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Research Focus

The development of Ca^{2+} indicators: a breakthrough in pharmacological research

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The development, beginning in 1979, of fluorescent Ca^{2+}-specific indicators as research tools has revolutionized transmembrane signaling studies. In this article, the state of the art in the 'pre-Ca^{2+}-indicator' era and the rationale for the development of indicators trapped in the cytosol to investigate the cytosolic concentration of Ca^{2+} in mammalian cells are summarized. Subsequent extension of these studies to the level of the single cell, together with the unique impact that Ca^{2+} indicators have had on signaling research and the introduction of specific, fluorescent gene constructs that provide direct, high-resolution information about the intracellular concentration of Ca^{2+}, are also discussed.

Beginning in 1979, a new class of experimental tools was developed that has revolutionized transmembrane signaling, one of the main fields of basic pharmacology. The discovery of these cytosolic Ca^{2+} indicators did not occur by serendipity. Instead, this discovery was a precisely planned and elegantly executed one-man enterprise, the man being Roger Y. Tsien, a young scientist who was studying for a PhD in Physiology and had an extraordinary 'touch' in organic chemistry. In this article, I illustrate this enterprise, starting with the state of the art at the time the work began.

The 'pre-quin2 era'

In the 1970s the role of Ca^{2+} as the most important second messenger was already generally accepted. Signaling events were known to be triggered and controlled by changes of the cytosolic concentration of free Ca^{2+} {$[Ca^{2+}]_c$}, in dynamic equilibrium not only with the extracellular $[Ca^{2+}]$ but also with intracellular Ca^{2+} stores. Knowledge about $[Ca^{2+}]_c$ was, however, confused and in many cases openly contradictory. This confusion arose because none of the procedures employed was fully satisfactory, and the cross-checking of the results obtained using different procedures was not common.

Let us consider some of the procedures that were available at the time. Subcellular fractionation, which had been instrumental in the study of protein and lipid distribution, provided misleading results with respect to Ca^{2+} because of the uncontrolled release and uptake events that occurred after cell homogenization. Analysis of ^{45}Ca fluxes into and from living cells is still employed, however, to investigate the dynamics of intracellular stores rather than the cytosolic pool. The study of other ions, such as ^{86}Rb, flowing through Ca^{2+}-dependent channels was risky because it depends not only on $[Ca^{2+}]_c$ but also on a variety of other processes. Direct assays were problematic on two levels: the introduction of the recording tools into the cells and the analysis of the signals. Therefore, as pointed out by Tsien shortly after his discovery [1] during this era, the study of $[Ca^{2+}]_c$ was 'limited to robust and well anchored, large cells that can tolerate insertion of ion-selection electrodes or microinjection of Ca^{2+} indicators or buffers into one cell at a time' (e.g. muscle fibers, squid giant axons and limulus photoreceptors). Smaller cells could also be studied, although only following traumatic treatments, such as fusion with erythrocyte ghosts or liposomes filled with the probe, hypo-osmotic shock or scraping. We now know that the consequences of these treatments on Ca^{2+} homeostasis were often devastating [2].

With respect to the probes, let us consider two types. (i) The photoprotein aequorin, at the time employed in only a few highly specialized laboratories, was resuscitated successfully in the 1990s, when gene-expression techniques enabled it to be used to study specific subcellular structures. (ii) Two azo dyes, arsenazo III and antipyrilazo III, were characterized by serious drawbacks such as low Ca^{2+} and Mg^{2+} selectivity, low affinity for Ca^{2+} and complex Ca^{2+} binding stoichiometry [2].

In 1979, Tsien was working in the Department of Physiology at the University of Cambridge (UK). From his previous experience with Ca^{2+}-selective microelectrodes [3–5] and fluorescent probes, used to investigate membrane potential [6], Tsien realized that new indicators were needed to monitor the kinetics of $[Ca^{2+}]_c$ changes. The properties required for these indicators were to affect cellular Ca^{2+} homeostasis only moderately and in a predictable way, generating at the same time easily detectable fluorescent signals that changed appropriately following binding of the cation. The great idea, worked on in 1979 and published in April 1980 [7], started from the best-known Ca^{2+} chelator EGTA, which, however, cannot be used as an indicator because it absorbs light in the far ultraviolet range and is not fluorescent. The result was the synthesis of 'rationally designed, high-affinity buffers and optical indicators for Ca^{2+} in which methylene links between oxygen and nitrogen of EGTA are replaced by benzene rings' [7]. The molecule that was better characterized in the initial studies, BAPTA [1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid] (Figure 1),

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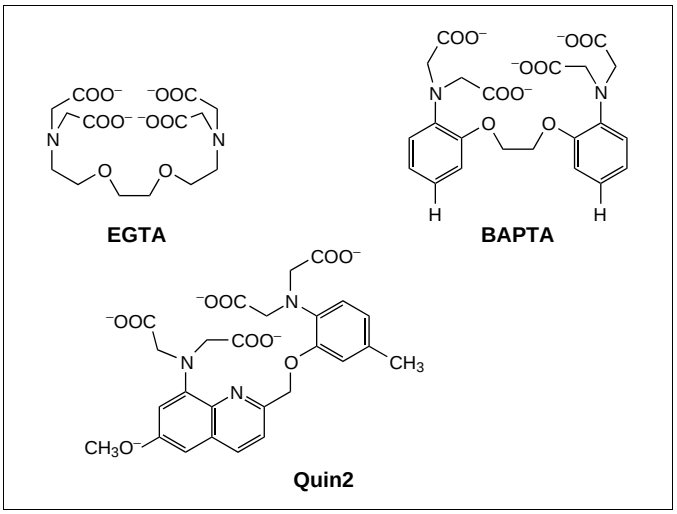

Figure 1. Compounds developed as Ca^{2+} chelators. BAPTA [1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid] is a derivative of EGTA, sharing the Ca^{2+} 'cage' formed by four carboxyl residues (which assures a 1:1 stoichiometry of Ca^{2+} binding) bound to two nitrogens. In BAPTA, two benzene rings replace the methylene groups connecting N to O; these rings have little effect on the overall geometry of the molecule. However, unlike EGTA, the ultraviolet spectrum of BAPTA changes significantly between the Ca^{2+}-free and Ca^{2+}-bound state, with a maximum excitation at 250 nm, which is not ideal for fluorescence measurements. The substitution of one oxy-benzene with a methoxyquinoline ring yields quin2, whose wavelength of excitation is shifted to 340 nm, and wavelength of emission is shifted to 492 nm. Following the binding of Ca^{2+} to quin2 there is virtually no shift of the excitation spectrum but the intensity of the fluorescence increases more than sixfold. As a result of these properties quin2 has revolutionized the cellular Ca^{2+} field by making it possible to monitor changes in the cytosolic concentration of Ca^{2+} in most types of mammalian cell. Reproduced, with permission, from [7].

although poor as a $[Ca^{2+}]_c$ indicator, is very popular in laboratories throughout the world as the ideal intracellular Ca^{2+} buffer. This is because, compared with EGTA, BAPTA is less affected by pH changes, has a higher Ca^{2+} and Mg^{2+} selectivity and is much faster at binding and releasing Ca^{2+}.

The appearance of quin2

In the article published in 1980 by Tsien [7], several analogs, distinct in terms of their Ca^{2+} affinity and Ca^{2+} and Mg^{2+} selectivity, were described in addition to BAPTA. These molecules were already matching, at least in perspective, many of the dreams of scientists studying Ca^{2+}, although with a big limitation. Similar to the azo dyes that were already available, these compounds were membrane impermeant. This was a problem that Tsien solved in a surprisingly short time. In April 1981, he demonstrated that Ca^{2+} chelators, when administered to the cell in a membrane-permeant form with the four carboxylic residues masked by acetoxymethyl groups, accumulate progressively within the cytosol. These acetoxymethyl groups are, in fact, cleaved off by unspecific esterases, and the resulting membrane-impermeant indicators remain trapped within the cytosolic compartment of the cell [1]. This idea was not entirely new because biacetylated pH-specific probes had been shown previously to accumulate within Ehrlich ascites cells as a consequence of de-acetylation [8]. The hydrophobic derivatives described by Tsien, however, were unique. Their acronym (AM, for acetoxymethyl) is now generally known as a magic bullet that directs to the cytosol of vertebrate cells not only Ca^{2+} indicators but also a variety of other molecules.

A second problem was the choice among BAPTA analogs of the best indicator for $[Ca^{2+}]_c$ studies. The quinolinic derivative quin2, sharing the tetracarboxyl specificity of EGTA (Figure 1), was quickly recognized as a breakthrough (Figure 2). Quin2 studies were carried out in collaboration with Tim Rink, a former student of Peter Baker and, at the time, Tsien's advisor, and with Tullio Pozzan, a young postdoctoral student from the University of Padova who was working in Cambridge in the Department of Biochemistry. The two historical papers that arose from this collaboration, a communication to *Nature* in January 1982 [9] and a comprehensive article published in the *Journal of Cell Biology* in August of the same year [10], not only opened the way to the study of $[Ca^{2+}]_c$ events in any cell, including cells as small as platelets [11], but revealed a variety of new, crucial aspects of Ca^{2+} homeostasis that until then had remained unknown.

New indicators, growing success

Since the initial work of Tsien, the story of $[Ca^{2+}]_c$ has been an uninterrupted series of successes. The introduction by Tsien in 1985 of new indicators that, upon Ca^{2+} binding, exhibit shifts of the excitation (fura-2) and/or emission (indo-1) spectra opened the way to studies at the single-cell level [12] (Figure 2). Fura-2 has additional advantages over quin2, including a slightly lower affinity for Ca^{2+} and a much higher fluorescence intensity. Because of these advantages fura-2 has progressively taken over from quin2, not only in research but also in the development of drugs that affect Ca^{2+} homeostasis. Today, the number of available Ca^{2+} indicators is impressive: 36 in the catalogue of Molecular Probes, where quin2 and fura-2 appeared initially, matching the needs of most (if not all) experimentalists. The affinity of these indicators for Ca^{2+} varies from a maximum K_d of 110 nM (quin2) to a minimum K_d of 90 000 nM (Fluo-5N).

The extraordinary importance of Ca^{2+} indicators also emerges from published articles. The number of published studies in which fura-2 ($\sim$75% of the total), quin2 and indo-1 have been used is $>$12 000, with these studies covering all disciplines of biological and biomedical sciences. Pharmacology is one of the best represented research areas, and $>$80% of these published studies include at least a mention of their relevance to drugs and/or pharmacological research.

From chemical indicators to gene products

The secret of this unusual success story lies in the introduction into cellular research of a series of specific molecules, beginning initially with chemical indicators and then more recently with fluorescent gene constructs, that provide direct, high-resolution information about $[Ca^{2+}]$ in the cytosol and, more recently, in the nucleus, intracellular organelles and membranes and their domains in most types of mammalian cells. During the past ten years, gene constructs, developed by Tsien in Berkeley and San Diego, in friendly and often collaborative competition with the group of his former collaborator

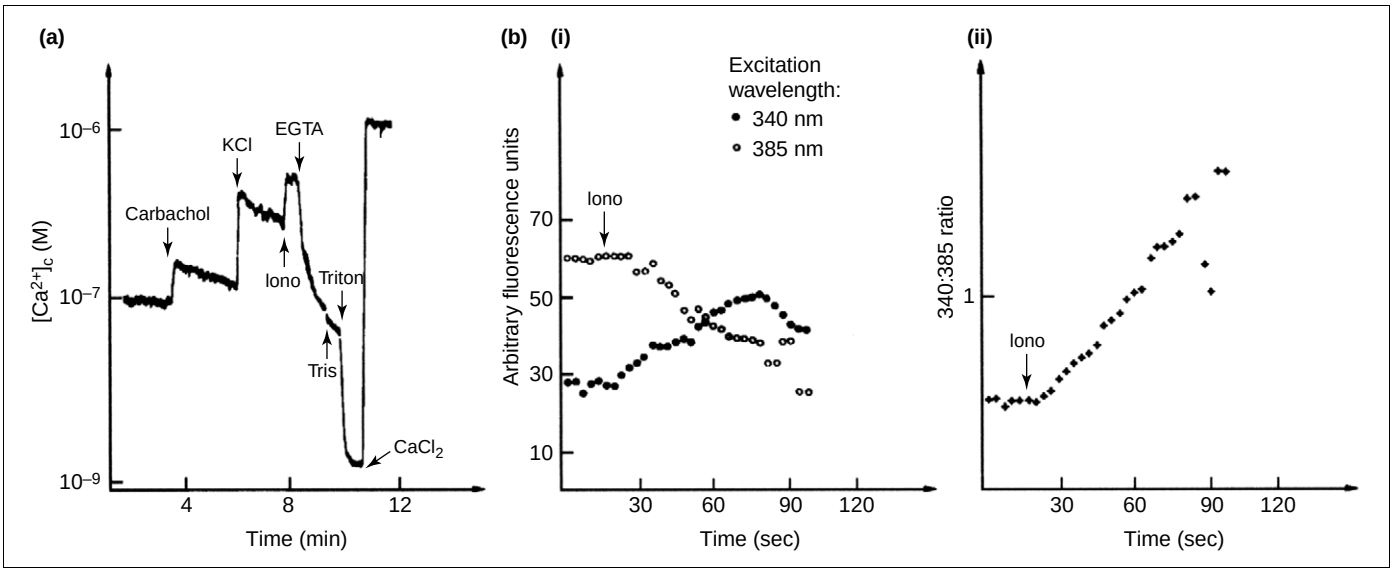

Figure 2. Typical experiments monitoring changes in the cytosolic concentration of Ca^{2+} {$[Ca^{2+}]_c$} using **(a)** quin2 in a suspension of PC12 cells and **(b)** fura-2 in a single PC12 cell. (a) Increases in $[Ca^{2+}]_c$ were induced by the sequential application of the M_1 muscarinic acetylcholine receptor agonist carbachol (0.1 mM), KCl (50 mM, inducing depolarization) and the Ca^{2+} ionophore ionomycin (Iono; 3 μM). The subsequent treatments with Tris (30 mM), Triton X-100 (0.1%) and $CaCl_2$ (4 mM) were applied to establish the minimal and maximal fluorescence (F) of the analyzed sample, which is necessary to calibrate the $[Ca^{2+}]_c$ and its changes according to the equation $[Ca^{2+}] = (110\ nM)\ (F - F_{min})/(F_{max} - F)$ [9]. (b) The fura-2 traces show, in a single cell exposed to ionomycin (Iono; 3 μM): (i) the changes of fluorescence at 340 nm (solid circles) and 385 nm (open circles), which increase and decrease on Ca^{2+} binding to the indicator, respectively; and (ii) the corresponding 340:385 ratios. The ratio values are proportional to the $[Ca^{2+}]_c$ values, which can therefore be established by making reference to a simple calibration curve. The results of both (a) and (b) are from [17].

Tullio Pozzan in Padova, have enjoyed and are still enjoying a success similar to the chemical indicators that were developed a decade before [13,14]. Discoveries have been almost infinite and, to mention a few, include: the continuous functional interactions between the endoplasmic reticulum and mitochondria, which are indispensable for the signaling, energy metabolism and survival of cells; the mechanisms of Ca^{2+}-dependent gene expression and cell growth; and the regulation of synaptic transmission and its plasticity. Moreover, the combined use of probes for other messengers and key cellular agents [15,16] has further expanded the impact of the indicators and fostered our understanding beyond the signaling field to complex processes such as enzyme activation and protein–protein interactions in many research areas that are often apparently distant from each other. The story, therefore, is not finished, and instead is growing more and more. The field promises to remain 'hot' in the future, and might deserve a second coverage in 25 years time, for the 50th birthday of *TiPS*!

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