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Ca^{2+} homeostasis of intracellular compartments: measurements using the jellyfish photo-protein aequorin

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The development of molecular biology techniques (which enable the modification and expression of exogenous cDNA in heterologous cell types), has been responsible in recent years for the widespread use of protein probes by cell biologists, for the measurement of biological parameters. To this end, 2 main types of proteins are used, isolated from a wide variety of luminescent organisms. The first group consists of chemiluminescent proteins. These are proteins that emit light, often associated to physiological parameters of interest, such as changes in ATP or Ca^{2+} concentration. Since mammalian cells do not possess endogenous lumiunescent molecules, the use of these proteins is normally associated to an excellent signal-to-noise ratio, as exemplified by aequorin, a photoprotein isolated from Aequorea victoria. The second group consists of fluorescent proteins. Among these, Aequorea victoria's green fluorescent protein (GFP) has attracted much attention, since its expression (or that of fusion products with proteins of interest) generates an intense signal, which enables complex phenomena (such as organelle structure and their dynamic changes, protein targeting etc.) to be followed in real time in living cells. This review presents some of the results obtained using aequorin for studying intracellular Ca^{2+} homeostasis.

Key words: Calcium - Organelles - Aequorin - Luminescence.

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The jellyfish *Aequorea victoria* expresses 2 proteins, aequorin and green fluorescent protein (GFP) that represent optimal probes for the study of Ca^{2+} homeostasis and for the labelling of cellular components, respectively. The use of protein as probes offers the possibility of targeting them exclusively to a specific cellular compartment. Indeed, the intracellular destiny of a protein depends on specific sequences of targeting, necessary and sufficient for its correct localization. It is possible to construct chimeric proteins that comprise one of these sequences signals fused to a heterologous protein (i.e. the luminescent or fluorescent probe) obtaining an exclusive cellular distribution to the compartment of interest. By this approach, in fact, we have created chimeras of aequorin specifically addressed to various cellular compartments (e.g. mitochondria, nucleus, endoplasmic reticulum and the Golgi apparatus). This review presents some of the results obtained using aequorin to study intracellular Ca^{2+} homeostasis.

An important improvement in the study of Ca^{2+} homeostasis has been represented from the discovery, at the beginning of the 1980s, of the intracellularly trappable fluorescent dyes for Ca^{2+}, such as fura-2. Their ability to cross the plasma membrane and to

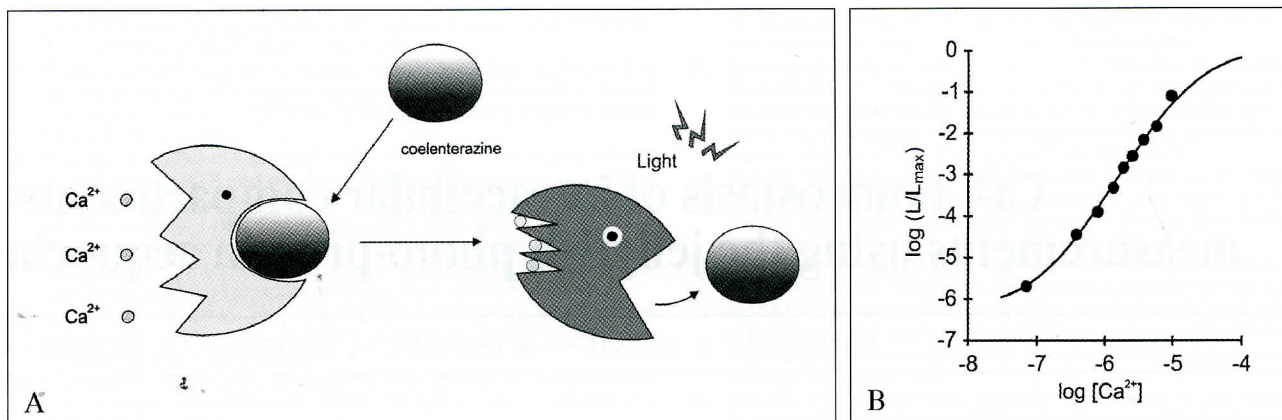

Figure 1.—A) Schematic model of the irreversible reaction of aequorin. When Ca^{2+} ions bind to the EF-hand binding sites of reconstituted aequorin (the prosthetic group, coelenterazine, is indicated) a photon is emitted and that molecule of aequorin is irreversibly discharged. (B) [Ca^{2+}] response curve of recombinant expressed aequorin. L, light emission immediately after adding the buffered Ca^{2+} solution; Lmax, integral of aequorin counts from the mixing to the end of the experiment (i.e. after aequorin consumption with excess Ca^{2+}), for details see text.

be blocked in the cytoplasm of the cells,[1, 2] has provided a board series of information on intracellular Ca^{2+} homeostasis in many cellular types and has pivotal in demonstrating the nearly ubiquitarious role of second messenger of this cation.[3]

While the chemical dyes are very powerful and versatile tools, they suffer of a major shortcoming: they are mostly accumulated in the cytoplasm and it is virtually impossible to selectively accumulate them in other cellular compartments, such as the membrane bound organelles. Conversely, this is a desired application. Indeed, numerous evidence demonstrates the involvement of the Ca^{2+} in phenomena that happen inside of cellular compartments, occur within organelles (see, for example, the regulation of dehydrogenases of the mitochondrial matrix or the post-translational modifications of proteins in the Golgi apparatus), and thus there is a strong interest also in the study of the concentration of Ca^{2+} inside of different organelles.

In our laboratory, the development of techniques for the specific intracellular targeting of a Ca^{2+} indicator (a Ca^{2+}-sensitive photoprotein: the aequorin) has been one of the main interests in order to solve this methodological problem.[4-10]

Aequorin, as isolated from the jellyfish *Aequorea victoria*[11] is composed of a 21 kDa apoprotein and a hydrophobic prosthetic group, coelenterazine (MW ~400 Da). The 2 components must be associated for the Ca^{2+}-triggered light emission to occur. The holoprotein possesses 3 high affinity Ca^{2+} binding sites (homologous to the sites present in other Ca^{2+} binding proteins, such as calmodulin). Upon binding of Ca^{2+} ions, aequorin undergoes an irreversible reaction in which one photon is emitted (Figure 1A). The rate of this reaction depends on the Ca^{2+} concentration ([Ca^{2+}]) to which the photoprotein is exposed. In particular, at [Ca^{2+}] between 10^{-7} and 10^{-5} M (the concentration normally occurring in the cytoplasm of living cells), there is a direct relationship between [Ca^{2+}] and the fractional rate of consumption of the photoprotein. Figure 1B shows the Ca^{2+} response curve of aequorin, at physiological conditions of pH, temperature, and ionic strength. It is apparent that the fractional rate of aequorin consumption expressed as the ratio between the emission of light at a defined Ca^{2+} concentration (L) and the maximal rate of the light emission at saturating [Ca^{2+}] (Lmax) is proportional to the 2nd-3rd power of [Ca^{2+}]. This is the basis of the use of aequorin as a Ca^{2+} probe. Indeed, if all the light emitted by the photoprotein throughout an experiment, as well as that discharged at the end are collected it is possible to estimate L_{max} and then calculate back the [Ca^{2+}] to which the photoprotein is exposed in every moment. As to the applicability of the approach, until the isolation of the aequorin cDNA[12], the use of aequorin was limited to the cell types in which the photoprotein could be microinjected and to the laboratories mastering the latter technique. Now the possibility of recombinant expres-

sion has drastically increased the applications and the number of users. Indeed, it is possible not only to express this protein as a calcium sensor in a variety of cell types, but also to design Ca^{2+} probes specifically targeted to defined subcellular locations.

In the following paragraphs we will focus on the main characteristics that make aequorin such a flexible and powerful intracellular calcium sensor.

Advantages

Development of targeted probes

Whereas recombinantly expressed wild-type aequorin is exclusively cytosolic, the intracellular fate of the photoprotein can be modified by adding specific targeting sequences. With this strategy, we have constructed aequorin chimeras destined to a wide variety of cell locations (mitochondrial matrix,[4] and intermembrane space,[9] nucleus,[5] endoplasmic reticulum,[6] sarcoplasmic reticulum,[7] Golgi apparatus [10] and subplasmamembrane region [8]).

Excellent signal to noise ratio

Virtually all mammalian cells show a very dim luminescent background. Given the steep response of aequorin, an excellent signal-to-noise ratio is obtained and minor variations in the amplitude of agonist-induced $[Ca^{2+}]$ changes can be easily detected.

Low buffering capacity

The binding of Ca^{2+} by aequorin could, in principle, affect intracellular Ca^{2+} homeostasis by acting as a buffer system. In practice, however, recombinant aequorin exhibits a very low Ca^{2+} buffering capacity, since it is usually expressed at a concentration in the range of 0.1-1 μM. Even at this low concentration (which is 2-3 orders of magnitude lower than that of the commonly employed fluorescent indicators), aequorin's excellent signal-to-noise ratio enables measurements to be made in cell populations.[13]

Wide dynamic range

Aequorin is capable of measuring calcium concentrations across a wide spectrum. Figure 2 shows that accurate measurements of $[Ca^{2+}]$ can be obtained from 0.5 μM to 10 μM, reaching values at which most fluorescent indicators are already saturated.

Possibility of co-expression with proteins of interest

A powerful approach for investigating the role, and the properties, of the various molecular components of the Ca^{2+} signalling apparatus is either the overexpression of the heterologous protein, followed by the study of the molecularly modified cell[14,15].

Disadvantages

Low light emission

In distinction to the fluorescent dyes (where up to 10^4 photons can be emitted by a single molecule, before photobleaching occurs), only 1 photon can be emitted by an aequorin molecule. Moreover, the principle of the use of aequorin for Ca^{2+} measurements is that only a small fraction of the total pool emits its photon every second. This is not a major limitation in population studies. Conversely, single cell imaging requires very high expression and special apparatuses.[16]

Expression may be inefficient and/or require time

In the case of recombinant aequorin, transfection is the simplest loading procedure. In this respect, it should be remembered that i) some cell lines may be quite resistant to transfection (although a wide range of procedures is now available, ranging from calcium phosphate to liposomes, electroporation and gene-gun); in many cases, time is required to find the appropriate transfection protocol and ii) time for protein expression must be waited before carrying out the Ca^{2+} measurements.

Overestimation of average value in dishomogeneous environment

A disadvantages of the steepness of the Ca^{2+} response curve of aequorin is that, if the increase of the $[Ca^{2+}]$ is not homogeneous, the average estimate will be based towards the highest values.

Chimeric Aequorin cDNAs

Below we briefly describe the constructs produced in our laboratory (Figure 3).

We routinely included in all the recombinant aequorins the HA1 epitope-tag that facilitates the immunocytochemical localization of the recombinant protein in the cell.

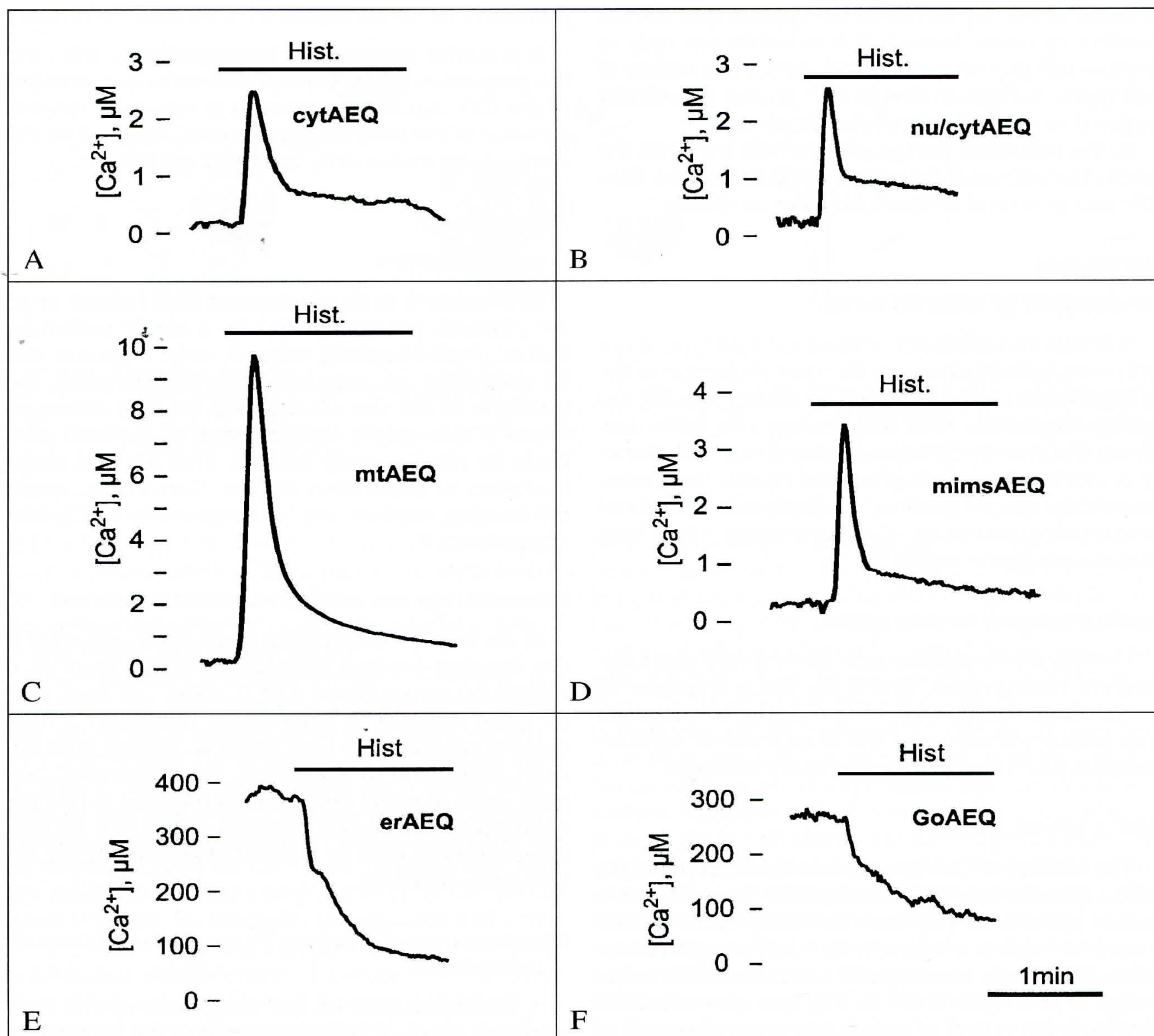

Figure 2.—Typical aequorin traces. Measurements of agonist-dependent variations in Ca^{2+} concentrations of different intracellular compartments in HeLa cells expressing the various aequorin targeted probes. Where indicated, the cells were challenged with 100 μM histamine (Hist).

Cytoplasm (cytAEQ)

An unmodified aequorin cDNA encodes a protein that, in mammalian cells is located in the cytoplasm and, given its small size, also diffuses into the nucleus. An alternative construct is also available that is located on the outer surface of the ER and of the Golgi apparatus.[9] This construct was intended to drive the localization of aequorin to the inner surface of the plasma membrane given that it derives from the

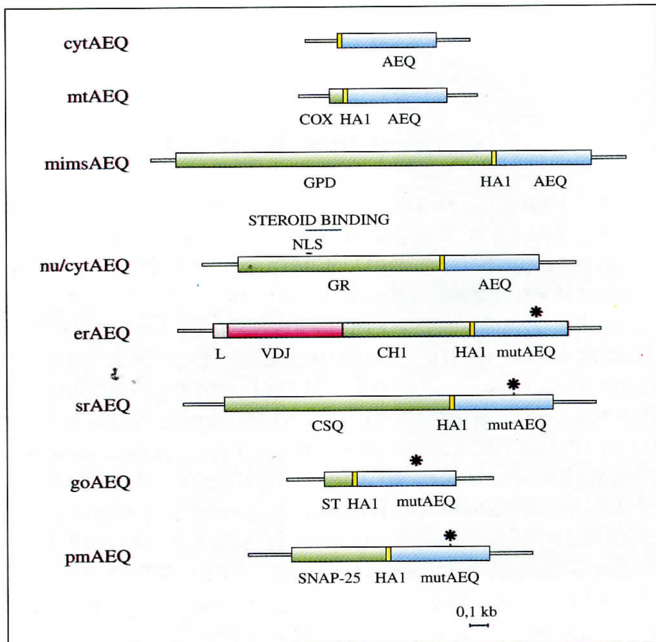

Figure 3. — Aequorin chimeras targeted to different intracellular compartments. The chimeras represented localize to the cytoplasm (cytAEQ), the mitochondrial matrix (mtAEQ), the mitochondrial intermembrane space (mimsAEQ), the cytosol or nucleus, depending on presence of glucocorticoids (cyt/nuAEQ), the endo- and sarcoplasmic reticulum (erAEQ and srAEQ, respectively), the Golgi apparatus (goAEQ) and the subplasmalemma region (pmAEQ). An asterisk within the aequorin portion designates the D119A mutation (mutAEQ). Abbreviations are as follows: HA1, hemagglutinin epitope (for immunolocalization); COX 8, N-terminal fragment of subunit 8 of cytochrome c oxidase; GPD, glycerol phosphate dehydrogenase; L-VDJ-CH1, domains of an Igg2b heavy chain; CSQ, calsequestrin; ST-tm, sialyltransferase transmembrane domain; SNAP-25, synaptosomal-associated protein; NLS, nuclear localisation signal; SB, steroid binding domain; GR, glucocorticoid receptor.

fusion of the aequorin cDNA with that encoding a truncated metabotropic glutamate receptor (mgluR1). The encoded chimeric protein, however, remains trapped on the surface of the ER and Golgi apparatus, with the aequorin polypeptide facing the cytoplasmic surface of these organelles. The cytoplasmic signal revealed by this chimeric aequorin is indistinguishable from that of a cytoplasmic aequorin, but it has the advantage of being membrane bound and excluded from the nucleus.

Mitochondria (mtAEQ)

mtAEQ was the first targeted aequorin generated in the laboratory, which has been successfully employed to measure the $[Ca^{2+}]$ of the mitochondrial matrix of various cell types. This construct includes the targeting presequence of subunit VIII of human cytochrome c oxidase fused to the aequorin cDNA.[4]

Mitochondrial intermembrane space (mimsAEQ)

For the delivery of aequorin to the mitochondrial intermembrane space (MIMS), we exploited the characteristics of another mitochondrial protein. Glycerol phosphate dehydrogenase (GPD) is an enzyme present in the mitochondrial inner membrane with a C-terminal domain protruding into the MIMS. To target aequorin to this space, we fused the photoprotein to the C-terminal portion of GPD, thus maintaining aequorin's C- terminus unaltered, since it is essential for its luminescent properties.[9]

Nucleus (nuAEQ)

Two constructs are presently available. The first consists in a hybrid cDNA coding for aequorin and the nuclear localization signal of the glucocorticoid receptor (excluding the hormone binding domain). The expressed protein is constitutively located in the nucleus[5]. The second construct contains a much larger portion of the same receptor, including the hormone binding domain. In the presence of glucocorticoids, the chimeric protein is translocated to the nucleus, while in its absence it is predominantly cytoplasmic. The advantage of this construct is that it allows the measurement of the cytoplasmic and nucleo-plasmic $[Ca^{2+}]$ in the same transfected cell population, depending on the addition of the hormone. Obviously, careful controls must be carried out to verify that glucocorticoids do not interfere with the Ca^{2+} response.

Endoplasmic reticulum (erAEQ)

The erAEQ includes the leader (L), the VDJ and Ch1 domains of an Ig2b heavy chain fused at the N-terminus of aequorin. Retention in the ER depends on the presence of the Ch1 domain that is known to interact with high affinity with the luminal ER protein BiP.[6]

Sarcoplasmic reticulum (srAEQ)

The chimera results from the fusion of aequorin with calsequestrin, a protein confined in the terminal cisternae of striated muscle SR.[7]

Golgi (GoAEQ)

To drive the expression of the aequorin in the Golgi lumen, the aequorin cDNA has been fused to the cDNA encoding the transmembrane portion of sialyl-transferase, a resident protein of the lumen of the medium-trans-Golgi[10].

Sub-plasmamembrane region (pmAEQ)

This construct derives from the fusion of the aequorin cDNA with that of SNAP25. The latter protein is part of the neurosecretory machinery and is recruited to the inner surface of the plasma membrane after palmitoylation of specific cysteine residues.[8]

To expand the range of Ca^{2+} sensitivity that can be monitored with the different targeted aequorins we have also employed in many of our constructs a mutated form of the photoprotein (asp119 → ala). This point mutation affects specifically the second EF hand motive of wild type aequorin.[6] The affinity for Ca^{2+} of this mutated aequorin is about 20 fold lower than that of the wild type photoprotein. Chimeric aequorins with the mutated isoform are presently available for the cytoplasm, the mitochondrial matrix, the ER and SR, the Golgi apparatus and the sub plasmamembrane region.

Aequorin expression

Although in a few cases the aequorin cDNA has been microinjected, the most commonly employed method to obtain expression of the recombinant protein is transfection. The transfection procedures mainly depends on the cell type employed. The calcium phosphate procedure is by far the simplest and less expensive and it has been used successfully to transfect a number of cell lines, including HeLa, L929, L cells, Cos 7, A7r5 and PC12 cells, as well as primary cultures of neurons and skeletal muscle myotubes. Other transfection procedures have been also employed, such as liposomes, the gene gun and electroporation. Viral constructs for some aequorins are also available.

After expression, the recombinant apoprotein must be reconstituted into functional aequorin. This can be accomplished by incubating transfected cells with the chemically synthesised prosthetic group, coelenterazine. Coelenterazine is freely permeable across cell membranes and reconstitution may occur within all intracellular compartments to which the photoprotein has been targeted.

Luminescence detection

The aequorin detection system (Figure 4) is based on the use of a low noise photomultiplier placed in close proximity (2-3 mm) of aequorin expressing cells. The cell chamber, which is on the top of a hollow cylinder, is adapted to fit 13-mm diameter coverslip. The volume of the perfusing chamber is kept to a minimum (about 200 ml). The chamber is sealed on the top with a coverslip, held in place with a thin layer of silicon. Cells are continuously perfused via a peristaltic pump with medium thermostated via a water jacket at 37°C. The photomultiplier (EMI 9789 with amplifier-discriminator) is kept in a dark box and cooled at 4°C. During manipulations on the cell chamber, the photomultiplier is protected from light by a shutter. During aequorin experiments, the shutter is opened and the chamber with cells is placed in close proximity of the photomultiplier. The output of the amplifier-discriminator is captured by an EMIC600 photon-counting board in an IBM compatible microcomputer and stored for further analysis. In order to calibrate the crude luminescent signal in terms of $[Ca^{2+}]$ an algorithm has been developed that takes into account the instant rate of photon emission and the total number of photons that can be emitted by the aequorin of the sample[13]. In order to obtain the latter parameter, at the end of each experiment the cells are lysed by perfusing them with a hyposmotic medium containing 10 µM $CaCl_2$ and a detergent (100 µM digitonin) in order to discharge all the aequorin that was not consumed during the experiment.

Analysis of Ca^{2+} response after agonist stimulation in living HeLa cells

Figure 2 shows the traces of the $[Ca^{2+}]$ measurement obtained in HeLa cells transfected with the aequorin probes targeted to cytosol, nucleus, mitochondrial matrix, ER lumen, mitochondrial intermembrane space and Golgi apparatus lumen. The coverslips with the cells were transferred to the luminometer chamber and recording was started.

In our experiments we have induced variations of $[Ca^{2+}]$ by stimulating the cells with an agonist, (hista-

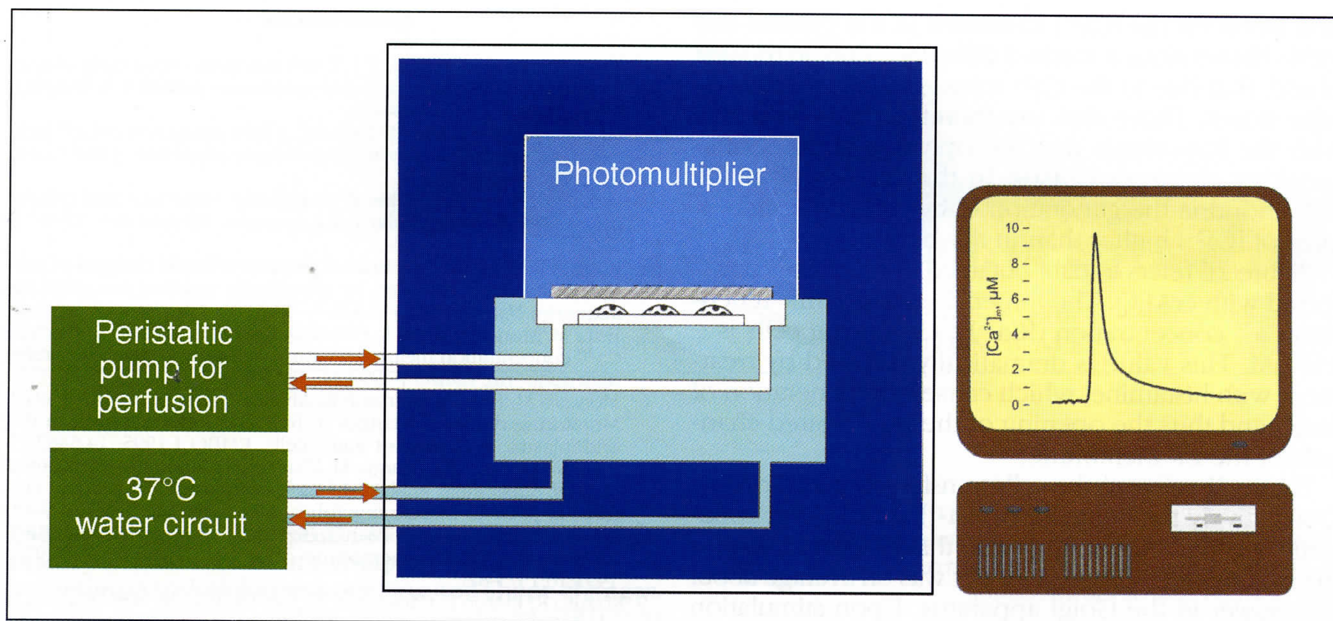

Figure 4. — Schematic representation of the aequorin measuring system. Cells expressing functional aequorin probe are incubated in a perfusion chamber, at 37°C, in close proximity to a photon-counting tube. The complete assemblage is kept in the dark to minimize extraneous signals. Acquisition of the data and subsequent calculations to transform light emission into $[Ca^{2+}]$ are performed by a dedicated computer.

mine 100 µM) added to the KRB (Krebs-Ringer modified buffer: 125 µM NaCl, 5 µM KCl, 1 µM Na_3PO_4, 1 µM MgSO4, 5.5 µM glucose, 20 µM HEPES, pH 7.4, 37°C). Histamine, by acting on a receptor coupled to Gq stimulation (hence PLC activation and production of inositol 1,4,5 triphosphate, IP_3) causes a sustained variation of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) by 2 mechanisms: Ca^{2+} release from the intracellular stores, triggered by IP3, and Ca^{2+} influx through the capacitative Ca^{2+} channels of the plasma membrane, i.e. those opened by the emptying of intracellular Ca^{2+} stores.

Figure 2A shows that cytAEQ reveals, upon histamine stimulation of HeLa cells, a transient rise in $[Ca^{2+}]$c. After the peak (~2.5 µM), which is mostly contributed by the release of Ca^{2+} from intracellular stores, $[Ca^{2+}]_c$ declines to a sustained plateau, due to the influx from the plasma membrane channels, and returns to basal values upon agonist washout.

Figure 2B refers to the cells transfected with nu/cytAEQ. Upon stimulation with the InsP3-generating agonist histamine, nuclear $[Ca^{2+}]_n$ surges to a peak approximately 2.5 µM, and then rapidly reaches a plateau of approximately 0.4 µM, which declines slowly under continuous agonist stimulation. The kinetic behavior and the absolute $[Ca^{2+}]$ values before or after stimulation are very similar of those observed in the cytosol.

Figure 2C shows the trace $[Ca^{2+}]$ measurements in HeLa cells expressing mtAEQ. Upon stimulation with an InsP3-generating agonist, mitochondria undergo a much higher $[Ca^{2+}]$ change than the bulk cytosol. The mitochondrial Ca^{2+} concentration, ($[Ca^{2+}]_m$), reaches a maximum value of approximately 10µM, then rapidly returns to values close to basal. The high rate of Ca^{2+} mitochondrial uptake after stimulation with agonists coupled to $InsP_3$ generation, was completely unexpected, given the low affinity of the mitochondrial uniporter. Our explanation for this finding is that the efficiency of mitochondrial Ca^{2+} accumulation depends on the generation of domains of high $[Ca^{2+}]$ close to the source of the Ca^{2+} rise[17] which could be sensed by neighboring mitochondria. More recently, this hypothesis has been further supported by the demonstration of the close proximity between the ER and mitochondria.[9]

Figure 2D shows the trace $[Ca^{2+}]$ measurements in HeLa cells expressing mimsAEQ. The MIMS shows a comparable behavior of the cytosol; the peak, however, is significantly higher (~3.5 µM), and then declines to the same steady-state plateau. The compar-

ison between the [Ca^{2+}] variations in the cytosol and MIMS shows since a marked difference only in the first phase, that due to the Ca^{2+} release from the intracellular stores. These data are therefore in accordance with the hypothesis that the opening of the $InsP_3$-sensitive channels causes, in the proximity of the mitochondria the generation of cytosolic microdomains, of [Ca^{2+}] higher than in the bulk cytosol.

Figure 2E refers to data obtained in HeLa cells transfected with erAEQ. The lumenal endoplasmic reticulum Ca^{2+} concentration ($[Ca^{2+}]_{er}$) in resting cells is ~ 450 µM. This value is dramatically reduced by treatment with histamine, which causes the generation of $InsP_3$ and thus the opening of the $InsP_3$-gated channels of the ER membrane.

Figure 2F refers to the cells transfected with GoAEQ. Qualitatively the behavior of [Ca^{2+}] in the Golgi apparatus was remarkably similar to that of the ER, but for the maximum level observed that was on average about 30% lower in the Golgi apparatus. Upon stimulation with histamine, a large, rapid decrease in $[Ca^{2+}]_{Golgi}$ is observed. The amplitude and rate of the [$cation^{2+}$] drop caused by histamine in the Golgi is slightly smaller and slower than that observed in the ER.

In summary, recombinant aequorin is capable of being targeted to virtually any subregion of the cell. With the large number of transfection methods currently available, essentially all cell types are amenable to the use of such chimeras. Once specifically localized, this chemiluminescent probe is capable of providing precious information regarding Ca^{2+} concentration and dynamics.

New chimeras may be constructed with proteins of interest (receptors, channels), thereby opening the possibility of a more precise molecular targeting that may allow eventually of monitoring of [Ca^{2+}] in any cellular microenvironments.

Recently the group of Tsien [18] has introduced a new class of GFP mutant as Ca^{2+} indicators named cameleons, kangaroo and pericam, molecularly engineered GFP proteins capable of coupling the advantages of aequorins in terms of selective targeting to the high signal characteristics of fluorescent molecules.

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