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Targeted Recombinant Aequorins: Tools for Monitoring [Ca^{2+}] in the Various Compartments of a Living Cell

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ABSTRACT In the last decade, the study of Ca^{2+} homeostasis within organelles in living cells has been greatly enhanced by the utilisation of a recombinant Ca^{2+}-sensitive photoprotein, aequorin. Aequorin is a Ca^{2+} sensitive photoprotein of a coelenterate that, in the past, was widely employed to measure Ca^{2+} concentration in living cells. In fact, the purified protein was widely used to monitor cytoplasmic [Ca^{2+}] changes in invertebrate muscle cells after microinjection. However, due to the time-consuming and traumatic procedure of microinjection, the role of aequorin in the study of Ca^{2+} homeostasis remained confined to a limited number of cells (giant cells) susceptible to microinjection. Thus, in most instances, it was replaced by the fluorescent indicators developed by Roger Tsien and coworkers. The cloning of aequorin cDNA [Inouye et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:3154–3158] and the explosive development of molecular biology offered new possibilities in the use of aequorin, as microinjection has been replaced by the simpler technique of cDNA transfection. As a polypeptide, aequorin allows the endogenous production of the photoprotein in cell systems as diverse as bacteria, yeast, slime molds, plants, and mammalian cells. Moreover, it is possible to specifically localise it within the cell by including defined targeting signals in the amino acid sequence. Targeted recombinant aequorins represent to date the most specific means of monitoring [Ca^{2+}] in subcellular organelles. In this review, we will not discuss the procedure of aequorin microinjection and its use as purified protein but we will present the new advances provided by recombinant aequorin in the study of intracellular Ca^{2+} homeostasis, discussing in greater detail the advantages and disadvantages in the use of this probe. *Microsc. Res. Tech. 46:380–389, 1999.*

INTRODUCTION

The wide expansion of molecular biology techniques, with the possibility of modifying and expressing exogenous cDNAs in virtually all cell types, has been responsible for the growing success in the use of protein probes in cell biology. Two groups of reporter proteins are currently employed, derived from the wide variety of bioluminescent organisms: the chemiluminescent proteins (e.g., the different types of luciferases and aequorin), and the fluorescent proteins (e.g., the green fluorescent protein, GFP). The latter will not be discussed here, but the explosive success of GFP in cell biology clearly demonstrates their potential usefulness.

Luminescent proteins emit light, usually in response to changes in physiological parameters, such as the Ca^{2+} or ATP concentration. The use of luminescence in cellular biology is usually associated with an excellent signal-to-noise ratio, since no such protein is naturally present in mammalian cells and thus the background signal is negligible. Conversely, because the signal reflects only the light emitted from the recombinant protein, it is low and must be collected either by integrating the signal coming from at least 10^3–10^4 cells or, at the single cell level, using single cell imaging analysis instruments of high sensitivity and high cost.

The isolation of aequorin cDNA (Inouye et al., 1985) has elicited a renewed interest in this Ca^{2+} probe. On the one hand, by opening the possibility of recombinant expression, it greatly simplified the loading procedure in mammalian cells. On the other, it opened new exciting applications: such as that of specifically targeting a Ca^{2+} probe to different cellular locations, and thus monitoring selectively the dynamic of [Ca^{2+}] with unprecedented spatial resolution. We will summarize here the strategies employed for targeting aequorin to the various cell compartments, thus constructing organelle-specific Ca^{2+} probes. We will then provide the relevant information for using these probes, presenting the experimental procedures and reviewing the advantages and the disadvantages, compared to the classical Ca^{2+} probes, the fluorescent dyes.

The fluorescent probes, developed in the early eighties by Tsien and colleagues (Tsien et al., 1982), are the most common Ca^{2+} indicators. Thanks to them, and to

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the development of optical and computer technology, which allowed the introduction of highly sophisticated microscopes and imaging systems, the temporal and spatial complexity of Ca^{2+} homeostasis was widely explored.

When a Ca^{2+} channel opens, a highly concentrated Ca^{2+} environment forms around its mouth and then dissipates. This means that in the cytoplasm of the cell, there are continuous fluctuations of $[Ca^{2+}]$ depending on the spatio-temporal patterns of opening and closing Ca^{2+} channels. This complexity is functionally important, given that various, often contrasting, cell functions can be activated by Ca^{2+} ions (Pozzan et al., 1994). In this context, the possibility of directly studying Ca^{2+} homeostasis in the organelles, such as mitochondria or endoplasmic reticulum, involved in the regulation of Ca^{2+} homeostasis appeared an important, albeit difficult, task.

Furthermore, the study of organelle Ca^{2+} homeostasis is important to better understand the physiology of these organelles. $[Ca^{2+}]$ inside organelles, such as mitochondria, endoplasmic reticulum, and nucleus, modulates their specific functions, e.g., in the mitochondria three enzymes of the Krebs cycle are Ca^{2+} dependent (McCormack et al., 1990), in the ER Ca^{2+} controls the process of synthesis, assembly, and folding of the proteins (Sitia and Meldolesi, 1992), and in the nucleus Ca^{2+} is involved in the regulation of gene expression, breakdown of nuclear envelope, and apoptosis (Collart et al., 1991; Gaido and Cidlowski, 1991; Tombes et al., 1992).

The fluorescent indicators are not ideally suited for monitoring $[Ca^{2+}]$ in defined cellular compartments. They are, in fact, mostly trapped in the cytoplasm of intact cells, given that the esterases are primarily located in this compartment (Grynkiewicz et al., 1985).

In some cell types they are, to some extent, sequestered within organelles, but this non-specific process represents a nuisance in the measurement of cytosolic Ca^{2+}, rather than a way to measure Ca^{2+} in other compartments.

The recombinant expression of a protein from its cDNA eliminates one of the major limitations to the use of photoproteins, i.e., the need of microinjecting a molecule that cannot freely cross the plasma membrane of living cells. This approach has already been successfully employed in a large variety of cell models, differing both in morphology and embryological origin, such as epithelial cell lines (HeLa, CHO), primary cultures of skeletal muscle, neurons, etc. Secondly, probes, which allow the monitoring of Ca^{2+} concentration within defined organelles, can be obtained by specifically targeting the recombinantly expressed photoprotein. This can be accomplished by constructing chimeric cDNAs, encoding a recombinant polypeptide composed of the Ca^{2+} sensitive photoprotein and a targeting signal (i.e., the amino acid sequence that directs the correct sorting of organelle proteins to the proper intracellular location). Moreover, an aequorin based Ca^{2+} probe could be, in principle, targeted through suitable chimeras, to specific proteins, which are modulators or effectors of the Ca^{2+} signal.

Aequorin, as isolated from the jellyfish *Aequorea*, is composed of a 21 kDa apoprotein and of a hydrophobic prosthetic group, coelenterazine (MW ~400 Da). Both constituents need to be present for the Ca^{2+}-triggered light emission to occur.

Recombinant aequorin, as expressed in a variety of cell types, includes only the protein moiety, but the holoprotein can be reconstituted, either in vitro or in vivo, by the simple addition of the prosthetic group. When Ca^{2+} ions bind to the high-affinity Ca^{2+} binding sites (homologous to the sites present in other Ca^{2+} binding proteins, such as calmodulin), aequorin undergoes an irreversible reaction, in which a photon is emitted (Fig.1). The rate of the reaction depends on the $[Ca^{2+}]$ 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 relationship between $[Ca^{2+}]$ and the fractional rate of consumption of the photoprotein.

Figure 2 shows this relationship: at physiological conditions of pH, temperature, ionic strength, light emission is proportional to the 2^{nd}–3^{rd} power of $[Ca^{2+}]$. The fractional rate of aequorin consumption is 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 $[Ca^{2+}]$ (L_{max}) (Blinks et al., 1978; Cobbold, 1980; Ridgway and Ashley, 1967; Ridgway et al., 1977).

APPLICATIONS

Advantages and Disadvantages

Today, recombinant aequorin is the tool of choice for selectively monitoring Ca^{2+} concentration in the intracellular compartments.

As discussed below, one of the main advantages of the use of aequorin as intracellular Ca^{2+} probe is the possibility of targeting it to a specific intracellular compartment and thus of selectively measuring the $[Ca^{2+}]$ concentration in that district.

This, together with other aequorin characteristics, opens the possibility of a variety of applications. In this section we will analyze aequorin' s properties in more detail to better understand its applications.

Very Low Ca^{2+} Buffering Capacity. Although the binding of Ca^{2+} by aequorin may, in principle, affect intracellular Ca^{2+} homeostasis, this undesired effect is less relevant than with fluorescent indicators. Recombinant aequorin is usually expressed at a concentration in the range of 0.1–1 μM, which is 2–3 orders of magnitude lower than dyes, and thanks to the excellent signal-to-noise ratio, this amount of protein is sufficient to carry out Ca^{2+} measurements in cell populations (Brini et al., 1995).

Absence of Chemiluminescent Proteins in Living Mammalian Cells. Due to the low luminescence background of cells and the steepness of the Ca^{2+} response curve of aequorin, minor variations in the amplitude of the agonist-induced $[Ca^{2+}]$ changes can be easily appreciated with aequorin. On the other, if the $[Ca^{2+}]$ is dishomogeneous it tends to bias the average towards the higher values.

Possibility of Measuring a Wide Spectrum of $[Ca^{2+}]$ Values. It is clearly evident from Figure 2 that aequorin can accurately measure $[Ca^{2+}]$ ranging from 0.5 μM to 10 μM, i.e., reaching concentrations at which most fluorescent indicators are saturated. Thanks to these properties, it is possible to estimate the large cytosolic Ca^{2+} concentration rises, ($[Ca^{2+}]_c$), which oc-

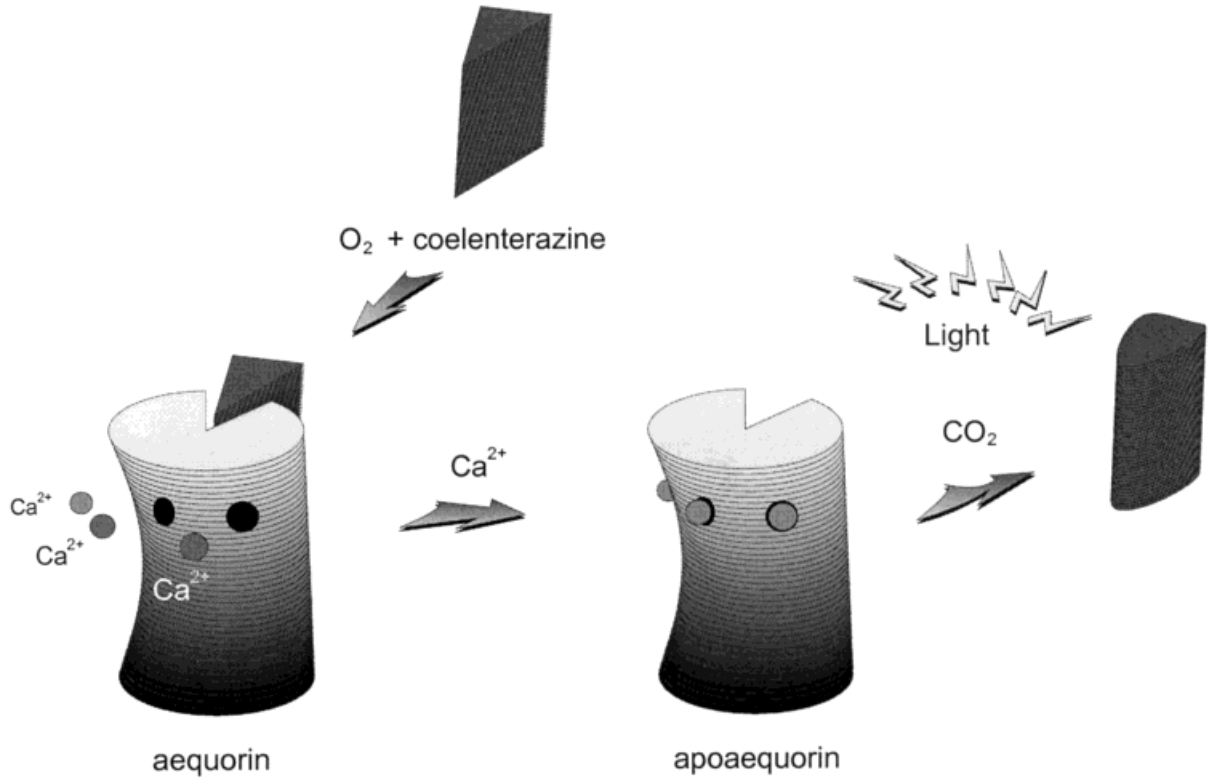

Fig. 1. Schematic model of the irreversible reaction of aequorin. When Ca^{2+} ions bind to the EF-hand binding sites of the reconstituted aequorin, a photon is emitted and that molecule of aequorin is irreversibly discharged.

cur, for example, in neurons (Fig. 3) (Brini et al., 1995). Moreover by introducing points-mutations in the Ca^{2+} binding sites (Kendall et al., 1992), using surrogate cations such as Sr^{2+} (Montero et al., 1995), and/or modified prosthetic groups (Barrero et al., 1997) (see Measurament of Ca^{2+} Concentration in the Intracellular Compartments With High Calcium), the sensitivity of the recombinant photoprotein can be further reduced, and thus the $[Ca^{2+}]$ can be monitored in intracellular compartments endowed with high $[Ca^{2+}]$ (e.g., the lumen of the ER, Golgi apparatus) (Montero et al., 1995; Pinton et al., 1998). On the other hand, aequorin is not ideally suited for measuring $[Ca^{2+}]$ in the resting condition; in fact, the calibrated signal at low $[Ca^{2+}]$ appears noisy, as expected from the steep Ca^{2+} response curve of aequorin.

As far as the disadvantages are concerned:

1. Low light emission. One of the major limitations of recombinant aequorin as an alternative method to the fluorescent probes is that only one photon can be emitted by an aequorin molecule in contrast to the fluorescent dyes (where up to 10^4 photons can be emitted by a single molecule). Moreover, only a small fraction of the total aequorin pool (varying, in a typical physiological experiment, from 10^{-7} to 10^{-2}) emits its photon every second. This means that, out of the 10^4–10^5 molecules/cell of a typical aequorin transfection, light emission will vary from nil to 1,000 photons at most. This is not a major limitation in population studies, (typically 10^3–10^4 cells) as in this case, the light

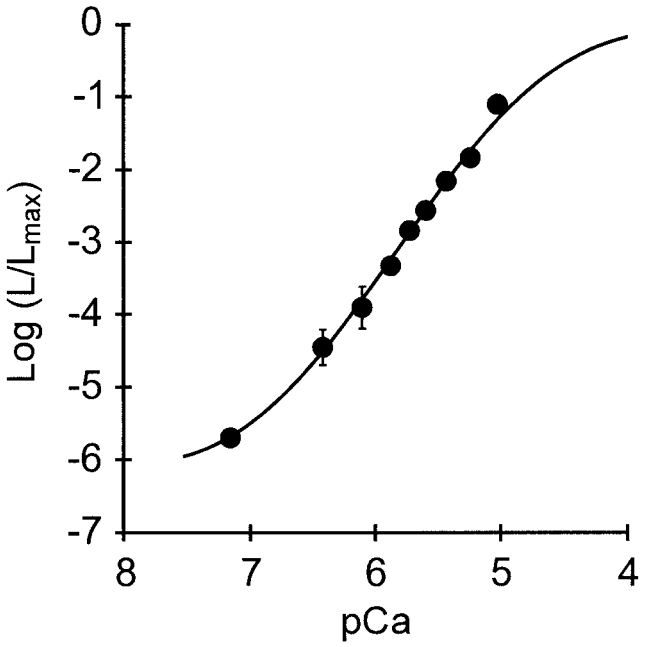

Fig. 2. $[Ca^{2+}]$ response curve of recombinant expressed aequorin. L, Light emission immediately after exposing recombinant expressed aequorin to solution with known $[Ca^{2+}]$; L_{max}, integral of aequorin counts from the mixing to the end of the experiment, i.e., after aequorin consumption with excess Ca^{2+}.

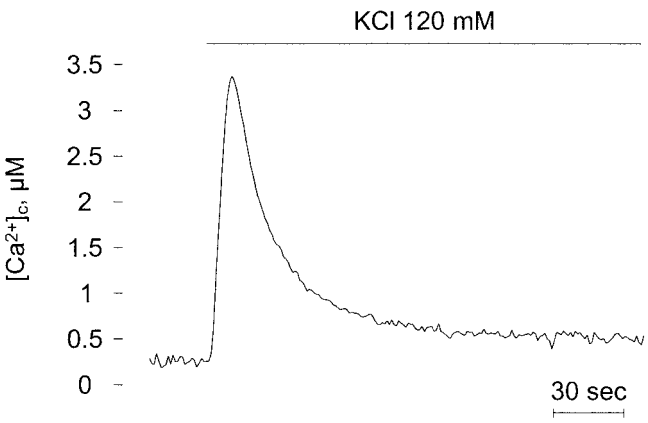

Fig. 3. $[Ca^{2+}]_c$ monitoring with cytAEQ in primary cultures of neurons. After reconstitution of the photoprotein, the coverslip with the cells were transferred to the thermostatted (37°C) chamber of the luminometer and perfused with modified Krebs-Ringer buffer (KRB) (see the text). Where indicated, the cells were depolarized with KCl 120 mM added to KRB.

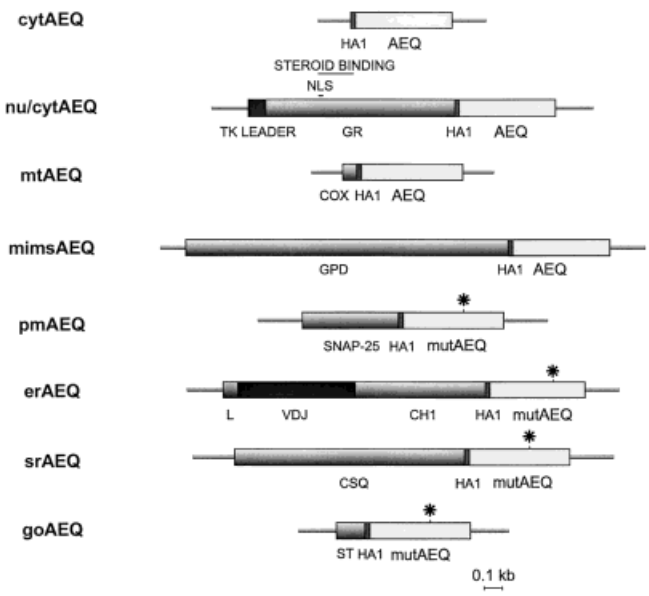

Fig. 4. Schematic representation of the chimeric aequorins. The details of the targeting strategies are explained in the text. cytAEQ, cytosolic aequorin; nu/cytAEQ, nucleus/cytosol hormone dependent shuttling aequorin chimera; mtAEQ, matrix mitochondrial targeted aequorin; mimsAEQ, inner mitochondrial membrane targeted aequorin; pmAEQ, inner subplasmalemmal space targeted aequorin; erAEQ, endoplasmic reticulum targeted aequorin; srAEQ, sarcoplasmic reticulum targeted aequorin; goAEQ, Golgi apparatus targeted aequorin. Coding and non coding regions of the cDNA are indicated as boxes and lines, respectively. Asterisk indicates the position of the Asp119→Ala mutation of the aequorin cDNA (mutAEQ). HA1, epitope tag.

signals vary from 20–30 photons (at resting $[Ca^{2+}]$) to 10^5 photons/sec.

Conversely, single cell imaging requires very high expression and special apparatuses (Rutter et al., 1996) and is endowed with lower spatial and temporal resolution than that obtained with fluorescent indicators.

2. Difficulties in transfection. Another point to be remembered is that some cell lines may be quite resistant to transfection (although a wide range of procedures are now available, ranging from calcium phosphate co-precipitation to liposomes, electroporation, and particle guns); in many cases, finding the appropriate transfection protocol may be time-consuming. Moreover after transfection, time must be allowed before carrying out Ca^{2+} measurements.

Although we detected reasonable aequorin expression 4 hours after transfection, this interval can prevent the use of aequorin in primary cultures with limited life span.

3. Difficulties in reconstitution. The recombinantly expressed aequorin does not emit light if the cells are not supplemented externally with the coenzyme. Reconstitution with coelenterazine is necessary to generate the functional photoprotein and usually this procedure requires at least 1 hour. Conversely, the loading of fluorescent dyes is easier and takes 20–30 minutes. Moreover, in the case of the aequorins trapped in the lumen of the ER or targeted to a high Ca^{2+} compartment, it is necessary to first reduce the luminal or local Ca^{2+} concentration and then reconstitute the functional photoproteins. This means that it is necessary manipulate the cells with ionophores or inhibitors of Ca^{2+} ATPases or other agents to open Ca^{2+} channels (Montero et al., 1995; Pinton et al., 1998).

Measurements of Ca^{2+} Concentration in Various Intracellular Compartments

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 targeted to the mitochondria (Rizzuto et al., 1992, 1993, 1994), the nucleus (Brini et al., 1993, 1994), the endoplasmic (Montero et al., 1995) and sarcoplasmic reticulum (Brini et al., 1997), the subplasmalemma region (Marsault et al., 1997), the Golgi apparatus (Pinton et al., 1998), and the outer surface of the Ca^{2+} impermeable inner mitochondrial membrane (Rizzuto et al., 1998). The details of the construction of the chimeric cDNAs prepared for targeting aequorin to the various intracellular compartments (Fig. 4) are described in Experimental Procedures. Here we just remind you of the different targeting mechanisms pursued by the cells to distribute the different proteins to the different locations. Thanks to molecular biology techniques, today it is possible to utilize these strategies to target exogenous recombinant proteins.

Mitochondrial Proteins. Only thirteen polypeptides are encoded from the mitochondrial genome. All the others are encoded by the nucleus, translated by cytosolic ribosomes and then imported into the organelle. In most cases, the recognition as a mitochondrial protein and the import into the organelle depends on a signal sequence rich in basic residues which is located at the N-terminus of the protein, and is then removed after import by proteases of the mitochondrial matrix (Hartl et al., 1989).

Nucleus. The transport of large proteins or of small proteins whose function is exclusively nuclear is an active process and requires suitable nuclear localization signals (NLSs). Nuclear localization could be tran-

sient and this could happen because the protein may change its conformation and expose only in some cases the NLS. NLS does not have a preferential position in the sequence and it is not removed after the import as the mitochondrial sequence (Kalderon et al., 1984; Dingwall and Laskey, 1991).

Endoplasmic Reticulum. The proteins are retained in this compartment because they usually contain a double targeting signal. A hydrophobic sequence located at the N-terminus of the protein causes its translation on membrane-bound ribosomes and its insertion in the endoplasmic reticulum. The retention of proteins within the endoplasmic reticulum depends on a second signal, which retrieves the proteins escaping into later compartments. The best characterised of these signals is the tetra-peptide KDEL, localized at the C-terminus of the protein (Munro and Pelham, 1987).

All the sequences and the mechanisms utilized by the cells to distribute their proteins to these three organelles are well known and it is possible, in theory, simply by adding these sequences, to direct aequorin to mitochondria, nucleus, and endoplasmic reticulum. This was true for the first two compartments, but not for the targeting to the ER. In fact, the modification of the C-terminus of the photoprotein drastically impairs its chemiluminescent properties. It was thus necessary to devise an alternative strategy, which takes advantage of a retention signal localized in a different portion of the protein. In particular, aequorin was fused to the signal that allows the heavy chain of immunoglobulins to be retained within the endoplasmic reticulum until assembly with the light chains occurs (Sitia and Meldolesi, 1992).

Other Cell Compartments. Alternative strategies could also be employed when the targeting sequence responsible for the localization of the resident proteins are not as well known as those of the compartments described above. In the case of aequorin targeted to the plasma membrane, to the Golgi apparatus, to the sarcoplasmic reticulum, and to the mitochondrial intermembrane space, in fact, we took advantage of the possibility of fusing the aequorin cDNA to the cDNA coding for a resident protein. Certainly this approach could be extended to construct targeted aequorins to monitor Ca^{2+} homeostasis in the different microenvironments of the cell.

Measurement of Ca^{2+} Concentration in the Intracellular Compartments With High Calcium

Aequorin is well suited for measuring $[Ca^{2+}]$ between 0.5 and 10 μM. However, in some intracellular compartments or regions the $[Ca^{2+}]$ is much higher (e.g., the lumen of ER and of the SR, near Ca^{2+} channels, pumps, etc.). In this case, it is necessary to reduce the affinity of aequorin. There are at least three different ways to reduce the Ca^{2+} affinity of the photoprotein:

1. introduce a point mutation in one or more of the Ca^{2+} binding sites (Kendall et al., 1992),
2. use surrogate cations that elicit a slower rate of photoprotein consumption than Ca^{2+} itself, e.g., Sr^{2+} (Montero et al., 1995)
3. use modified prosthetic groups that decrease the affinity of aequorin for Ca^{2+} (Barrero et al., 1997).

These three approaches can be combined to obtain a clear shift in the Ca^{2+} affinity of the photoprotein. Because of the cooperativity between the three Ca^{2+} binding sites of aequorin, the point mutation we generated (Asp119→Ala), which affects the second EF-hand domain, produces a mutated aequorin which can measure $[Ca^{2+}]$ in the range of 10 to 100 μM. The range of aequorin sensitivity can be expanded further by employing divalent cations other then Ca^{2+}. We utilize Sr^{2+}, which is known to be a suitable Ca^{2+} surrogate. Sr^{2+} permeates across the Ca^{2+} channels and is actively transported, although with lower affinity, by both the plasma membrane and the sarco-endoplasmic Ca^{2+} ATPases (SERCAs). Altogether, by combining the two approaches, a mutated aequorin-probe can measure $[cation^{2+}]$ ranging from the μM to the mM range.

In order to avoid possible discrepancies between the behavior of the two cations and to provide a more accurate estimate of the $[Ca^{2+}]$ in compartments with high $[Ca^{2+}]$, it is now possible to use Ca^{2+} and a low-affinity coelenterazine analogous (coelenterazine n) (Barrero et al., 1997).

The lower rate of aequorin-coelenterazine n consumption at high Ca^{2+} concentration allows the monitoring of millimolar concentration of Ca^{2+} for relatively long periods of time.

Possibility of Coexpression 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 the overexpression of the heterologous protein, followed by the study of the molecularly modified cell. In particular, channels, receptors, and other proteins involved in Ca^{2+} signalling have been expressed in a variety of cell types (Camacho and Lechleiter, 1993; He et al., 1997), with the goal of studying their function and/or their coupling mechanisms. $[Ca^{2+}]$ is a key parameter to be monitored in these types of studies, and fluorescent dyes have traditionally been employed. However, when clones are generated that express a foreign protein, functional data rely on the comparison with the parental line and/or control clones, and the high variability between cell clones complicates the interpretation of the results. On the other hand, when the cells are transiently transfected, there is obviously no way to load the dye selectively in the transfected cells (which usually represent about 10–20% of total); the signal originates from the whole cell population, and the effect of the gene manipulation can be easily overlooked. Single cell imaging must, therefore, be performed, and, even if positive cells can be easily identified via a cotransfected reporter (e.g., GFP), a large statistical analysis is necessary for minimizing variabilities in cell responses. In contrast, recombinant aequorin can be cotransfected with the gene of interest, taking advantage of the fact that in the commonly employed transfection protocols, the DNA is taken up by the same subset of cells (Brini et al., 1995).

EXPERIMENTAL PROCEDURES

Construction of the Chimeric Aequorin Probes

As previously stated, the expression of recombinant photoprotein inside the organelles is possible due to the

proteinaceus nature of aequorin and to biomolecular technology. In fact, since the intracellular fate of a protein depends on specific targeting sequences (necessary and sufficient for the correct localization), it's possible to construct a chimera protein that includes any one of these signal sequences and a heterologous protein. In this way, the protein probe will acquire a cellular distribution that is exclusive of the selected organelle/region. In the specific case, chimeric cDNAs were constructed by fusing in frame the aequorin cDNA with the cDNA of a mammalian protein, which includes specific targeting sequences. These chimeras, recombinantly expressed in living cells, preserve the ability to measure the $[Ca^{2+}]$ and are localized only in the cellular compartment defined by the targeting sequence. In Figure 4, the strategies used for the construction of our probes are shown.

Cytosol (cytAEQ). Recombinantly expressed wild-type aequorin is exclusively cytosolic and, therefore, it doesn't require any modification to measure $[Ca^{2+}]$ in this compartment. The only modification made has been at the 5' end of the coding region to include the HA1 epitope tag in order to verify the correct localization of the probe (Brini et al., 1995).

Nucleus (nu/cytAEQ). The chimeric cDNA encodes a fusion protein composed of a portion of the glucocorticoid receptor GR, including the nuclear localization signal (NLS), the hormone binding domain, and the HA1 epitope tagged aequorin.

This polypeptide is localized in the cytosol in the absence of glucocorticoids, and translocated to the nucleus upon hormone treatment.

Mitochondria (mtAEQ). A mitochondrially-targeted aequorin was constructed by fusing in frame the cDNA of the HA1-aequorin (HA1/AEQ) to the cDNA of the mitochondrial presequence derived from subunit VIII of the human cytochrome c oxidase (Rizzuto et al., 1992). The encoded polypeptide is composed of the cleavable mitochondrial presequence, six aminoacids of the mature mitochondrial polypeptide, plus the whole photoprotein.

Mitochondrial Intermembrane Space (MimsAEQ). The cDNA encoding HA1-tagged aequorin was fused in frame with that encoding glycerol phosphate deydrogenase (GPD), an integral protein of the inner mitochondrial membrane, with a large C-terminal tail protruding on the outer side of the membrane, i.e., the intermembrane space.

Plasma Membrane (pmAEQ). The chimeric cDNA was constructed by fusing in frame the cDNA encoding SNAP-25 and that encoding HA1-tagged aequorin (Marsault et al., 1997). The targeting of aequorin to the subplasmalemmal space was based on the fusion with SNAP-25, a protein that is synthesized on free ribosomes and recruited to the inner surface of the plasma membrane after palmitoylation of specific cysteine residues.

Endoplasmatic Reticulum (erAEQ). In our chimera, retention in the endoplasmic reticulum (ER) does not depend on the typical C-terminal sequence KDEL, but on the presence of the CH1 domain at the N-terminus of aequorin. The encoded polypeptide includes the leader sequence (L), the VDJ and CH1 domains of an Igγ2b heavy chain and aequorin at the C-terminus. The chimera is retained in the ER because of the binding of the CH1 domain to the resident endogenous ER protein BiP (Sitia and Meldolesi, 1992). This binding is displaced only by the light chain, while in absence of this (i.e., not in plasma cells) the CH1 domain is expected to be selectively retained in the ER. The aequorin used to measure $[Ca^{2+}]$ in this compartment was also modified by introducing an epitope tag and a point mutation (Asp119→Ala), which reduces the Ca^{2+} affinity of the photoprotein (Montero et al., 1995).

Sarcoplasmic reticulum (srAEQ). This chimera results from the fusion of aequorin to the endogenous SR protein calsequestrin (CS), thus including the relevant targeting information for addressing the heterologous polypeptide to the SR.

Golgi Apparatus (goAEQ). The chimeric cDNA was constructed by fusing in frame the cDNAs encoding the transmembrane portion of sialyltransferase (ST) and HA1-tagged aequorin. The fusion polypeptide is retained in the Golgi apparatus because of the 17 amino acid membrane-spanning domain of the sialyltransferase, a resident protein of the lumen of the *trans*-Golgi and *trans*-Golgi network (TGN).

Cell culture and Transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) in 75 cm^2 Falcon flasks. In transient expression experiments, the cells were seeded onto glass coverslips (diameter 13 mm) and allowed to grow to 50% confluence. At this stage, transfection with the appropriate plasmid (4μg) using the calcium phosphate procedure, was carried out as previously described (Rizzuto et al., 1995). The cells were analyzed for aequorin expression 36 hours after transfection. The calcium phosphate procedure was used also in the transfection of primary cultures of cortical neurons and skeletal myotubes.

Other transfection procedures could be employed for cell types in which calcium phosphate proved to be quite ineffective, i.e., we use electroporation with GH3 and Ins1 cells and PEI (polyethylenimine) with Jurkat.

For all probes produced, we carried out the immunocytochemical analysis in order to investigate the correct localization. Figure 5 shows, for example, the correct sorting of the aequorin chimeras targeted to the cytosol, mitochondria, and endoplasmic reticulum, respectively.

Aequorin Reconstitution

Recombinantly expressed aequorin is only the polypeptide portion of the photoprotein. In order to measure $[Ca^{2+}]$ changes, it is necessary to reconstitute it in the active form. To do this, it is sufficient to add the prosthetic group, coelenterazine, to the incubation medium for a few hours in order to allow the diffusion of coelenterazine through the cellular membranes. Coelenterazine employed in our experiments is a synthetic hydrophobic prosthetic group.

In order to reconstitute the cytosol, nucleus, and mitochondrially targeted aequorin, the coverslip with the transfected cells is incubated with 5μM coelenterazine for 2 hours in DMEM supplemented with 1% FCS at 37°C in 5% CO_2 atmosphere. Then, the coverslip is directly transferred to the luminometer chamber, where it is perfused with KRB saline solution (Krebs-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM

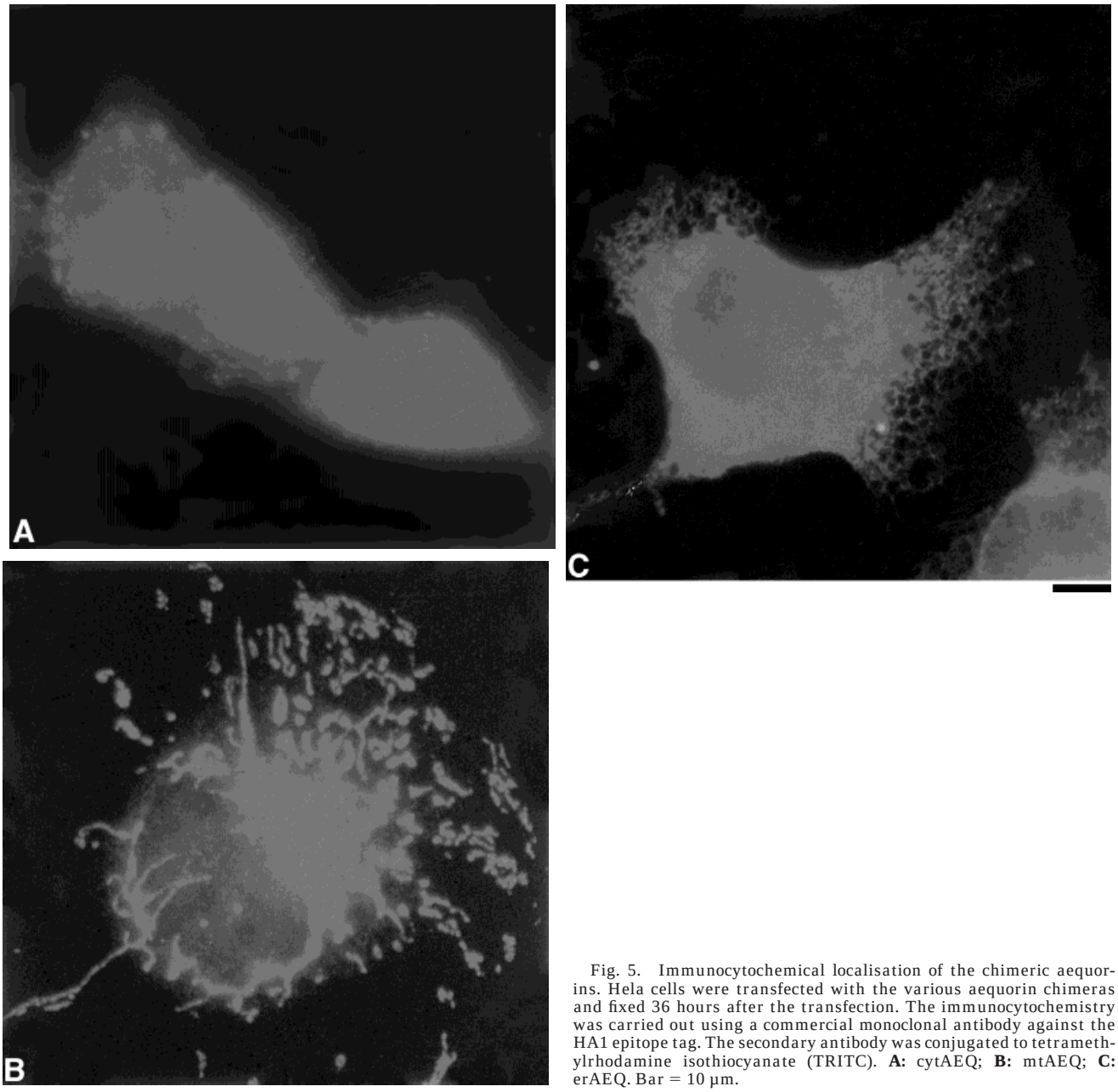

Fig. 5. Immunocytochemical localisation of the chimeric aequorins. Hela cells were transfected with the various aequorin chimeras and fixed 36 hours after the transfection. The immunocytochemistry was carried out using a commercial monoclonal antibody against the HA1 epitope tag. The secondary antibody was conjugated to tetramethylrhodamine isothiocyanate (TRITC). **A:** cytAEQ; **B:** mtAEQ; **C:** erAEQ. Bar = 10 µm.

Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37°C).

Concerning the ER, SR, and Golgi apparatus targeted aequorin, in order to obtain a sufficient amount of active protein, it is necessary to make a drastic reduction of the lumenal Ca^{2+} before the reconstitution. Otherwise, the high rate of aequorin consumption strongly counteracts the process. To this end, the cells are incubated for 1 hour at 4°C, in KRB supplemented with coelenterazine (5µM), the Ca^{2+} ionophore ionomycin (5µM), and 600µM EGTA. After this incubation, cells are extensively washed with KRB supplemented with 2% BSA and 1 mM EGTA before being transferred to the luminometer chamber.

In contrast to cytAEQ, the reconstitution of the subplasmamembrane region targeted aequorin is reduced drastically in media containing physiological Ca^{2+} concentrations (1 mM). In order to increase the amount of active pmAEQ, the transfected cells are transferred to modified KRB supplemented with 100 µM EGTA and 5 µM coelenterazine. After 45 minutes of incubation at 37°C, the coverslip is transferred to the luminometer chamber.

Measurements

Aequorin Measuring System. The aequorin measuring apparatus is schematically shown in Figure 6 and it was built on the model of that described by

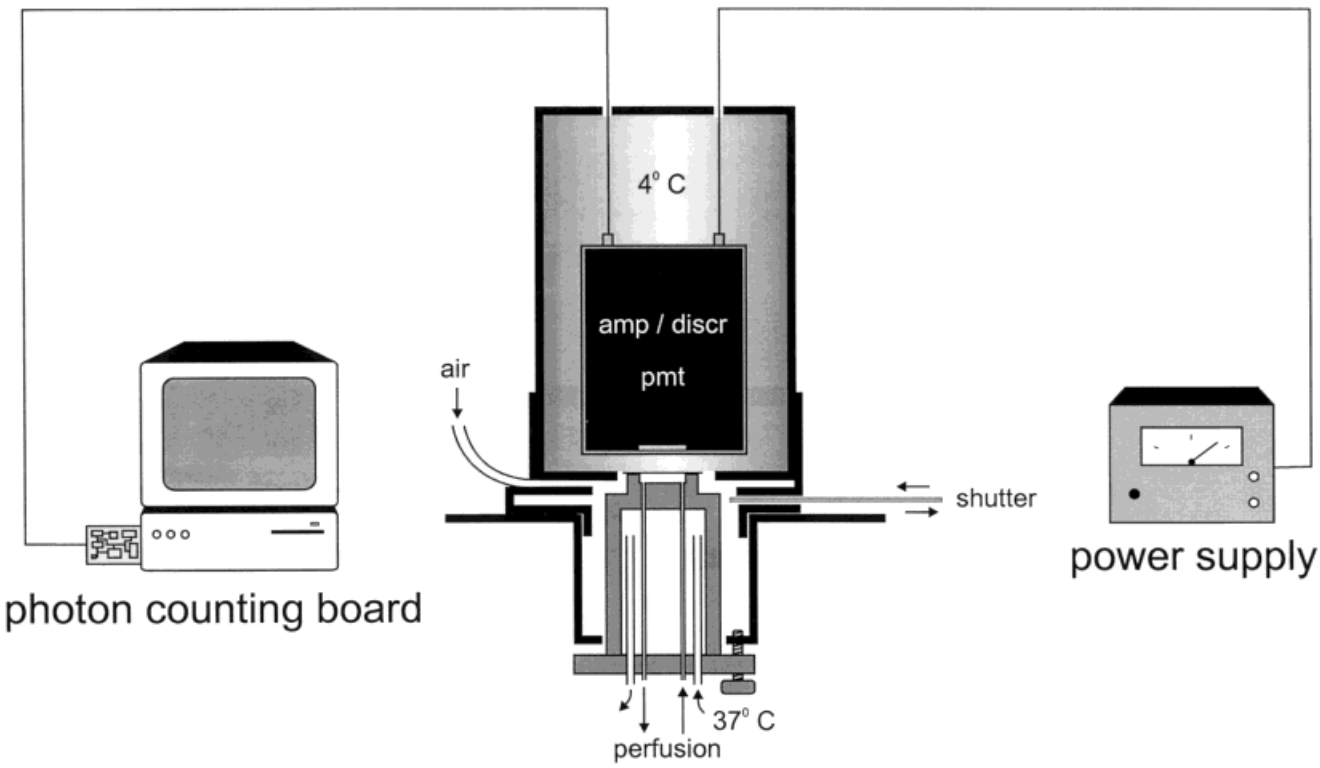

Fig. 6. The aequorin measuring system. pmt=photomultiplier; amp/discr = amplifier/discriminator. The photomultiplier is kept in a cooled dark box, the cell chamber at room temperature. During manipulations on the cell chamber, the photomultiplier is protected from light by a shutter. During aequorin measurements, the shutter is open and the chamber held in position (in close proximity of the photopmultiplier window) by a O-ring. An amplifier-discriminator is built in the photomultiplier housing. The photon counting board allows the storing of the data in the computer memory for further analyses.

Cobbold and Lee (1991). Therefore, only the principles are hereinafter described, referring to the above article for any technical details. The 13 mm coverslips with the cells are placed inside a 37°C thermostatted perfusion chamber (diameter 15 mm, height 2 mm). During aequorin measurement, the cell chamber is held in close proximity to a photomultiplier, which is kept in a dark refrigerated box (4°C). An amplifier discriminator is built in the photomultiplier housing; the pulses generated by the discriminator are captured by a Thorn EMI photon counting board, installed in an IBM-compatible computer. The board allows the storing of the data in the computer memory for further analyses. During the experiment, the thermostatted chamber is continuously perfused with buffer via a Gilson peristaltic pump. In order to obtain a more rapid equilibration of the perfusing medium inside the chamber, the flow rate can be increased during the changes of medium. At the end of each experiment, the cells are lysed by perfusing them with a hyposmotic medium containing 10 mM $CaCl_2$ and a detergent (100μM digitonin) in order to discharge all the aequorin that was not consumed during the experiment. This allowed us to estimate the total aequorin content, which must be known for converting the luminescence data into $[Ca^{2+}]$ values. At the end of the experiment, the luminescence data can be directly converted into free $[Ca^{2+}]$, by means of a program that estimates the $[Ca^{2+}]$ to which the photoprotein is exposed, based on the calculated fractional rate of consumption of aequorin and the Ca^{2+} response curve at physiological conditions of pH, ionic strength, and $[Mg^{2+}]$ (Brini et al., 1995).

Aequorin Measurements

For the monitoring of $[Ca^{2+}]$, the cells were plated onto the coverslips and transfected with the aequorin chimera expression plasmids. Thirty-six hours after transfection, aequorin was reconstituted by adding the prosthetic group. After reconstitution, the coverslip with the cells was transferred to the measuring apparatus, and aequorin light output was monitored. During the experiment, the cell monolayer was continuosly perfused with buffer. Agonists and drugs were added to the same medium. Figure 7 reports an example of the calibration of the light (photons) data into $[Ca^{2+}]$ values; in Figure 7 (top), the light emission from a monolayer of HeLa cells transiently expressing mitochondrial aequorin is shown, and in Figure 7 (bottom) the conversion of the light data into $[Ca^{2+}]_m$ values carried out by a computer algorithm as described above is depicted. Figure 8 shows the traces of the $[Ca^{2+}]$ measurement obtained in HeLa cells transiently transfected with the aequorin probes targeted to cytosol, mitochondrial matrix, and ER lumen. The coverslips with the cells were transferred to the luminometer chamber, and recording was started. Figure 8A refers to the cells transfected with cytAEQ. In our experiments, we have induced a rise in $[Ca^{2+}]_c$ by stimulating the

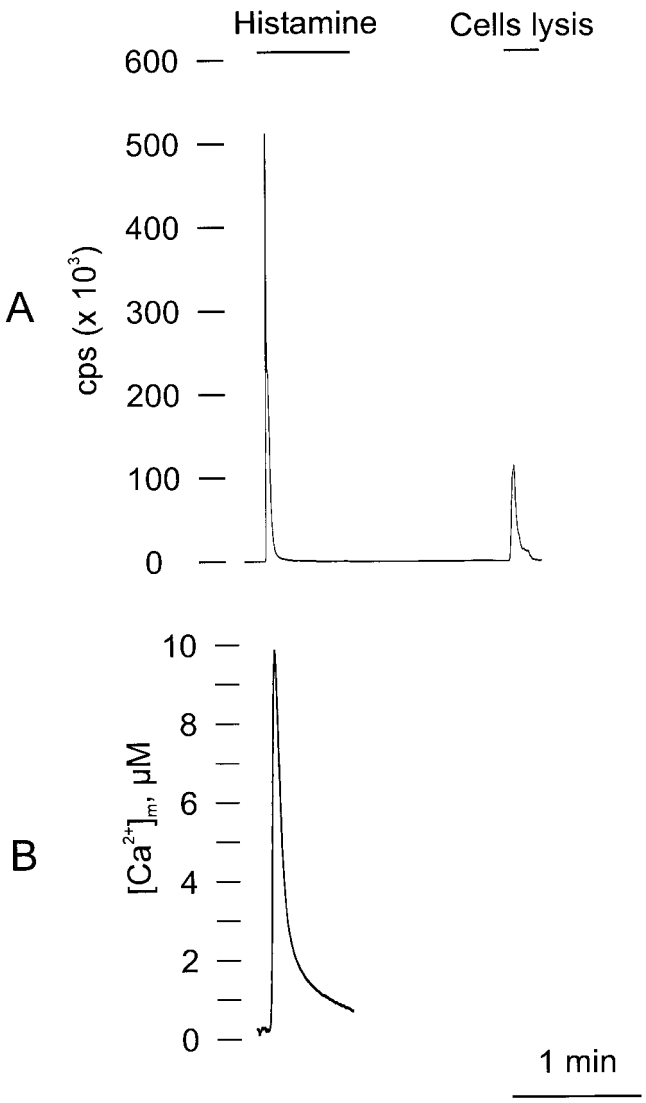

Fig. 7. Calibration of the light data in $[Ca^{2+}]$ values. Light emission (**A**) and calculated $[Ca^{2+}]_m$ values (**B**), from a monolayer of HeLa cells expressing mtAEQ. Where indicated, the cells were challenged with 100µM histamine. At the end of the experiments the cells were lysed with 100µM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to estimate the total photoprotein. cps, counts per second.

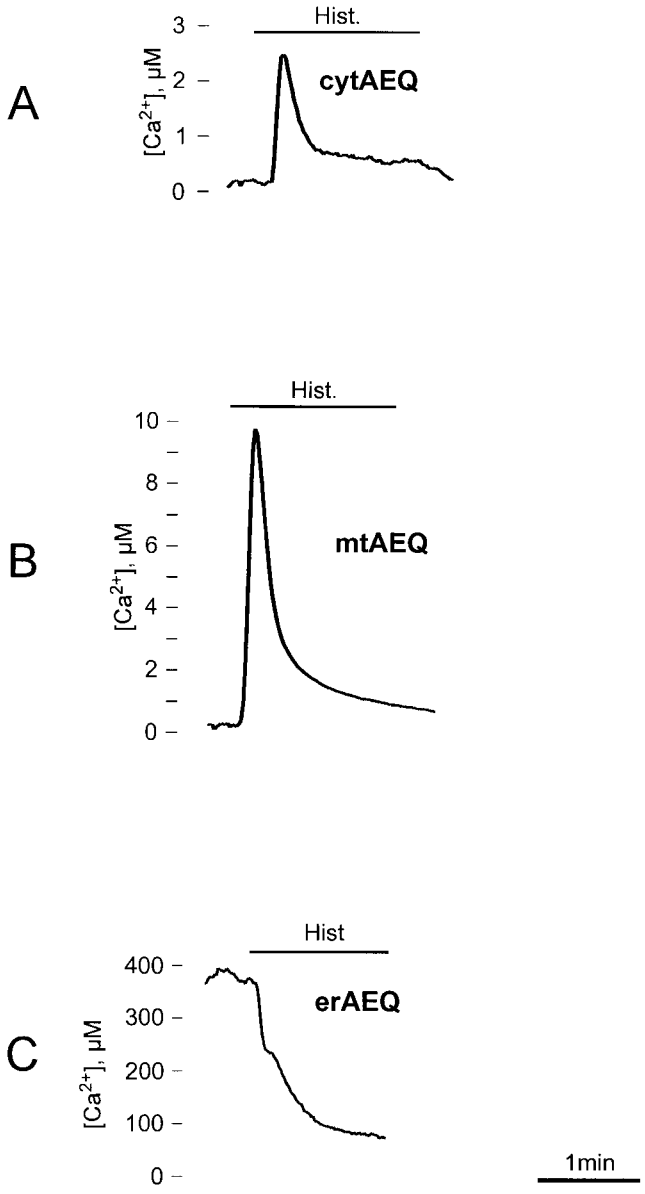

Fig. 8. A typical experiment. Measure of $[Ca^{2+}]$ obtained with the probes cytAEQ (**A**), mtAEQ (**B**), and erAEQ (**C**). Where indicated, the cells were challenged with 100µM histamine (see the text).

cells with an agonist (histamine 100µM) added to the KRB buffer, which is able to induce variation of $[Ca^{2+}]_c$ by two mechanisms: the release of the Ca^{2+} from the intracellular stores, through $InsP_3$ generation, and the Ca^{2+} influx through the plasma membrane channels. Figure 8A shows that cytAEQ reveals, upon histamine stimulation of HeLa cells, a transient rise in $[Ca^{2+}]_c$. After the peak (2.5 ± 0.3µM, n = 10), 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 8B shows the trace $[Ca^{2+}]$ measurements in HeLa cells expressing mtAEQ. Upon stimulation with an $InsP_3$-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 (9.7 ± 1.5µM, n = 10), 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 (Rizzuto et al., 1993), 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 (Rizzuto et al., 1998). Figure 8C refers to data obtained in HeLa cells transfected with erAEQ. The lumenal endoplasmic reticu-

lum Ca^{2+} concentration ($[Ca^{2+}]_{er}$) at steady state is $\sim$ 450 μM (440 ± 50μM, n = 5). 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.

PERSPECTIVES

We have described a method for measuring Ca^{2+} concentrations in defined cellular compartments that is based on the specific targeting and recombinant expression of the coelenterate protein aequorin in mammalian cells. We have successfully followed this approach in several cell types (HeLa, fibroblast, endothelial cells, neurons, myotubes, etc.) and for several subcellular locations (mitochondria, nucleus, endoplasmic reticulum, subplasmalemma region, Golgi apparatus, and mitochondrial intermembrane space) thus indicating that it may represent a general method for designing subcellular Ca^{2+} probes.

New chimeras may be constructed with proteins of interest (receptors, channels), thereby opening the possibility of a more precise "molecular" targeting that may allow, in principle, monitoring of Ca^{2+} concentrations in highly defined cellular microenviroments. Two appealing applications appear to be an important goal for the future: single-cell imaging and the generation of transgenic animals expressing chimera aequorins. As to the first, the low-light emission of photoproteins represents a major problem; work needs to be done, both in the development of suitable apparatuses designed for low-light imaging and in the enhancement of the levels of aequorin expression. As to transgenic animals, they should open the fascinating possibility of studying Ca^{2+} homeostasis in situ.

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