vitro*. It is

Fig. 3. Relationship between the rate of aequorin consumption and $[Ca^{2+}]$ *in vitro* in 2 mM-EGTA buffers containing 150 mM-KCl, 10 mM-Pipes and $[Mg^{2+}]_{free}$ as indicated, at 37 °C

Replotted from Cobbold *et al.* (1986) and Woods *et al.* (1987). The points are individual determinations. The broken line represents a slope of 3.

essential that this buffer mimics the intracellular milieu in terms of ionic strength [see section 3.1(g)], free $[Mg^{2+}]$ [see section 3.1(h)] and temperature. pH is important only insofar as it has a profound effect on EGTA buffering. Curves showing the effects of altering one parameter have been published (Allen & Blinks, 1979), but cannot be used to predict the curve in any one set of conditions. Fig. 3 shows the effect of changing free $[Mg^{2+}]$ on signals *in vitro*; the 5 mM- Mg^{2+} curve was used on studies of heart myocytes (Cobbold & Bourne, 1984), and the 1 mM curve for hepatocytes (Woods *et al.*, 1986, 1987). The higher free $[Mg^{2+}]$ shifts the curve towards higher $[Ca^{2+}]_i$ values and depresses the rate of aequorin consumption in the absence of calcium ('the calcium-independent light'). The curves show that aequorin cannot be used to measure $[Ca^{2+}]_i$ below 10^{-7} M and that it is imprecise on the heel of the curve, up to about 3×10^{-7} M. However, the signal rises about 200-fold if $[Ca^{2+}]_i$ rises from 10^{-7} to 10^{-6} M, and a further about 1000-fold in going from 10^{-6} to 10^{-5} M. The slope of the curves in this region is very nearly 3, in agreement with the three calcium-binding sites predicted by Moisescu *et al.* (1975) and described by sequence analysis of the gene (Inouye *et al.*, 1985; Tsuji *et al.*, 1986; Prasher *et al.*, 1985). Clearly, the percentage changes in the signal for a

small change in $[Ca^{2+}]_i$ (within the range 3×10^{-7} to 10^{-5} M) are large, giving good precision. Of course, at the higher $[Ca^{2+}]_i$ values the rate of consumption becomes appreciable and will limit the duration of the measurements, but such conditions will probably only arise in pathological studies; in physiological experiments less than 10% of all the aequorin is normally consumed.

3.4.3. Predicted signals. The curves shown in Fig. 3 can be used to predict the size of the signal to be expected for a given $[Ca^{2+}]_i$, given knowledge of the total quantity of aequorin luminescence that can be detected from a cell. Thus, we routinely inject about 3×10^5 detected counts into a single hepatocyte (equivalent to about 2 μ M cytoplasmic aequorin concentration; section 3.2.3) hence the signals to be expected at different $[Ca^{2+}]_i$ values will be (interpolating on the 1 mM- Mg^{2+} curve): 10^{-7} M, 1.0 count/s; 10^{-6} M, 190 counts/s; 3×10^{-6} M, 3800 counts/s. Clearly, detection of the signal at 10^{-7} M (1 count/s) above the background level (also about 1 count/s) will require about 15 min, dictated by the counting statistics. So rapid small-magnitude changes in resting $[Ca^{2+}]_i$ will not be resolved. However, a rise in $[Ca^{2+}]_i$ to 10^{-6} M will generate a 200-fold larger signal that is easily resolved from background even if the rise is transient, lasting only a fraction of a second.

3.4.4. Localized luminescence from zones of high $[Ca^{2+}]_i$. Aequorin is sometimes criticized on the basis that the cube-law relationship with $[Ca^{2+}]$ (Fig. 3) will exaggerate signals arising from locally-raised $[Ca^{2+}]_i$. This is undoubtedly true, and favours detection of a cellular response, even if the rise in $[Ca^{2+}]_i$ does subsequently prove to be localized. Photomultiplier measurements preclude visualization of the high- $[Ca^{2+}]_i$ zone, so homogeneity of $[Ca^{2+}]_i$ is usually assumed in the calibration procedure. Nonetheless, if localization is suspected, the extent of the $[Ca^{2+}]_i$ rise in that zone can be estimated (for example, Cobbold, 1979, 1980). Imaging techniques will ultimately be needed to establish firmly the existence of a localised zone.

When populations of aequorin-laden cells are being studied, it must be remembered that the same property of aequorin will exaggerate signals from a few unhealthy cells with raised $[Ca^{2+}]_i$ (see section 3.2.3).

3.5. Future possibilities for photoproteins

The separation of milligram quantities of the more active isoequorins (Shimomura, 1986), together with chemical modification such as acetylation (Shimomura & Shimomura, 1982, 1985) promise to improve the sensitivity of aequorin, and to eliminate uncertainties caused by heterogeneity of the native protein.

The cloning of an aequorin gene (Inouye *et al.*, 1985; Tsuji *et al.*, 1986; Prasher *et al.*, 1985) and the chemical synthesis of coelenterazine (Inouye *et al.*, 1977) should promote the simplest, least stressful and most effective way of obtaining high intracellular concentrations of active aequorin, by transfecting cultured cells with the gene in an appropriate expression vector and later immersing the cells in a solution of coelenterazine. This procedure should eliminate both the stress of reversible permeabilization and the intricacies of microinjection, while producing intracellular concentrations of aequorin comparable with microinjection and, hence, suitable for single cell recordings.

The production of transgenic animals strains in which the aequorin gene is expressed in various tissues will, when coupled with coelenterazine loading, open up exciting possibilities, including measurements in primary isolated single cells. The possibility of imaging free $[Ca^{2+}]_i$ levels in a slice of tissue is an equally exciting possibility, particularly when applied to the brain.

4. CONCLUSIONS

We have outlined the two techniques which have provided most of the important results measuring $[Ca^{2+}]_i$ in cell suspensions and in single cells. The relative advantages and disadvantages of the luminescent and fluorescent probes are contained in the foregoing account. We remain dubious about the reliability of measurements with aequorin introduced into populations of cells by various forms of reversible lysis; the inter- and intracellular distribution of the protein has not been worked out in any instance. For recording overall $[Ca^{2+}]_i$ transients in moderately large single cells, e.g. myocytes and hepatocytes, microinjected aequorin still holds the edge by virtue of its large dynamic range (i.e. it does not saturate at about $5 \mu M$ $[Ca^{2+}]_i$) and lack of cytosolic Ca^{2+} buffering. Although technically quite demanding the procedure is not fearsomely difficult and the apparatus required is not all that expensive. To get comparable sensitivity with fura-2 or indo-1 one needs dual wavelength, epifluorescence microscopy, i.e. rather more complex and expensive equipment and loading of at least tens of micromolar which may significantly perturb $[Ca^{2+}]_i$ transients. Ester-loading is a major advantage of the dyes provided the hazards of compartmentation and leakage are avoided or accounted for. Of course microinjection overcomes many of the potential drawbacks of ester loading.

For most purposes the fluorescence dyes are the method of choice: for ease of use, for ability to quantify at and below resting $[Ca^{2+}]_i$, and for the enormous photoflux they can provide to permit imaging of $[Ca^{2+}]_i$ within one small cell.

The reader might expect us to end with comments on direct comparisons between aequorin and fluorescent dye results. Unfortunately this is not appropriate at present, since the critical experiment of directly comparing signals from aequorin and fura-2 or indo-1 microinjected into the same cells has not yet been done. All other comparisons are contaminated with variations in loading methods, location of probe and different preparations.

We thank Maureen Bowden and Bea Leigh for help in preparing the manuscript and Ron Jacob, Tullio Pozzan and Roger Tsien for their helpful comments and permission to cite unpublished observations.

REFERENCES

- Allen, D. G. & Blinks, J. R. (1978) *Nature (London)* **273**, 509–513
- Allen, D. G. & Blinks, J. R. (1979) in *Detection and Measurement of free Ca^{2+} in Cells* (Ashley, C. C. & Campbell, A. K., eds), pp. 159–174, Elsevier/North-Holland, Amsterdam
- Allen, D. G., Blinks, J. R. & Prendergast, F. G. (1977) *Science* **196**, 996–998
- Allen, D. G., Eisner, D. A. & Orchard, C. H., (1984) *J. Physiol. (London)* **352**, 113–128
- Allen, T. J. & Baker, P. F. (1985) *Nature (London)* **315**, 755–756
- Allshire, A., Piper, H. M., Cuthbertson, R. & Cobbold, P. H. (1987) *Biochem. J.* **244**, 381–385
- Almers, W. & Neher, E. (1985) *FEBS Lett.* **192**, 13–18
- Alvarez-Leefmans, F. J., Rink, T. J. & Tsien, R. Y. (1981) *J. Physiol. (London)* **315**, 531–548
- Arlsan, P., Di Virgilio, F., Beltrame, M., Tsien, R. Y. & Pozzan, T. (1985) *J. Biol. Chem.* **260**, 2719–2727
- Ashley, C. C. & Campbell, A. K. (eds) (1979) *Detection and Measurement of Free Ca^{2+} in Cells*, Elsevier/North-Holland, Amsterdam
- Berlin, J. R., Cannell, M. B., Goldman, W. F., Lederer, W. V., Marban, E. & Weir, W. G. (1986) *J. Physiol. (London)* **371**, 200p
- Blinks, J. R. (1978) *Photochem. Photobiol.* **27**, 423–432
- Blinks, J. R., Prendergast, F. G. & Allen, D. G. (1976) *Pharmacol. Rev.* **28**, 1–93
- Blinks, J. R., Mattingly, P. H., Jewell, B. R., van Leeuwen, M., Harrer, G. C. & Allen, D. G. (1978) *Methods Enzymol.* **57**, 292–328
- Blinks, J. R., Weir, W. G., Hess, P. & Prendergast, F. G. (1982) *Prog. Biophys. Mol. Biol.* **40**, 1–114
- Borle, A. B. & Snowdowne, K. W. (1982) *Science* **217**, 252–254
- Borle, A. B., Freudenrich, C. C. & Snowdowne, K. W. (1986) *Am. J. Physiol.* **251**, c323–c326
- Brownlee, C. & Wood, J. W. (1986) *Nature (London)* **320**, 624–625
- Cadenas, E. & Sies, H. (1984) *Methods Enzymol.* **105**, 221–231
- Campbell, A. K. (1974) *Biochem. J.* **143**, 411–418
- Campbell, A. K. & Dormer, R. L. (1975) *Biochem. J.* **152**, 255–265
- Campbell, A. K. & Dormer, R. L. (1978) *Biochem. J.* **176**, 53–66
- Campbell, A. K., Daw, R. A., Hallet, M. B. & Luzio, J. P. (1981) *Biochem. J.* **194**, 551–560
- Campbell, A. K., Dormer, R. L. & Hallett, M. B. (1985) *Cell Calcium* **6**, 69–82
- Cannell, M. B. & Allen, D. G. (1983) *Pflügers Arch.* **398**, 165–168
- Cobbold, P. H. (1979) in *Detection and Measurement of Free Ca^{2+} in Cells* (Ashley, C. C. & Campbell, A. K., eds.), pp. 383–394, Elsevier/North-Holland, Amsterdam
- Cobbold, P. H. (1980) *Nature (London)* **285**, 441–446
- Cobbold, P. H. & Bourne, P. K. (1984) *Nature (London)* **312**, 444–446
- Cobbold, P. H., Cuthbertson, K. S. R., Goyns, M. H. & Rice, V. (1983) *J. Cell Sci.* **61**, 123–136
- Cobbold, P. H., Cheek, T. R., Cuthbertson, K. S. R. & Burgoyne, R. D. (1987) *FEBS Lett.* **211**, 44–48
- Connor, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6179–6183
- Conrad, G. W. & Rink, T. J. (1986) *J. Cell. Biol.* **103**, 439–450
- Cork, R. J. (1986) *Plant Cell Environ.* **9**, 157–161
- Cuthbertson, K. S. R. & Cobbold, P. H. (1985) *Nature (London)* **316**, 541–542
- Cuthbertson, K. S. R., Whittingham, D. G. & Cobbold, P. H. (1981) *Nature (London)* **294**, 754–757
- Defeo, T. T. & Morgan, K. G. (1986) *J. Gen. Physiol.* **88**, 18a–19a
- Di Virgilio, F., Viccatini, L. M., Treres, S., Riz, G. & Pozzan, T. (1985) *Biochem. J.* **229**, 361–367
- Di Virgilio, F., Lew, D. P. & Pozzan, T. (1984) *Nature (London)* **310**, 691–693
- Eisen, A., Kiehart, D. R., Wieland, S. J. & Reynolds, G. T. (1984) *J. Cell Biol.* **99**, 1647–1654
- Eisen, A. & Reynolds, G. T. (1984) *J. Cell Biol.* **99**, 1878–1882

- Fried, R. M. & Tashjian, A. H. (1986) *J. Biol. Chem.* **261**, 7669–7674
- Gilkey, J. C., Laffe, L. F., Ridgway, E. B. & Reynolds, G. T. (1978) *J. Cell. Biol.* **76**, 448
- Goligorsky, M., Hruska, K., Cofrus, G. & Elson, E. (1986) *J. Cell. Physiol.* **128**, 466–474
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Hallam, T. J. & Pearson, J. D. (1986) *FEBS Lett.* **207**, 95–99
- Hallam, T. J. & Rink, T. J. (1985) *FEBS Lett.* **186**, 175–179
- Hallam, T. J., Daniel, J. L., Kendrick-Jones, J. & Rink, T. J. (1985) *Biochem. J.* **232**, 373–377
- Hallam, T. J., Peonie, M. & Tsien, R. Y. (1986) *J. Physiol. (London)* **377**, 123p
- Hallett, M. B. & Campbell, A. K. (1982) *Nature (London)* **295**, 155–158
- Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J. C. (1983) *J. Biol. Chem.* **258**, 4876–4882
- Inouye, S., Taguchi, H., Murata, M., Kakoi, H. & Goto, T. (1977) *Chem. Lett.*, 259–262
- Inouye, S., Hoguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T. & Tsuji, F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3154–3158
- Himpens, B. & Somlyo, A. P. (1987), *Biophys. J.* **51**, 100a
- James-Kracke, M. R. (1986) *Am. J. Physiol.* **251**, C512–C523
- Kojima, I. & Ogata, E. (1986) *J. Biol. Chem.* **261**, 9832–9838
- Lattanzio, F. A. & Pressman, B. C. (1986) *Biochem. Biophys. Res. Commun.* **139**, 816–821
- Lazzari, K. G., Proto, P. J. & Simon, E. R. (1986) *J. Biol. Chem.* **261**, 9710–9713
- Lemasters, J. J., DiGiuseppi, J., Nieminen, A.-L. & Herman, B. (1987) *Nature (London)* **325**, 78–81
- Luckhoff, A. (1986) *Cell Calcium* **7**, 233–248
- Malgaroli, A., Milani, D., Meldolesi, J. & Pozzan, T. (1987) *J. Cell Biol.*, in the press
- McNaughton, P. A., Ceretto, L. & Nunn, B. J. (1986) *Nature (London)* **322**, 261–263
- McNeil, P. L. & Taylor, D. L. (1985) *Cell Calcium* **6**, 83–93
- McNeil, P. L., McKenna, M. P. & Taylor, D. L. (1985) *J. Cell Biol.* **101**, 372–379
- Merritt, J. E. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 4958–4960
- Metcalfe, J. C., Hesketh, T. R. & Smith, G. A. (1985) *Cell Calcium* **6**, 183–195
- Moiescu, D. G., Ashley, C. C. & Campbell, A. K. (1975) *Biochim. Biophys. Acta* **396**, 133–140
- Morgan, J. P. & Morgan, K. G. (1982) *Pflugers Arch.* **395**, 75–77
- Morgan, J. P. & Morgan, K. G. (1984) *J. Physiol. (London)* **357**, 539–551
- Neering, I. R. & McBurney, R. N. (1984) *Nature (London)* **309**, 158–160
- Neering, I. R., & Morgan, K. G. (1980) *Nature (London)* **288**, 585–587
- Neher, E. & Almers, W. (1986) *EMBO J.* **5**, 51–53
- Nemeth, E. F. & Scarpa, A. (1986) *FEBS Lett.* **203**, 15–19
- Poenie, M. & Tsien, R. Y. (1986) *Prog. Clin. Biol. Res.* **210**, 53–56
- Poenie, M., Alderton, J., Tsien, R. Y. & Steinhardt, R. A. (1985) *Nature (London)* **315**, 147–149
- Poenie, M., Alderton, J. A., Steinhardt, R. A. & Tsien, R. Y. (1986) *Science* **233**, 886–889
- Pollock, W. K. & Rink, T. J. (1986) *Biochem. Biophys. Res. Commun.* **139**, 308–314
- Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) *Biochem. J.* **235**, 869–877
- Pozzan, T., Arslan, I., Tsien, R. Y. & Rink, T. J. (1982) *J. Cell. Biol.* **xx**, 335–340
- Prasher, D., McCann, R. O. & Cormier, M. J. (1985) *Biochem. Biophys. Res. Commun.* **126**, 1259–1268
- Rabinovitch, P. S., June, C. H., Grossmann, A. & Ledbetter, J. A. (1986) *J. Immunol.* **137**, 952–961
- Rapp, P. E. & Berridge, M. J. (1981) *J. Exp. Biol.* **93**, 119–132
- Ratan, R. R., Shelenski, M. L. & Maxfeld, F. R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5136–5140
- Reiss, H. D. & Nobiling, R. (1986) *Protoplasma* **131**, 244–246
- Reynolds, G. T. (1972) *Q. Rev. Biophys.* **5**, 296–349
- Ridgway, E. B. & Ashley, C. C. (1967) *Biochem. Biophys. Res. Commun.* **29**, 229–234
- Ridgway, E. B., Gilkey, J. C. & Jaffe, L. F. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 623–627
- Rink, T. J. (1983) *Pure Appl. Chem.* **55**, 1977–1988
- Rink, T. J. & Hallam, T. J. (1984) *Trends Biochem. Sci.* **9**, 215–219
- Rink, T. J. & Pozzan, T. (1985) *Cell Calcium* **6**, 133–144
- Rink, T. J., Tsien, R. Y. & Warner, A. E. (1980) *Nature (London)* **283**, 658–660
- Rink, T. J., Smith, S. W. & Tsien, R. Y. (1982) *FEBS. Lett.* **148**, 21–26
- Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319
- Rose, B. & Loewenstein, W. R. (1975) *Nature (London)* **254**, 250–252
- Rose, B. & Loewenstein, W. R. (1976) *J. Membr. Biol.* **28**, 87–119
- Sage, S. O. & Rink, T. J. (1986a) *Biochem. Biophys. Res. Commun.* **136**, 1124–1129
- Sage, S. O. & Rink, T. J. (1986b) *Eur. J. Pharmacol.* **128**, 99–107
- Shimomura, O. (1986) *Biochem. J.* **234**, 271–277
- Shimomura, O. & Johnson, F. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2611–2615
- Shimomura, O. & Shimomura, A. (1982) *FEBS Lett.* **138**, 201–204
- Shimomura, O. & Shimomura, A. (1984) *Biochem. J.* **221**, 907–910
- Shimomura, O. & Shimomura, A. (1985) *Biochem. J.* **228**, 745–749
- Shimomura, O., Johnson, F. H. & Saiga, Y. (1962) *J. Cell. Comp. Physiol.* **59**, 223–239
- Simpson, A. W. M., Hallam, T. J. & Rink, T. J. (1986) *FEBS Lett.* **201**, 301–305
- Snowdowne, K. W. & Borle, A. B. (1984a) *Am. J. Physiol.* **246**, E198–E201
- Snowdowne, K. W. & Borle, A. B. (1984b) *Am. J. Physiol.* **247**, C396–C408
- Taylor, D. L., Blinks, T. R. & Reynolds, G. (1980) *J. Cell. Biol.* **86**, 599–607
- Thomas, M. V. (1982) *Techniques in Calcium Research*, Academic Press, New York
- Tsien, R. Y. (1980) *Biochemistry* **19**, 2396–2404
- Tsien, R. Y. (1981) *Nature (London)* **290**, 527–528
- Tsien, R. Y. & Rink, T. J. (1985) *Curr. Methods Cell. Neurobiol.* **3**, 249–312
- Tsien, R. Y. & Peonie, M. (1986) *Trends Biochem. Sci.* **11**, 450–455
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982a) *J. Cell. Biol.* **94**, 325–334
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982b) *Nature (London)* **295**, 68–71
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1984) *Trends Biochem. Sci.* **9**, 263–266
- Tsien, R. Y., Rink, T. J. & Poenie, M. (1985) *Cell Calcium* **6**, 145–157
- Tsuji, F. I., Inouye, S., Goto, T. & Sakaki, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8107–8111

- Tucker, R. W., Snowdowne, K. W. & Borle, A. B. (1986) *Eur. J. Cell. Biol.* **41**, 347–351
- Ware, J. A., Johnson, P. C., Smith, M. & Salzman, E. W. (1985) *Biochem. Biophys. Res. Commun.* **133**, 98–104
- White, J. G. & Amos, W. B. (1987) *Nature (London)* **328**, 183–184
- Williams, D. A., Fogarty, K. E., Tsien, R. Y. & Fay, F. S. (1985) *Nature (London)* **318**, 558–561
- Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602
- Woods, N. M., Cuthbertson, R. & Cobbold, P. H. (1987) *Cell Calcium* **8**, 79–100