M.Sc. 2nd Semester Study Material (I)

Subject: HUMAN PHYSIOLOGY

Topic : Molecular Pharmacology	
Paper	**PHY 202**
Unit	**16 (Module IV)**
Name of Guest Lecturer	**Suparna Majumder**

A. MEASUREMENT OF INTRACELLULAR Ca^{2+} LEVEL :

- Calcium is ubiquitous in living systems, and diverse in its roles as an intracellular signal. The heterogeneity of calcium function can be appreciated in the elaborate neurons and glia of the vertebrate nervous system, where many intracellular signalling cascades are regionally distinct and can be studied in isolation.

- As one of the alkaline earth metals, calcium has a high dehydration rate, is extremely reactive, and possesses an irregular coordination sphere that forms numerous metal complexes with other neutral or negatively charged molecules (proteins, sugars, etc.).

- In eukaryotic signalling, perhaps the most familiar of these calcium coordination compounds are the members of more than 120 families of EF-hand proteins that contain a helix-loop-helix structure of 12 or 14 amino acids to bind calcium (Ca2+) ions the most familiar of which is calmodulin. After binding calcium, the conformation and function of EF-hand proteins such as calmodulin (CaM) are changed, and thus a calcium-triggered signal is born.

- Given the number and diversity of potential partners, calcium within cells is rarely found in isolation. Though the total intracellular calcium may be a few millimolar (mM), in most cells the free cytoplasmic calcium, [Ca2+]c, is only tens of nanomolar (nM), which is four orders of magnitude less than the 2–2.5 mM extracellular calcium, [Ca2+]o, found in blood or CSF. This extraordinary difference between total and free [Ca2+] reflects in part the rapid sequestration of Ca2+ into intracellular compartments, efflux through the plasma membrane via transporters or pumps, and interactions with the aforementioned binding proteins as well as other cytoskeletal and diffusible buffers. As a consequence of this highly reactive environment, the free path length for Ca2+ is often less than 75 nm.

- Its ability to serve as a signalling molecule in a cell that may be tens of microns in extent implies that there must be an array of cellular elements that allow transient local changes of [Ca2+] c via routes other than its influx and efflux through the plasma membrane. In this chapter we will come to appreciate that at least two intracellular organelles have been implicated as local and highly dynamic reservoirs from which Ca2+ is released by signalling molecules that diffuse through the cytoplasm. Though far from being the sole intracellular second messenger, it is now clear that over the course of eukaryote evolution Ca2+ has been selected as an ideal, rapid and localized signalling component of many cellular processes.

CALCIUM MEASUREMENT

- ✓ **Much of our understanding of the essential role of Ca^{2+} in cellular physiology has been indirect:**
  - During extra-vital investigations of the perfused frog heart by Sydney Ringer in the 1880s, the accidental use of London tap water to make his experimental salt solutions led to an extraordinary discovery.
  - The abnormal function of the heart observed in isotonic NaCl could be corrected and dramatically extended by inclusion of calcium in the bathing solution at a concentration similar to that found in blood (Ringer, 1883).
  - The major technical challenge in the subsequent century was to develop methods by which [Ca2+]c could be rapidly, reliably and non-destructively measured during physiological activity. Early attempts to measure calcium flux or concentration relied on monitoring the movement of radioactive [45Ca2+], and the miniaturization of ion-sensitive electrodes whose sub-micron tips could impale cells and be used to estimate the average [Ca2+]c.
  - Higher spatial resolution was achieved by electron microscopy using energy dispersive X-ray microanalysis of neuronal compartments, but this provided only static information. To examine [Ca2+] dynamics, tissue was prepared by rapid freezing and fixation at precise moments following physiological stimulation. When combined with fluorescent imaging, the results from these heroic experiments supported the notion that the neuronal endoplasmic reticulum (ER) acts as a rapid source and sink of cytoplasmic Ca2+ following activation of voltage- or ligand operated Ca2+ entry at synapses (Pozzo-Miller et al., 1999).

- ✓ **Current optical methods to measure calcium use chemical or protein-based fluorescent indicators:**

  - Most recent efforts to demonstrate variations in [Ca2+]c are inspired by early imaging experiments on muscle and nerve using aequorin, a calcium-sensitive protein from a luminous jellyfish that emitted light in response to Ca2+ binding. Based on the power of this approach, the revolution over the last 30 years in characterizing calcium signalling at the subcellular level originated with the pioneering work of Nobel laureate Roger Tsien and colleagues (Miyawaki et al., 1997; Tsien, 1980). By clever chemical and molecular tricks they created chemical or protein- based compounds that permit optical detection of [Ca2+] even within cellular micro-domains.

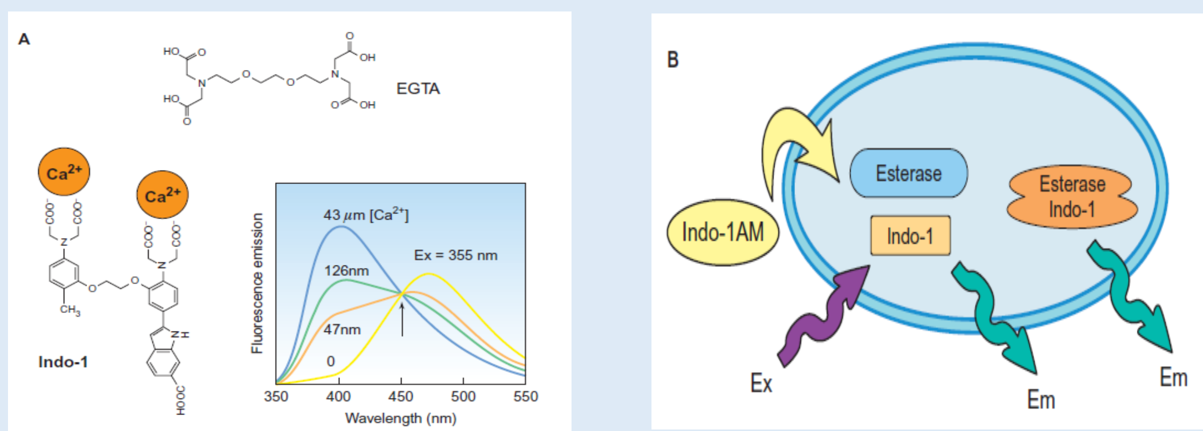
The optical monitoring of [Ca2+] relies on indicators whose fluorescence changes upon binding to calcium

- Many of the chemical probes used to measure [Ca2+] are tetracarboxylate dyes, derivatives of the well-known calcium chelator EGTA [ethylene glycol bis(2-aminoethyl ether) *N*,*N*,*N*',*N*'-tetraacetic acid] that have been hybridized with aromatic rings to impart fluorescence. The enormous utility and popularity of these dyes is due largely to the fact that they can be made membrane permeable by esterification of the calcium-binding sites, and loaded by diffusion into cells of any size.

- Once inside, the dyes are trapped by endogenous esterases that remove the masking acetoxymethyl groups and render them functional indicators again. Substitution of different functional groups on the aromatic rings creates indicators with a wide range

of spectral properties and disassociation constants, kd, for calcium binding that range from 100 nM to 400 µM. A rule of thumb is that indicators can be used to quantify [Ca2+] over a range from one-tenth to 10 times their kd. So in principle it should be possible to measure the full range of intracellular [Ca2+] by using multiple dyes of overlapping affinities. Some care needs to be taken in following rapid changes in Ca2+ in neurons and glia, however, since the observed changes in fluorescence will be slower with higher-affinity dyes.

- Variations of this technique use the salts or dextran-bound versions of the dyes introduced into individual cells via patch pipette. This can be used to enhance the detectability of the cellular fluorescence against a dark field, and to avoid the uptake of dye into organelles. Further, the use of patch pipettes to load the dye makes it possible to simultaneously measure [Ca2+] while recording ionic currents through voltage- or ligand-operated channels.



A. Many chemical indicators of [Ca2+] are based on EGTA.
A. One example, Indo-1, has two Ca-binding sites, and when excited with 355 nm light the resulting fluorescence spectra is the sum of the curves for Ca-Indo-1 (peak, 398 nm) and apo-Indo-1 (peak,482 nm). The ratio of the fluorescence at these wavelengths is used to estimate free Ca2+. All spectra regardless of [Ca2+] cross at 447 nm, the isobestic point (arrow).
B. Membrane-permeant Indo-1 AM is free to diffuse across membranes and is trapped inside cells and their organelles by endogenous esterases. The fluorescence, shown emanating from the cytoplasm and the endoplasmic reticulum, can be used to estimate the [Ca2+] in each region. AM, acetoxymethyl; Ex, excitation; Em, emission.

✓ ***Increased resolution can be accomplished optically or by targeting indicator proteins***

- In conventional fluorescence microscopy, the spatial resolution is limited to an oblate spheroid roughly 200 nm in diameter in the plane of view, and extending 600 nm along the optical axis. These dimensions are much larger than the granularity required to measure Ca2+ dynamics within synaptic boutons, dendritic spines, astrocytic processes, or even the domain immediately subjacent to the plasma membrane.

- In special cases when cells and their regions of interest are adherent to a flat optical surface, total internal reflectance fluorescence (TIRF) microscopy can be used to image restricted regions that extend less than 100 nm into the sample. As useful as this is, it fails to solve the problem of tracking calcium dynamics in even smaller

volumes within a particular organelle or region, particularly those that are not adherent to the surface.

- This problem is currently addressed by using one of the genetically encoded Ca2+ indicators (GECIs). These indicators can be selectively targeted to a precise locale (plasma membrane, nucleus, ER, mitochondria, Golgi, secretory vesicles or gap junctions) by inclusion of an appropriate localization signal sequence as part of the indicator.

- Alternatively, GECIs can be fused to a protein of interest and report the [Ca2+] in neighboring nanodomains. These indicators are generally of three types. Some are derivatives of aequorin, and generate their bioluminescence via a chemical reaction upon binding to calcium. Others, like camgaroos and pericams, use Ca2+-response elements such as calmodulin linked to circularly permute green fluorescent protein (GFP); Ca2+ binding changes the protonation and spectral properties of the indicator.

- In the final category of GECIs a calcium response element is inserted between two different fluorescent proteins chosen so that the emission spectra of one overlap the excitation spectra of the other. Upon Ca2+ binding the distance between the two proteins changes, and the indicator fluorescence is altered by changes in the efficiency of Fluorescence resonance energy transfer (FRET) between two chromophores; for example, cyan and yellow fluorescent proteins. In principle these gene product based systems make it possible to detect [Ca2+] in any subcellular domain.

- Ultimately, the experimental challenge in the analysis of smaller and smaller volumes will reduce to whether it is possible to target and express sufficient dye to provide a detectable signal at the targeted location.

HOME ASSIGNMENT:

Prepare the note on Calcium in different organelles and their homeostasis in human body (briefly). You have to submit the assignments after opening the college.