have a phosphotyrosine at BCR position 177 that mediates the interaction with the Ras activator Grb-2/SOS.

Concluding Remarks

Artificial oligomerization of tyrosine kinases by fusion to GST or BCR could result in their activation. Molecular mechanisms may involve disruption of negative regulation or mimic physiological activation as in the case of receptor tyrosine kinases. In the BCR-ABL oncogene, oligomerization of ABL is necessary but not sufficient for its full activation. The artificial activation of tyrosine kinases of as yet unknown biological function may potentially give a clue to uncover their functional roles.

[33] Recombinant Aequorin as Tool for Monitoring Calcium Concentration in Subcellular Compartments

By VALERIE ROBERT, PAOLO PINTON, VALERIA TOSELLO, ROSARIO RIZZUTO, and TULLIO POZZAN

Aequorin as Ca^{2+} Indicator

Aequorin is a 21-kDa protein, isolated from jellyfish of the genus *Aequorea*, that emits blue light in the presence of calcium. The aequorin originally purified from the jellyfish is a mixture of different isoforms called "heterogeneous aequorin."[1] In its active form the photoprotein includes an apoprotein and a covalently bound prosthetic group, coelenterazine. As schematically shown in Fig. 1, when calcium ions bind to the three high-affinity E-F hand sites, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and emission of light. Although this reaction is irreversible, *in vitro* an active aequorin can be obtained by incubating the apoprotein with coelenterazine in the presence of oxygen and 2-mercaptoethanol. Reconstitution of an active aequorin (expressed recombinantly) can also be obtained in living cells by simple addition of coelenterazine to the medium. Coelenterazine is highly hydrophobic and has been shown to permeate cell membranes of various cell types, ranging

[1] O. Shimomura, *Biochem. J.* **234,** 271 (1986).

0076-6879/00 $30.00

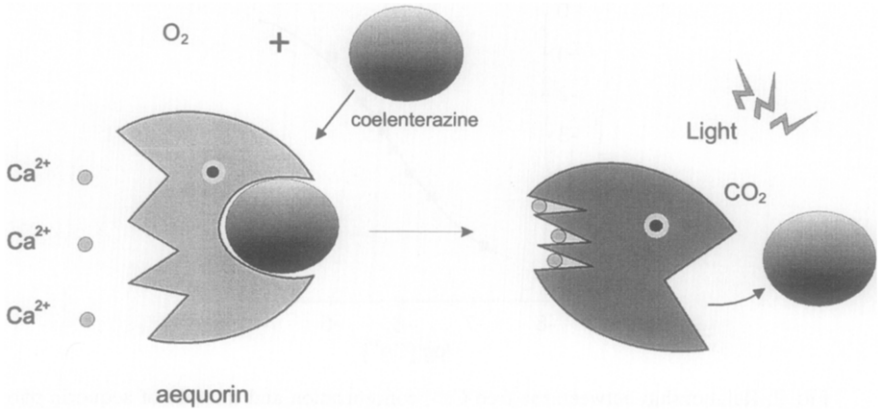

FIG. 1. Scheme of the Ca^{2+}-induced photon emission process.

from the slime mold *Dictyostelium discoideum* to mammalian cells and plants.[2]

Different coelenterazine analogs have been synthesized that confer to the reconstituted protein specific luminescence properties.[3] A few synthetic analogs of coelenterazine are now commercially available from Molecular Probes (Eugene, OR).

The possibility of using aequorin as a calcium indicator is based on the existence of a well-characterized relationship between the rate of photon emission and the free Ca^{2+} concentration. For physiological conditions of pH, temperature, and ionic strength, this relationship is more than quadratic in the $[Ca^{2+}]$ range of 10^{-5}–10^{-7} *M*. The presence of three Ca^{2+}-binding sites in aequorin is responsible for the high degree of cooperativity, and thus for the steep relationship between photon emission rate and $[Ca^{2+}]$ (Fig. 2). $[Ca^{2+}]$ can be calculated from the formula L/L_{max}, where L is the rate of photon emission at any instant during the experiment and L_{max} is the maximal rate of photon emission at saturating $[Ca^{2+}]$. The rate of aequorin luminescence is independent of $[Ca^{2+}]$ at high ($>10^{-4}$ *M*) and low $[Ca^{2+}]$ ($<10^{-7}$ *M*). However, as described below in more detail, it is possible to expand the range of $[Ca^{2+}]$ that can be monitored with aequorin. Although aequorin luminescence is not influenced by either K^+ or Mg^{2+} (which are the most abundant cations in the intracellular environment and thus the most likely source of interference in physiological experiments), both ions are competitive inhibitors of Ca^{2+}-activated luminescence. Aequorin

[2] T. Pozzan, R. Rizzuto, P. Volpe, and J. Meldolesi, *Physiol. Rev.* **74,** 595 (1994).
[3] O. Shimomura, B. Musicki, Y. Kishi, and S. Inouye, *Cell Calcium* **14,** 373 (1993).

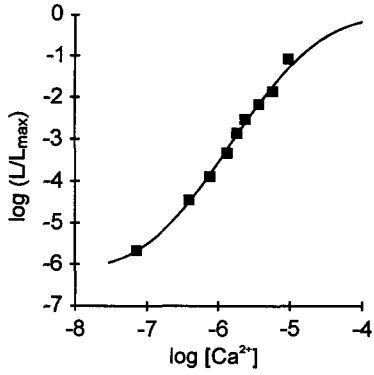

FIG. 2. Relationship between the free Ca^{2+} concentration and the rate of aequorin photon emission.

photon emission can also be triggered by Sr^{2+}, but its affinity is about 100-fold lower than that of Ca^{2+}, while lanthanides have high affinity for the photoprotein (e.g., are a potential source of artifacts when they are used to block Ca^{2+} channels). pH was also shown to affect aequorin luminescence but at values below pH 7. Because of the characteristics described above, experiments with aequorin need to be done under well-controlled conditions of pH and ionic concentrations, notably of Mg^{2+}.

Recombinant Aequorins

For a long time the only reliable way to introduce aequorin into living cells was to microinject the purified protein. This procedure is time consuming and laborious, and requires special care in handling of the purified photoprotein. Alternative approaches (scrape loading, reversible permeabilization, etc.) have been rather unsuccessful. These procedures are not described in this chapter. The cloning of the aequorin gene[4] has opened the way to recombinant expression and thus has largely expanded the applications of this tool for investigating Ca^{2+} handling in living cells. In particular, recombinant aequorin can be expressed not only in the cytoplasm, but also in specific cellular locations by including specific targeting sequencing in the engineered cDNAs.

Extensive manipulations of the N terminal of aequorin have been shown not to alter the chemiluminescence properties of the photoprotein and its Ca^{2+} affinity. On the other hand, even marginal alterations of the C terminal

[4] S. Inouye, N. Masato, Y. Sakaki, Y. Takagi, T. Miyaka, S. Iwanaga, T. Miyata, and F. I. Tsugi, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3154 (1985).

either abolish luminescence altogether or drastically increase Ca^{2+}-independent photon emission.[5] As demonstrated by Watkins and Campbell,[6] the C-terminal proline residue of aequorin is essential for the long-term stability of the bound coelenterazine. For these reasons, all targeted aequorins synthesized in our laboratory include modifications of the photoprotein N terminal. Three targeting strategies have been adopted.

1. Inclusion of a minimal targeting signal sequence with the photoprotein cDNA. This strategy was initially used to design the mitochondrial aequorin and was also followed to synthesize an aequorin localized in the nucleus and in the lumen of the Golgi apparatus.
2. Fusion of the cDNA encoding aequorin to that of a resident protein of the compartments of interest. This approach has been used to engineer aequorins localized in the sarcoplasmic reticulum (SR), in the nucleoplasm and cytoplasm (shuttling between the two compartments depending on the concentration of steroid hormones), on the cytoplasmic surface of the endoplasmic reticulum (ER) and Golgi, and in the subplasmalemma cytoplasmic rim.
3. Addition to the aequorin cDNA of sequences that encode polypeptides that bind to endogenous proteins. This strategy was adopted to localize aequorin in the ER lumen.

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

Chimeric Aequorin cDNAs

Below we briefly describe the constructs produced in our laboratory (Fig. 3). A few other constructs have been produced in other laboratories and are not dealt with in detail here.

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.[7] An alternative construct is also available that is located on the outer surface of the ER and of the Golgi apparatus.[8] This construct

[5] M. Nomura, S. Inouye, Y. Ohmiya, and F. I. Tsuji, *FEBS Lett.* **295,** 63 (1991).
[6] N. J. Watkins and A. K. Campbell, *Biochem. J.* **293,** 181 (1993).
[7] M. Brini, F. De Giorgi, M. Murgia, R. Marsault, M. L. Massimino, M. Cantini, R. Rizzuto, and T. Pozzan, *Mol. Biol. Cell.* **8,** 129 (1997).
[8] R. Rizzuto, P. Pinton, W. Carrington, F. S. Fay, K. E. Fogarty, L. M. Lifshitz, R. A. Tuft, and T. Pozzan, *Science* **280,** 1763 (1998).

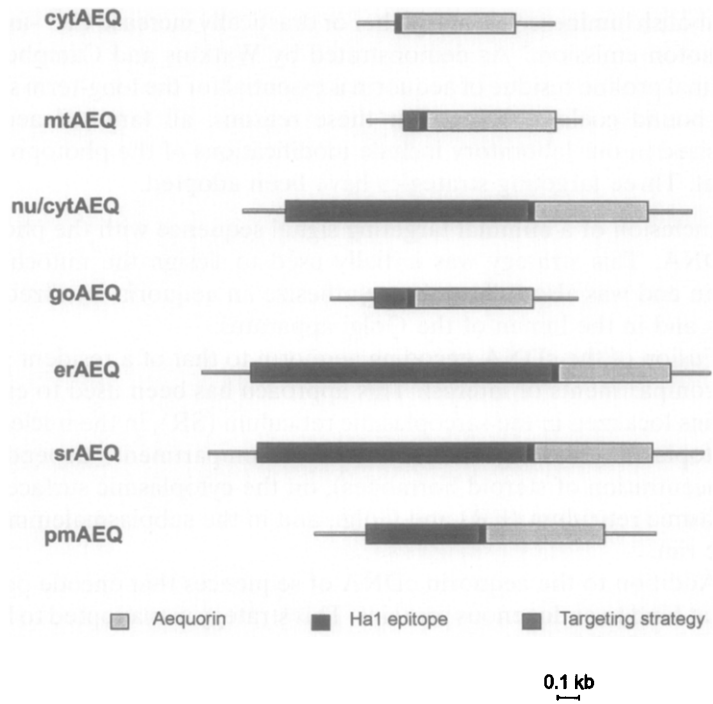

FIG. 3. Schematic representation of aequorin chimeras.

was intended to drive the localization of aequorin to the inner surface of the plasma membrane, given that it derives from the 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, and it 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.[9]

[9] R. Rizzuto, A. W. Simpson, M. Brini, and T. Pozzan, *Nature (London)* **358,** 325 (1992).

Nucleus (*nuAEQ*): Two nuAEQ constructs are presently available. The first consists of a hybrid cDNA encoding aequorin and the nuclear localization signal of the glucocorticoid receptor (excluding the hormone-binding domain). The expressed protein is constitutively located in the nucleus.[10] 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 measurement of the cytoplasmic and nucleoplasmic [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.[7]

Golgi (*goAEQ*): To drive the expression of aequorin in the Golgi lumen, the aequorin cDNA has been fused to the cDNA encoding the transmembrane portion of sialyltransferase, a resident protein of the lumen of the medium-*trans*-Golgi.[11]

Endoplasmic Reticulum (*erAEQ*): The erAEQ includes the leader (L) and the VDJ and Ch1 domains of an IgG_{2b} heavy chain fused at the N terminus of aequorin. Retention in the ER depends on the presence of the Ch1 domain, which is known to interact with high affinity with the lumenal ER protein BiP.[12]

Sarcoplasmic Reticulum (*srAEQ*): The srAEQ chimera results from the fusion of aequorin with calsequestrin, a protein confined in the terminal cisternae of striated muscle SR.[7]

Subplasma Membrane Region (*pmAEQ*): The pmAEQ 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.[13]

To expand the range of Ca^{2+} sensitivity that can be monitored with the various 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 motif of wild-type aequorin.[14] The

[10] M. Brini, M. Murgia, L. Pasti, D. Picard, T. Pozzan, and R. Rizzuto, *EMBO J.* **12,** 4813 (1993).

[11] P. Pinton, T. Pozzan, and R. Rizzuto, *EMBO J.* **18,** 5298 (1998).

[12] M. Montero, M. Brini, R. Marsault, J. Alvarez, R. Sitia, T. Pozzan, and R. Rizzuto, *EMBO J.* **14,** 5467 (1995).

[13] R. Marsault, M. Murgia, T. Pozzan, and R. Rizzuto, *EMBO J.* **16,** 1575 (1997).

[14] J. M. Kendall, G. Sala-Newby, V. Ghalaut, R. L. Dormer, and A. K. Campbell, *Biochem. Biophys. Res. Comm.* **187,** 1091 (1992).

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 subplasma membrane region.

Cell Preparation and Transfection

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. Various expression plasmids have been employed, some commercially available (pMT2, pcDNA1 and -3), others kindly provided by colleagues. It is not the purpose of this chapter to describe all the transfection procedures, the choice of which mainly depends on the cell type employed. The calcium phosphate procedure is by far the simplest and least expensive and it has been used successfully to transfect a number of cell lines, including HeLa, L929, L, Cos 7, A7r5, and PC12 cells, as well as primary cultures of neurons and skeletal muscle myotubes. Other transfection procedures have also been employed, such as liposomes, the "gene gun," and electroporation. Viral constructs for some aequorins are also available.[15,16]

In this section we briefly describe the calcium phosphate procedure, a simple and convenient transfection method for HeLa cells and rat myotubes. Whenever a new cell type is investigated, we always start with this procedure and only if problems arise do we adopt more sophisticated approaches.

HeLa Cells

One day before the transfection step, HeLa cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) are plated on a 13-mm round coverslip at 30–50% confluence. Just before the transfection procedure, cells are washed with 1 ml of fresh medium.

Skeletal Muscle Myotubes

Primary cultures of skeletal muscle are prepared from the posterior limb muscles of newborn rats. Primary cultures are initiated from satellite cells obtained after four successive treatments with 0.125% (w/v) trypsin in phosphate-buffered saline. Cells are then plated in DMEM supplemented

[15] C. M. Rembold, J. M. Kendall, and A. K. Campbell, *Cell Calcium* **21,** 69 (1997).
[16] M. T. Alonso, M. J. Barrero, E. Carnicero, M. Montero, J. Garcia Sancho, and J. Alvarez, *Cell Calcium* **24,** 87 (1998).

with 10% FCS in 10-cm petri dishes at a density of 10^6 cells/ml. After 1 hr of incubation at 37°, nonadherent cells are collected and seeded at a density of 2×10^5 cells onto 13-mm coverslips coated with gelatin [2% (w/v) in phosphate-buffered saline (PBS)]. The myoblasts are then transfected during the second day of culture, i.e., before fusion occurs.

Calcium Phosphate Transfection Procedure

The following stock solutions need to be prepared and kept at −20° until use.

$CaCl_2$, 2.5 *M*

HEPES-buffered solution (HBS): 280 m*M* NaCl, 50 m*M* HEPES, Na_2HPO_4 (pH 7.12), 1.5 m*M*

Tris–EDTA (TE): 10 m*M* Trizma base, EDTA (pH 8), 1 m*M*

All solutions are sterilized by filtration, using 0.22-μm pore size filters.

For one coverslip, 5 μl of 2.5 *M* $CaCl_2$ is added to the DNA dissolved in 45 μl of TE. Routinely, 4 μg of DNA is used to transfect one coverslip. The solution is then mixed by vortexing with 50 μl of HBS and incubated for 20 to 30 min at room temperature. The cloudy solution is then added directly to the cell monolayer. Eighteen to 24 hr after addition of the DNA, the cells are washed with PBS (two or three times until the excess precipitate is completely removed). Using this protocol the transfected cells are usually between 30 and 50%. Although an optimal transfection is obtained after an overnight incubation, we found that substantial aequorin expression, sufficient for most experimental conditions, is also obtained by incubation for only 6 hr with the calcium phosphate–DNA complex.

Stable clones can also be obtained by cotransfecting with the aequorin cDNA another plasmid encoding resistance to neomycin and then selecting the cells with neomycin at 0.8 mg/ml.[17]

After removing the calcium phosphate precipitate, myoblasts are cultured in DMEM supplemented with 2% (v/v) horse serum (HS) to stimulate the fusion of myoblasts into myotubes. The cell medium is then changed every 2 days. The myotubes can be maintained under these conditions for more than 10 days. The number of myotubes expressing the recombinant aequorin is usually about 50% and the cells continue to express the polypeptide during the whole period of culture.

Localization of Expressed Proteins

An essential aspect of targeted aequorin methodology is the accuracy of the subcellular localization. Although mistargeting has been rarely observed

[17] R. Rizzuto, M. Brini, and T. Pozzan, *Methods Cell. Biol.* **40,** 339 (1994).

with the constructs described above, it cannot be taken for granted that this will not occur in some cell model. We thus suggest that any time a new cell type is employed the subcellular localization be checked by immunocytochemistry (Fig. 4). The inclusion of the HA1 epitope tag in all the constructs described above is thus extremely convenient. Briefly, coverslips of transfected cells are fixed with 4% (v/v) formaldehyde in PBS for 20 min, washed two or three times with PBS and then incubated for 10 min in 50 m*M* NH_4Cl in PBS. Permeabilization of cells is obtained with 0.5% (v/v) Triton X-100 in PBS for 5 min followed by two or three washes with PBS. After a step of saturation of unspecific sites with 1% (w/v) gelatin for at least 30 min, cells are incubated with the anti-HA1 monoclonal antibody 12CA5 at 1:100 dilution for 1 hr in a wet chamber. The cells are

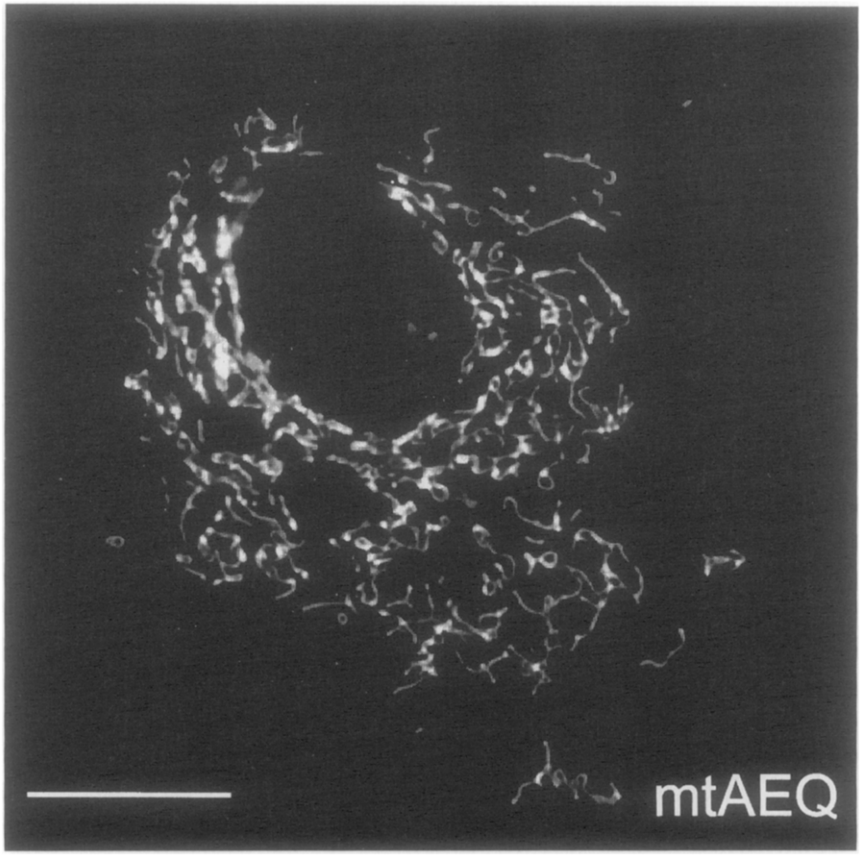

FIG. 4. Immunochemical analysis of mtAEQ localization in HeLa cells. Bar: 10 μm.

then washed with PBS and incubated with fluorescein- or rhodamine-labeled anti-mouse IgG antibodies.

A simple, indirect alternative method to verify the subcellular distribution of the recombinant aequorin is to use plasmids in which the cDNA encoding aequorin is substituted with cDNA encoding green fluorescent protein (GFP). The assumption is that aequorin and GFP behave as passive cargoes and the subcellular localization depends exclusively on the targeting strategy. So far this assumption has been verified for all the constructs described above.

Reconstitution of Functional Aequorin

Once expressed the recombinant aequorin must be reconstituted into the functional photoprotein. This is accomplished by incubating cells with the synthetic coelenterazine for variable periods of time (usually 1–3 hr) and under conditions of temperature and $[Ca^{2+}]$ that depend on the compartment investigated. Practically, coelenterazine is dissolved at 0.5 m*M* in pure methanol as a 100× stock solution kept at −80°. This solution tolerates several freeze–thaw cycles. However, we recommend that the supply of coelenterazine solution be split into small aliquots (50 μl). Coelenterazine must be protected from light.

For compartments with low $[Ca^{2+}]$ under resting conditions (cytosol, mitochondria, and nucleus) the cells transfected with the appropriate recombinant aequorins are simply incubated at 37° in fresh DMEM supplemented with 1% (v/v) FCS and 5 μM coelenterazine. Higher or lower coelenterazine concentrations can also be used, if necessary. Good reconstitution is achieved with 1 hr of incubation, but an optimal reconstitution requires 2–3 hr.

For compartments endowed with high $[Ca^{2+}]$ under resting conditions (ER, SR, and the Golgi apparatus), to obtain good reconstitution and interpretable data it is first necessary to reduce the $[Ca^{2+}]$ in the organelle, otherwise aequorin is immediately consumed after reconstitution and in steady state little functional photoprotein is present in cells. Depletion of Ca^{2+} from the organelles has been (and can be) achieved in different ways. Below we describe a few simple protocols.

Depletion protocol 1: Cells are incubated at 37° for 5 min in KRB solution (Krebs–Ringer modified buffer: 125 m*M* NaCl, 5 m*M* KCl, 1 m*M* Na_3PO_4, 1 m*M* $MgSO_4$, 5.5 m*M* glucose, 20 m*M* HEPES, pH 7.4) supplemented with 3 m*M* EGTA, 10 μM ionomycin, and 10 μM tBuBHQ (an inhibitor of the endosarcoplasmic reticulum Ca^{2+} ATPases). After washing with KRB containing 100 μM EGTA, 5% (w/v) bovine serum albumin,

and 10 μM tBuBHQ, cells are further incubated in the same medium supplemented with 5 μM coelenterazine for 1 hr, but at 4°.

Depletion protocol 2: All conditions are as described above, but ionomycin is omitted and cells are treated instead with an agent (histamine for HeLa cells) that induces the release of Ca^{2+} from the ER through opening of the inositol triphosphate (InsP3) receptors.

Depletion protocol 3: For skeletal muscle myotubes transfected with srAEQ we have designed a slightly different protocol that better preserves the functionality of the cells. Briefly, the depletion of the SR is carried out for 2 min at room temperature in a KRB solution containing 10 mM caffeine (to open the ryanodine receptor) and 30 μM tBuBHQ to prevent refilling of the SR. The cells are then incubated in a KRB solution containing 100 μM EGTA and 5 μM coelenterazine for 1 hr at 4°.

Slight variations in these depletion protocols have been used both by our group and other investigators. Here it is necessary to stress a few general aspects of the procedure: (1) The more efficient the Ca^{2+} depletion, the better the reconstitution; (2) some compartments (e.g., the Golgi and in part of the ER) can be grossly altered morphologically by the Ca^{2+} depletion protocol. The incubation at 4° largely prevents these morphological changes, without altering the efficacy of the reconstitution; and (3) if ionophores or sarcoplasmic-endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) inhibitors are employed for depletion they must be removed completely before starting the experiment. For this reason extensive washing of the cell monolayer with bovine serum albumin (BSA) is recommended at the end of the reconstitution procedure.

In the case of cells transfected with the aequorin targeted to the subplasmalemmal space, the protocol of reconstitution may require an incubation in Ca^{2+}-free medium. In A7r5 we have demonstrated that the efficiency of reconstitution is considerably augmented in media without extracellular Ca^{2+}.[13] This was not the case in rat myotubes.

Luminescence Detection

The aequorin detection system is derived as described by Cobbold and Lee[18] and is based on the use of a low-noise photomultiplier placed in close proximity (2–3 mm) to aequorin-expressing cells. The cell chamber, which is on the top of a hollow cylinder, is adapted to fit a 13-mm-diameter coverslip. The volume of the perfusing chamber is kept to a minimum (about 200 μl). The chamber is sealed on the top with a coverslip, held in

[18] P. H. Cobbold and J. A. C. Lee. J. C. McCormack and P. H. Cobbold, eds., p. 55. Oxford University Press, Oxford, 1991.

place with a thin layer of silicon. Cells are continuously perfused via a peristaltic pump with medium thermostatted via a water jacket at 37°. The photomultiplier (EMI 9789 with amplifier-discriminator) is kept in a dark box and cooled at 4°. 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 to the photomultiplier. The output of the amplifier-discriminator is captured by an EMI C600 photon-counting board in an IBM- compatible microcomputer and stored for further analysis.

Ca^{2+} Measurement

For the cells transfected with cytosolic, mitochondrial, or nuclear aequorins, the coverslip with the transfected cells is transferred to the luminometer chamber and perfused with KRB saline solution in the presence of 1 m*M* $CaCl_2$ to remove the excess coelenterazine. The stimuli or drugs to test are added to the perfusing medium and reach the cells with a lag time that depends on the rate of the flux and the length of the tubes. To make the stimulation more rapid and homogeneous the rate of the peristaltic pump is set to its maximum speed. Under these conditions we calculated that the whole monolayer is homogeneously exposed to the stimuli in 2 sec. At the end of the experiments, all the aequorin is discharged by permeabilizing the cells with a hypotonic solution containing digitonin (100 μM) and $CaCl_2$ (10 m*M*). A typical experiment in HeLa cells is presented in Fig. 5.

For erAEQ-, srAEQ-, or GoAEQ-transfected cells, unreacted coelenterazine and drugs are removed by prolonged perfusion (3–6 min) with a saline solution containing 600 μM EGTA and 2% (w/v) BSA. BSA is then removed from the perfusion buffer and the refilling of the compartments is started by perfusing the medium containing either 1 m*M* $CaCl_2$ or $SrCl_2$ (Fig. 6). Note that BSA increases the luminescence background level.

We found that, despite the depletion protocol and the use of a low Ca^{2+} affinity aequorin mutant, the rate of aequorin consumption on Ca^{2+} refilling is so rapid that most aequorin is consumed in 30 sec and the calibration of the signal in terms of [Ca^{2+}] becomes unreliable.[12] Two alternative solutions to this problem have been developed: (1) the use of Sr^{2+} as a Ca^{2+} surrogate and (2) the reconstitution not with the wild-type coelenterazine, but with the analog coelenterazine n, which reduces the rate of aequorin photon emission at high [Ca^{2+}]. In the latter case [Ca^{2+}] between 10^{-4} and 10^{-3} *M* can be reliably calibrated.[11,19] Finally it should be stressed that

[19] V. Robert, F. De Giorgi, M. L. Massimino, M. Cantini, and T. Pozzan, *J. Biol. Chem.* **273,** 30372 (1998).

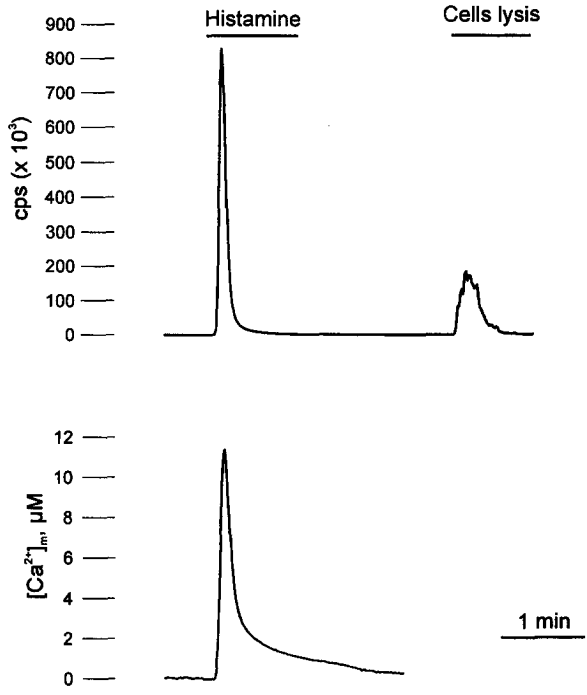

FIG. 5. Changes in mitochondrial $[Ca^{2+}]$ in HeLa cells. *Top:* Luminescence data. *Bottom:* Calibrated $[Ca^{2+}]$. Where indicated histamine (100 μM) was added. At the end of the experiments, cells were lysed by digitonin plus 10 mM $CaCl_2$. Cps, counts per second.

even a minor missorted fraction of aequorin in a low-$[Ca^{2+}]$ compartment, undetectable by morphological methods, can lead to a substantial artifactual underestimation of the Ca^{2+} levels in the high-$[Ca^{2+}]$ compartments.[20] A simple empirical solution to this problem has been described by Maechler *et al.*[21]

Conversion of Luminescent Signal into Ca^{2+} Concentration

To transform luminescence values into $[Ca^{2+}]$ values, we have used the method described by Allen and Blink.[22] The method relies on the relationship between $[Ca^{2+}]$ and the ratio between the light intensity re-

[20] M. Montero, J. Alvarez, W. J. J. Scheenen, R. Rizzuto, J. Meldolesi, and T. Pozzan, *J. Cell. Biol.* **3,** 601 (1997).

[21] P. Maechler, D. Kennedy, E. Sebo, T. Pozzan, and C. B. Wollheim. *J. Biol. Chem.* **18,** 12583 (1999).

[22] D. G. Allen and J. R. Blinks, *Nature (London)* **273,** 509 (1978).

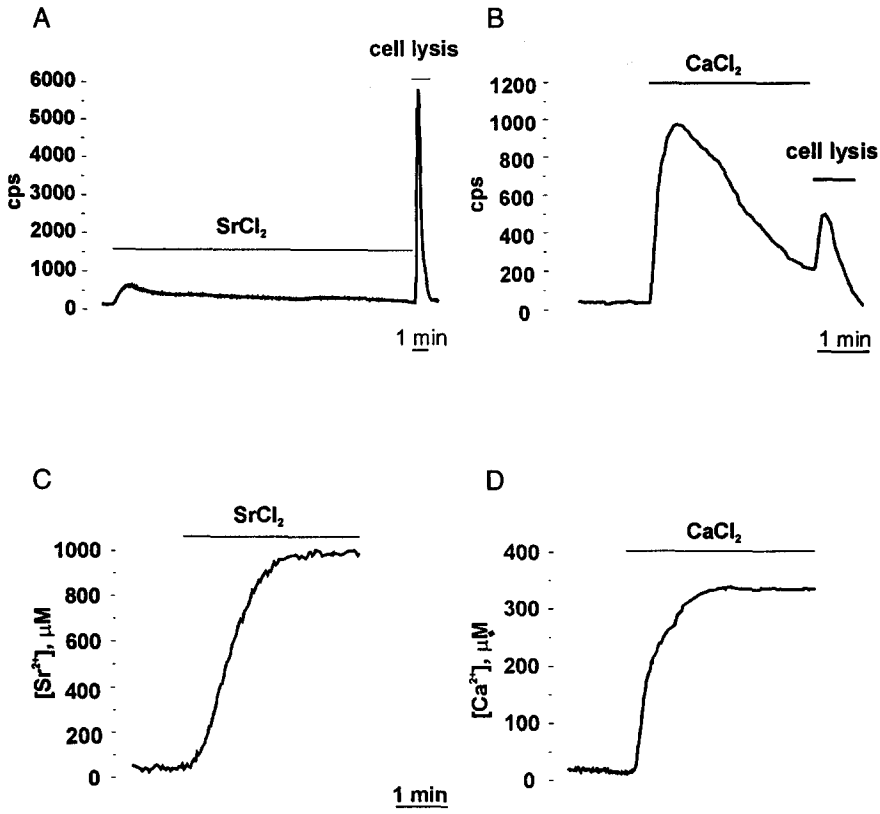

FIG. 6. Monitoring of $[Ca^{2+}]$ and $[Sr^{2+}]$ in rat myotubes. (A and B) Crude luminescence data. (C and D) Calibrated $[Sr^{2+}]$ and $[Ca^{2+}]$. The SR was depleted as described. Functional srAEQ was reconstituted with wild-type coelenterazine (A–C) or coelenterazine n (B–D). Where indicated, the perfusion medium was supplemented with 1 m*M* $SrCl_2$ or $CaCl_2$.

corded in physiological conditions (L, counts per second) and that which would have been reported if all the aequorin were instantaneously exposed to saturating $[Ca^{2+}]$ (L_{max}). Given that the rate constant of aequorin consumption at saturating $[Ca^{2+}]$ is 1.0 sec^{-1}, a good estimate of L_{max} can be obtained from the total aequorin light output recorded from the cells after discharging all the aequorin. This usually requires the addition of excess Ca^{2+} and detergents as shown in the preceding section. As aequorin is being consumed continuously, it must be stressed that, for calibration purposes, the value of L_{max} is not constant and decreases steadily during the experiment. The value of L_{max} to be used for $[Ca^{2+}]$ calculations at every time point along the experiment should be calculated as the total light

output of the whole experiment minus the light output recorded before that point.

The relationship between the ratio (L/L_{max}) and $[Ca^{2+}]$ has been modeled mathematically. The model postulates that each of the Ca^{2+}-binding sites has two possible states, T and R, and that light is emitted when all the sites are in the R state. Ca^{2+} is assumed to bind only in the R state. This model contains three parameters: KR, the Ca^{2+} association constant, KTR = [T]/[R], and n, the number of Ca^{2+}-binding sites. The values we obtained for the recombinantly expressed recombinant aequorin for each parameter are KR = 7.23 10^6 M^{-1}, KTR = 120, n = 3. The equation for the model reported by Allen *et al.*[23] provides the algorithm we used to calculate the $[Ca^{2+}]$ values at each point where the ratio = $(L/L_{max})1/n$.

$$Ca^{2+}\ (M) = \text{ratio} + (\text{ratio KTR}) - 1/\text{KR} - (\text{ratio KR})$$

Advantages and Disadvantages of Aequorin Compared with Other Ca^{2+} Indicators

Today numerous indicators are available to measure $[Ca^{2+}]$. In this section, we briefly describe some advantages and disadvantages of the photoprotein over the most widely used fluorescent indicators, the tetracarboxilate dyes such as Fura-2, Indo-1, and Fluo-3.

1. The photoprotein aequorin is exclusively localized in the cytosol and is completely excluded from organelles. The fluorescent dyes, if loaded using their intracellularly trappable AM-esters, are usually found also in intracellular organelles such as the mitochondria and the ER. This intracellular trapping can complicate the interpretation of the data obtained with the dyes. Furthermore, the dyes are usually slowly released into the medium, while aequorin loss requires the death of the cell.

2. Because of the low luminescence background of cells and the steepness of the Ca^{2+} dependence of aequorin luminescence, minor changes in $[Ca^{2+}]$ can be easily appreciated.

3. Obviously both the photoproteins and the fluorescent Ca^{2+} indicators increase the Ca^{2+}-buffering capacity of the cells and thus potentially may interfere with cellular Ca^{2+} homeostasis. The high signal-to-noise ratio characteristic of aequorin allows, however, the use of the photoprotein at concentrations that are two to three orders of magnitude lower than those necessary for the fluorescent indicators. The immediate advantage of using

[23] D. G. Allen, J. R. Blinks, and F. G. Prendergast, *Science* **195,** 996 (1971).

aequorin is thus a reduced Ca^{2+} buffering that minimizes artifactual quantitative and qualitative changes in Ca^{2+} dynamics.[24]

4. For many years, a major limitation of the use of aequorin has been the need to introduce the polypeptide into living cells by traumatic methods such as microinjection. Because of this restriction, the use of aequorin as a Ca^{2+} indicator has been limited to large and robust cells (eggs, muscle fibers, giant synapses of squid, etc.). The cloning of the aequorin cDNA[4] had offered the possibility of expressing the recombinant protein into virtually all cell types. In addition, aequorin cDNA can be cotransfected with a gene of interest and allows a simple and effective way to monitor in a large number of cells the effect of that gene on Ca^{2+} homeostasis. Such studies are far more complex and time consuming when using the Ca^{2+} indicators.[24]

5. The major impact of aequorins in the field of signaling mechanisms depends on the possibility of obtaining accurate targeting of the protein in subcellular regions. Such a selective localization has never been achieved with fluorescent dyes.

6. The most obvious disadvantage of aequorins with respect to the fluorescent Ca^{2+} indicators is the low number of photons (<1 photon per molecule compared to $>10^4$ photons in the case of fluorescent dyes) that can be emitted by aequorin and the irreversible nature of the photon emission reaction. For these reasons, single-cell analysis of Ca^{2+} transients with cytosolic aequorin (or with any of the targeted chimeras) requires expensive equipment and has intrinsically low time and spatial resolution. This type of analysis is far simpler and more accurate with the fluorescent dyes.

7. Calibration of aequorin signals in terms of [Ca^{2+}] most often requires cell lysis (but see Ref. 25 for an alternative approach). Many fluorescent Ca^{2+} indicators can be calibrated in intact cells with the "ratio mode." Furthermore, because of the steep relationship between [Ca^{2+}] and the rate of photon emission, the signal of aequorin is biased toward cells (or compartments) with higher [Ca^{2+}]. This disadvantage can be, in some cases, turned into a bonus.[13]

8. Aequorin is consumed while measuring Ca^{2+}, while this phenomenon is negligible with fluorescent dyes.

In summary, aequorins, and in particular targeted recombinant aequorins, may represent for some applications a useful, and sometime superior, tool (compared with fluorescent Ca^{2+} indicators) to investigate Ca^{2+} signal-

[24] M. Brini, R. Marsault, C. Bastianutto, J. Alvarez, T. Pozzan, and R. Rizzuto, *J. Biol. Chem.* **17,** 9896 (1995).

[25] M. R. Knight, N. D. Read, A. K. Campbell, and A. J. Trewavas, *J. Cell. Biol.* **1,** 83 (1993).

ing in living cells. Quite recently the group of Tsien[26] has introduced the Ca^{2+} indicators named "cameleons," molecularly engineered proteins capable of coupling the advantages of aequorins in terms of selective targeting to the high signal characteristics of fluorescent molecules. This technique is presently in its infancy, but its potential in this field is enormous.

[26] A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura, and R. Y. Tsien, *Nature (London)* **388,** 882 (1997).

[34] Recombinant Aequorin as Reporter of Changes in Intracellular Calcium in Mammalian Cells

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Introduction

The photoprotein aequorin, from the coelenterate jellyfish *Aequorea victoria,* is a bioluminescent complex formed from the 21-kDa apoaequorin, the luminophore cofactor coelenterazine, and molecular oxygen.[1] Apoaequorin is a 189-amino acid polypeptide chain containing three calcium-binding sites (EF-hand structures).[2] After the binding of calcium to these sites, aequorin undergoes a conformational change, converting into an oxygenase. The subsequent oxidation of coelenterazine by the bound molecular oxygen results in the production of apoaequorin, coelenteramide, CO_2, and light with a λ_{max} for emission of 469 nm, which can be detected by conventional luminometry. The kinetics of light emission after the binding of calcium to the aequorin complex is rapid and is typically complete in less than 10 sec.

Aequorin has been used for many years as a reporter of changes in intracellular calcium concentration in mammalian cells or *Xenopus* oocytes.[3] In these experiments loading of cells with aequorin has involved microinjection of purified protein. However, more recently the cloning of the aequorin cDNA has allowed expression of this protein, both transiently

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[2] S. Inouye, M. Noguchi, Y. Sakaki, Y. Tagaki, T. Miyata, S. Iwanaga, T. Miyata, and F. Tsuji, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3154 (1985).

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