3.8: Measurements of "Free" Calcium Concentrations

Much of our present knowledge of the biological role of Ca^{2+} ions in the regulation and modulation of cellular activities rests on the development of analytical techniques in three different areas: our ability to measure the low concentration levels in the cytoplasm of resting cells, follow the concentration changes, both temporally and spatially, that may occur as a result of an external stimulus, and measure the distribution of Ca^{2+} in various compartments of a cell. The last decade has seen the emergence of many such new techniques, and the improvement of old ones, which has had a major impact on our understanding of the detailed molecular mechanisms and dynamics of the Ca^{2+} messenger system. In this section, we will survey some of the most important techniques and results obtained using these. Broadly speaking there are two main groups of experimental techniques: those that aim at measuring the concentration of "*free*" (or uncomplexed) *Ca^{2+}-ion concentrations* (or activities), and those that measure *total calcium*.

Ca^{2+}-selective Microelectrodes

Ion-selective electrodes can be made from a micropipette (external diameter 0.1-1μm) with an ion-selective membrane at the tip.[18,19] For Ca^{2+} the membrane can be made of a polyvinyl chloride gel containing a suitable Ca^{2+}-selective complexing agent soluble in the polymer gel. A commonly used complexing agent is "ETH 1001" (see Figure 3.2A). An additional "indifferent" reference electrode is needed. For measurements inside cells, the reference electrode can also be made from a micropipette filled with an electrolyte gel. Often the ion-selective and reference electrodes are connected in a double-barrelled combination microelectrode.[21] The whole assembly can then be inserted, using a micromanipulator, into a single cell typically 30-50 μm across. The arrangement is depicted in Figure 3.2B. With proper care, Ca^{2+} microelectrodes can be used to measure Ca^{2+}-ion concentrations down to 10^{-8} M.[19,21] One limitation of the technique is that the response time is usually in seconds or even minutes, making rapid concentration transients difficult to follow.

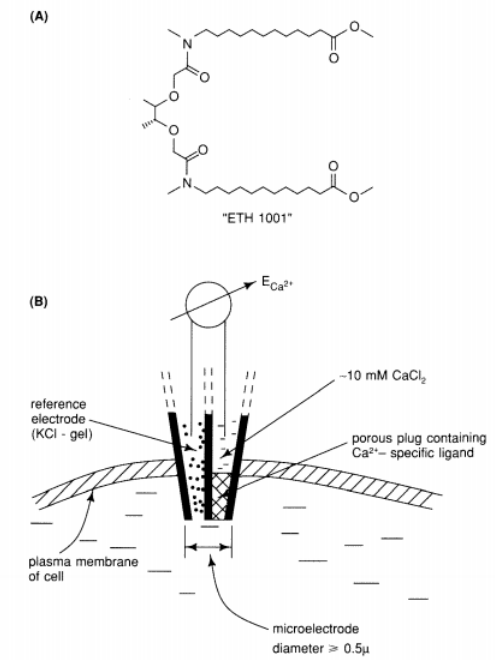

Figure 3.2 - (A) Structure of a commonly used neutral Ca^{2+} chelator in Ca^{2+}-selective electrodes, "ETH 1001" (N,N'-di[11-(ethoxycarbonyl) undecul]-N,N'-4,S-tetramethyl-3,6-dioxaoctan-1 ,8-diamide).[20] (B) Schematic arrangement for the measurement of the activity (or concentration) of Ca^{2+} ions in cells using a Ca^{2+}-selective double-barreled microelectrode. Frequently the microelectrode is supplemented by a third, indifferent, electrode inserted into the bathing medium surrounding the cell.[21]

Bioluminescence

Several living oganisms are able to emit light. The light-emitting system in the jellyfish (Aequorea) is a protein called aequorin ($M_r \simeq 20$ kDa). The light is emitted when a high-energy state involving a prosthetic group (coelenterazine) returns to the ground state in a chemical reaction that is promoted by Ca^{2+} ions. At Ca^{2+} concentrations below ~0.3 μM the emission is weak, but in the range 0.5-10 μM the emission is a very steep function of the concentration (roughly as $[Ca^{2+}]^{2.5}$).[18,19,22] The response to a Ca^{2+}-concentration transient is rapid ($\tau_{1/2}$ = 10 ms at room temperature), and the light emitted can be accurately measured even at very

low light levels by means of image intensifiers and/or photon counting. For measurements of Ca^{2+} concentrations inside cells, aequorin has usually been introduced either through microinjection or through some other means. A novel idea, however, is to utilize recombinant aequorin reconstituted within the cells of interest, thus circumventing the often difficult injection step.[174]

Complexing Agents with Ca^{2+}-dependent Light Absorption or Fluorescence

An important advance in the field of Ca^{2+}-ion detennination was made by R. Y. Tsien, who in 1980 described[23] the synthesis and spectroscopic properties of several new tetracarboxylate indicator dyes that had high affinity and reasonable selectivity for Ca^{2+}. All these dye molecules have a high UV absorbance that is dependent on whether Ca^{2+} is bound or not; a few also show a Ca^{2+}-dependent fluorescence. Tsien has also demonstrated that these anionic chelators can be taken up by cells as tetraesters, which, once inside the cells, are rapidly enzymatically hydrolyzed to give back the Ca^{2+}-binding anionic forms. Fluorescent tetracarboxylate chelators with somewhat improved Ca^{2+} selectivity such as "BAPTA," "Quin-2," and "Fura-2" (Figure 3.3) were later described.[24] These chelators are very suitable for measurement of Ca^{2+}-ion concentrations in the range 1 μM to 10 nM in the presence of 1 mM Mg^{2+} and 100 mM Na^+ and/or K^+—i.e., conditions typically prevailing in animal cells. Recently a new set of chelators that are more suitable for measurements of calcium concentrations above 1 μM was presented.[25] The most interesting of these is "Fluo-3," with a calcium-binding constant of 1.7 x 10^6.

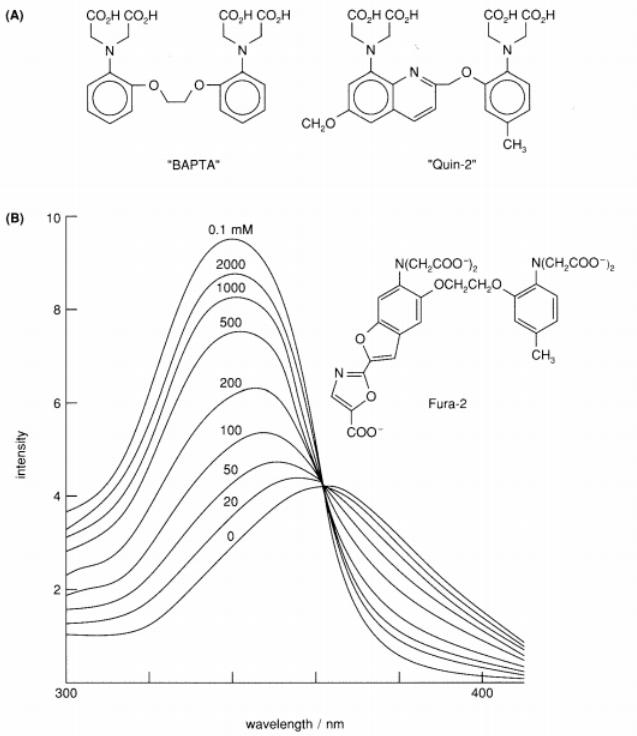

Figure 3.3 - Molecular structure of three chelators frequently used in measurements of "free" Ca^{2+}-ion concentrations. They may all be regarded as aromatic analogues of the classical chelator "EDTA"; their optical spectroscopic properties change upon binding Ca^{2+} ions. (A) For "BAPTA" the spectral changes are confined to the absorption spectrum, whereas "Quin-2" and the "Fura-2" in (B) show Ca^{2+}-dependent changes in their fluorescence spectra.[23, 24] (B) The relative fluorescence intensity of "Fura-2" at 505 nm as a function of the wavelength of the excitation light at different Ca^{2+} concentrations. The data[26] refer to a solution containing 115 mM KCI, 20 mM NaCl, and 1 mM Mg^{2+} at 37 °C and pH 7.05. At increasing Ca^{2+} concentration, the excitation efficiency at ~350 nm is increased, but that at ~385 nm is decreased. In order to eliminate (as much as possible) variations in fluorescence intensity in biological samples due to slight variations in dye concentrations and/or cell thickness, it is often advantageous to measure the intensity ratio at 345 and 385 nm excitation wavelengths.

Whereas the emission spectrum for Fura-2 (Figure 3.3B), which peaks at 505-510 nm, hardly shifts wavelength when Ca^{2+} is bound, the absorption spectrum shifts toward shorter wavelengths. In studies of free Ca^{2+} concentrations where internal referencing is necessary, for example, in studies of single cells, it is therefore advantageous to excite alternately at ~350 and 385 nm, and to measure the ratio of fluorescence intensity at ~510 nm.

The use of fluorescent chelators has recently permitted studies in single cells of rapid fluctuations or oscillations of free Ca^{2+} and the formation of Ca^{2+} concentration gradients. Using a fluorescence microscope coupled to a low-light-level television camera feeding a digital image processor, Tsien *et al.*[26] have been able to reach a time resolution of about 1 s in single-cell studies. The

results of some highly informative studies made using this instrument are shown in Figure 3.4. (See color plate section, page C-7.) The concentration of free Ca^{2+} is presented in pseudocolor, and the Fura-2 concentration inside cells is 50-200 μM, as indicated in the figures. We see a Ca^{2+} gradient diffusing through an entire sea-urchin egg (~120 μ across) in 30 s. The free Ca^{2+} concentration of the resting egg (~100 nM) is increased to about 2 μM as Ca^{2+} diffuses through the egg. The mechanism of propagation is believed to be a positive feedback loop with inositol trisphosphate releasing Ca^{2+} and vice versa (see Section V).

A pertinent question concerning the uses of intracellular Ca^{2+} chelators is whether or not the chelator significantly perturbs the cell. The chelator will obviously act as a Ca^{2+} buffer in addition to all other Ca^{2+}-binding biomolecules in the cell. The buffer effect is probably not of any major consequence, since the cell may adjust to the new situation by an increase in total Ca^{2+}, especially if the chelator concentration is in the μM range. The chelators could, however, interact with and inhibit intracellular enzymes or other molecules, an effect that could result in aberrant cellular behavior. It is not unlikely that BAPTA will bind to certain proteins.[27]

Complexing Agents with Ca^{2+}-dependent NMR Spectra

A series of symmetrically substituted fluorine derivatives of BAPTA (see Figure 3.3A) has been synthesized.[28,29] One of these chelators is 5F-BAPTA (Figure 3.5A), which has a binding constant for Ca^{2+}, K_B^{Ca}, of 1.4 x 10^6 M^{-1} and a ^{19}F NMR chemical shift, δ, that in the free ligand is different from that in the complex with Ca^{2+} ($\Delta\delta_{Ca^{2+}} \approx 6$ ppm). The rate of Ca $^{2+}$ dissociation, k_{off}, is 5.7 x 10^2 s^{-1}, which gives the rate of association, k_{on}, as 8 x 10^8 $M^{-1}s^{-1}$ according to

$$K_B = k_{on}/k_{off} \tag{3.2}$$

This exchange rate means that we are approaching the slow exchange limit in ^{19}F NMR, and in subsaturating concentrations of Ca^{2+} two ^{19}F signals are seen (see Figure 3.5B).

Since the areas of the NMR signals from the bound (B) and free (F) forms of the ligand are proportional to their concentration, the free Ca^{2+} concentration is obtained simply as

$$[Ca^{2+}]_{free} = \frac{B}{F} \cdot \frac{1}{K_B}. \tag{3.3}$$

An additional beneficial property of 5F-BAPTA and other fluorinated analogues of BAPTA is that they will also bind other metal ions with a ^{19}F chemical shift of the complex that is characteristic of the metal ion.[29] Under favorable circumstances, it is thus possible to measure simultaneously the concentrations of several cations.

For 5F-BAPTA the selectivity for Ca^{2+} over Mg^{2+} is very good ($K_B^{Mg2+} \approx 1$ M^{-1}). In applications of 5F-BAPTA to intracellular studies, the same protocol is used as with the parent compound and its fluorescent derivatives: some esterified derivative, e.g., the acetoxymethyl ester, is taken up by the cells and allowed to hydrolyze in the cytoplasm. The intracellular concentrations of 5F-BAPTA needed to get good ^{19}F NMR signals depend on the density of cells in the sample tube and the number of spectra accumulated. With accumulation times on the order of ten minutes (thus precluding the observation of concentration transients shorter than this time), Ca^{2+} concentrations of the order of 1 μM have been studied in perfused rat hearts using 5F-BAPTA concentrations of about 20 μM.[34]