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Aequorin Targeted to the Endoplasmic Reticulum Reveals Heterogeneity in Luminal Ca^{++} Concentration and Reports Agonist- or IP_3-induced Release of Ca^{++}

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A chimeric protein (ERaeq) comprised of the invariant chain (I_i) of class II major histocompatability complex (MHC-II) and aequorin was localized in the endoplasmic reticulum (ER) of transfected human embryonal kidney 293 cells. The targeted aequorin resided in the lumen of the ER membrane system, including the nuclear cistern, and following addition of the chromophore coelenterazine underwent Ca^{++}-activated chemiluminescence. The majority of chemiluminescence produced by coelenterazine treatment of ERaeq-expressing 293 cells was consumed rapidly (within 2–4 min) upon re-addition of Ca^{++} to coelenterazine-loaded cells, a finding consistent with very high Ca^{++} concentrations ($\sim 10^{-5}$-10^{-3} M Ca^{++} ion) inside the ER. However, following the initial rapid consumption of ERaeq chemiluminescence, the activity that remained (10–30% of total sample luminescence of permeabilized cells or 50–70% of total sample luminescence of intact cells) was found to produce a stable baseline corresponding to a Ca^{++} ion concentration $\leq$1–2 μM. The stable baseline of luminescence observed following rapid consumption of the majority of the sample's activity was not derived from re-binding of fresh chromophore to spent photoprotein, suggesting that a minority fraction of the ER membrane system within which the ERaeq chimera was distributed contained a relatively low Ca^{++} concentration. Addition of IP_3 to digitonin-permeabilized cells, or agonist treatment of intact cells decreased this residual signal. Luminescence recordings from cells expressing an ER-targeted aequorin with relatively high affinity for Ca^{++} thus reveal heterogeneity in luminal ER Ca^{++} concentration and permit observation of receptor- and IP_3-activated release of Ca^{++} from the ER membrane system.

INTRODUCTION

The mobilization and turnover of intracellular Ca^{++} is an integral aspect of cell regulation during responses to several classes of extracellular stimuli. The widespread use of fluorescent Ca^{++}-chelator dyes has resulted in an enormous body of work, part of which reveals that agonists produce many different patterns of change in cytosolic Ca^{++}. Responding cells can experience transient global elevations of Ca^{++}, oscillations in Ca^{++}, or propagation of Ca^{++} waves. These signals vary in amplitude and frequency and may also exhibit distinctive spatial patterns (for reviews, see Tsien and Tsien, 1990; Fewtrell, 1993; Clapham, 1995). The spatial and temporal complexities of intracellular Ca^{++} changes presumably reflect a need for specification of signals conveyed by a wide array of agonists that must operate in many different cell phenotypes.

Control of Ca^{++} signaling involves storage of Ca^{++} inside intracellular organelles, the stimulus-activated release of stored Ca^{++}, and efficient uptake of Ca^{++} from outside the cell for replenishment of Ca^{++} stores during prolonged response sequelae. The endoplas-

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mic reticulum (ER) is believed to be the primary agonist-sensitive Ca^{++} store and is the source of Ca^{++} that can be released by IP_3, Ca^{++} itself, and perhaps cADP-ribose (Galione, 1993; Hua *et al.*, 1994). Mitochondria and isolated nuclei also store and release Ca^{++} (Malviya *et al.*, 1990; Nicotera *et al.*, 1990; Rizzuto *et al.*, 1992; Gerasimenko *et al.*, 1995), and there is evidence that the Golgi apparatus (GA) may sequester and/or release Ca^{++} when cytosolic Ca^{++} levels increase (Connor, 1993; Zha *et al.*, 1995). Study of the participation of these and other organelles in Ca^{++} homeostasis is an area of intense research activity.

Aequorin is a Ca^{++}-activated luminescent protein from jellyfish that has been used to great advantage for several decades in measurements of intracellular Ca^{++} changes (Blinks *et al.*, 1976). Until recently the use of aequorin was limited to measurements of single or small numbers of relatively large cells microinjected with the purified photoprotein, or bulk-loaded cells. For this reason its use was largely eclipsed by fluorescent probes that are much easier to place inside cells and offer vastly greater photon yield. However, aequorin users have recently taken advantage of expression of the apo-protein in cells transfected with the aequorin cDNA, followed by luminescence measurements on cultures pre-treated with the required chromogenic co-factor, coelenterazine.

The groups of Pozzan and Campbell have pioneered this strategy with measurements of Ca^{++} changes in specific microdomains of cells by targeting the aequorin luminescence activity to mitochondria and ER, respectively. The use of mitochondrial-targeted aequorin has shown that Ca^{++} uptake occurs at mitochondria even when agonists produce average cytosolic Ca^{++} increases that are lower than the K_m for mitochondrial Ca^{++} transport (Rizzuto *et al.*, 1993). This mitochondrial Ca^{++} reporter has also revealed that in pancreatic β cell lines, mitochondrial Ca^{++} uptake is tightly linked to Ca^{++} influx, as well as release of Ca^{++} from internal stores (Rutter *et al.*, 1993). This finding is helpful for understanding how depolarizing stimuli are integrated with metabolic and secretory processes. These important results underscore the advantage of using a targeted reporter capable of sensing a locally controlled event that is difficult or impossible to resolve by bulk loading and fluorescence imaging methods. An ER-retained aequorin that contains the tetrapeptide ER retention signal Lys-Asp-Glu-Leu (KDEL) at its C-terminus has yielded an estimate of the ER Ca^{++} concentration in the range of 0.3–1.0 uM (Kendall *et al.*, 1994). This form of targeted aequorin unfortunately displays elevated basal activity, an outcome presumably attributable to modification of aequorin at its C-terminus (Nomura *et al.*, 1991).

In an effort to learn more about the mechanisms that control uptake and release of intracellular Ca^{++} we have developed novel targeted aequorin forms that reside predominately inside either the GA or the ER. We now report results from a chimeric form of aequorin in which the ER-retained invariant chain (I_i) of class II major histocompatability complex (MHC-II) is fused at its COOH-terminus to the NH-teminus of aequorin. The reporter is discretely localized in the ER (as opposed to the distinctive Golgi localization of a galactosyltransferase-aequorin, GT-aequorin, fusion protein), it displays Ca^{++}-sensitive luminescence, and can be used to monitor changes in Ca^{++} concentration inside the lumen of the ER.

MATERIALS AND METHODS

Construction of Chimeric cDNAs

Chimeric I_i-aequorin (ERaeq) and GT-aequorin (GOLaeq) were made using a two-stage polymerase chain reaction strategy. First, the two coding sequences of each chimera were independently amplified using primers that introduced exact-match overlaps where in-frame fusion of the coding sequences occurs. Stage two reactions were then performed using dilutions of the first stage products (after removal of residual free nucleotide with Wizard PCR Preps resin; Promega, Madison, WI) and primers appropriate for generating full length chimeric product.

The I_i portion of the I_i-aequorin chimera was made from the p33-ATG1 I_i cDNA template (Strubin *et al.*, 1986) (generously provided by Dr. Eric Long, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) using an upstream primer that includes sequence from the first three codons of I_i (I-UP: 5′ AGC TAG CGG CCG CCA CCA TGC ACA GGA GG), and a downstream primer encoding the last four codons of I_i in-frame with codons 2–5 of aequorin (I-DN: 5′ TTG TTC GCT GGT CAT GGG GAC TGG 3′). The GT portion of the GT-aequorin chimera was amplified from the GT cDNA template (Aoki *et al.*, 1992) (generously provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation, San Diego, CA) using an upstream primer that includes GT cDNA nucleotides −34 to −16 (GT-UP: 5′ ATA AGA ATG CGG CCG CTA GCC CAC ACC CTT CTT AA 3′) and a downstream primer encoding GT residues 55–60 fused in-frame with aequorin codons 2–7 (GT-DN: 5′ TGA GTA TTG TTC GCT GGT CTG CAG CGG TGT GGA GAC 3′). The aequorin portion of both chimeras is composed of aequorin residues 2–196 amplified from the pCDM.AEQ template (Button and Brownstein, 1993) using upstream primers specific for fusion with either I_i or GT (i.e., upstream aequorin primers used in stage one reactions are reverse complements of I-DN or GT-DN, respectively) and a downstream primer that encodes an intact aequorin C-terminus (AEQ-DN: 5′ GCG CGC TCT AGA GTT TCT TAG GGG ACA G). Stage two reactions that produced full length I_i-aequorin required use of the I-UP and AEQ-DN primers; amplification of the full length GT-aequorin sequence required use of GT-UP and AEQ-DN primers.

Full length chimeric open reading frames (ORFs) were cloned in the CMV-promoter-driven expression vector pCMV.IRES (Button and Brownstein, 1993). Using a Ca^{++}-phosphate procedure (Chen and Okayama, 1988), human embryonal kidney 293 (293) cells were transfected with the chimeric aequorin expression vectors. Clones and populations of cells expressing high levels of aequorin luminescence were isolated by selection in media containing 0.4 mg/ml G418.

Immunohistochemistry

Cells expressing aequorin were seeded on plastic, 8-chamber slides. Sub-confluent cultures were rinsed once with 0.3 ml of phosphate-buffered saline (PBS) and then fixed by incubating for 30 min in 0.3

ml PBS + 4% paraformaldehyde. All procedures were performed at room temperature (RT). After fixation cells were rinsed once with 0.3 ml PBS and then incubated for 30–60 min with 0.2 ml block solution (PBS supplemented with 1% bovine serum albumin [BSA] and 3.3% normal goat serum) containing 0.1% Triton X-100. Detergent-containing block solution was then aspirated and replaced with 0.2 ml fresh block solution with no detergent for a 1- to 2-h incubation. After removal of block solution, anti-aequorin primary antibody (1:500–1:1000 dilution of immune serum in block solution) was then added (0.1 ml per chamber) for a 1-h incubation. Anti-aequorin rabbit serum was produced using purified aequorin photoprotein from Friday Harbor Photoproteins (Friday Harbor, WA) as antigen. After incubation with primary antibody, chambers were rinsed three times with 5-min incubations of 0.15 ml of block solution and then incubated with a 1:300–1:500 dilution of a CY3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in block solution for 1 h. After secondary antibody binding chambers were rinsed three times with 5-min incubations of 0.15 ml of block solution and coverslips were then placed on two drops of Slowfade anti-fade reagent (Molecular Probes, Eugene, OR). Staining with fluorescein isothiocyanate (FITC)-conjugated *Lens culinaris* agglutinin (LCA; Vector Laboratories, Burlingame, CA) was performed on paraformaldehyde-fixed cells (already stained with the aequorin antiserum) by incubation with a 50- to 100-fold dilution of the commercially supplied reagent in PBS containing 0.1 mM $CaCl_2$. Observation of the fluorescence staining patterns for preparations that were double labeled by both the aequorin antibody (indirectly coupled to a CY3 fluorophore) and FITC-conjugated LCA was performed with a laser scanning microscope (Zeiss, LSM 410, at the Light Imaging Facility, NINDS, Bethesda, MD) with alternating acquisition of the red and green fluorescence channels.

Subcellular Fractionation and Marker Enzyme Assays

293 cultures ($4–8 \times 10^7$ cells) expressing targeted aequorins were collected in PBS from 15-cm culture dishes after repeated rinsing with PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were sedimented by centrifugation, rinsed one time each with PBS and 0.25 M sucrose in 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), resuspended in 2 ml 0.3 M sucrose, and then pressurized at 4°C in a nitrogen cavitation chamber, 100 psi for 30 min. Cavitated cells were disrupted on ice with 20 strokes in a tight-fitting dounce homogenizer and the sample was centrifuged at $750 \times g$ for 15 min. The post-nuclear supernatant was layered on top of a discontinuous sucrose gradient prepared, centrifuged, and processed as described (Bole *et al.*, 1986). Mannosyltransferase and galactosyltransferase activities (2–10 μl of gradient fraction per assay) were measured in 100-μl reactions also as described (Bole *et al.*, 1986). Luminescence in 2–10 μl of each gradient fraction was determined by incubating gradient fractions with 100 mM KCl, 10 mM Tris (pH 7.5), 10 mM EDTA, 1% β-mercaptoethanol (β-ME), and 1 μM coelenterazine (50 μl final volume), in 6 ml polypropylene tubes (Falcon, no. 2063) on ice for 4–6 h. Activity was then measured during a 30-s interval in a Berthold LB9051 luminometer after automated injection of 100 μl of activating solution containing 1 M $CaCl_2$, 10 mM Tris (pH 7.5), and 0.1% Triton X-100.

Metabolic Labeling of Cells and Analysis of Endo-β-N-acetyl glucosaminidase H (Endo H) Sensitivity

Confluent cultures of ERaeq-expressing cells ($\sim 2 \times 10^6$) were treated with methionine- and cysteine-free Dulbecco's modified Eagle's medium (DMEM-met/cys) for 15 min, followed by a 20-min pulse labeling with 0.1 mCi of [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-label, ICN Pharmaceuticals, Irvine, CA) in the same media. Label media was then aspirated and fresh DMEM plus 10% fetal calf serum (FCS) was added for "chase" intervals of variable duration. At the end of the chase interval cells were collected in 1 ml of ice-cold PBS containing 1% Triton X-100 plus 1 mM [4-(2-aminoethyl)-benzenesulfonylfluoride, HCl] (AEBSF, Calbiochem, La Jolla, CA), vortexed, held on ice for 20 min, and then centrifuged at $1300 \times g$ for 10 min. Supernatants were divided into equal portions and incubated overnight at 4°C on a mixing platform with either 2 μl of aequorin antiserum or 2 μl of ascites containing monoclonal antibody (mAb) W6/32 (Barnstable *et al.*, 1978; kindly provided by Dr. Paul Roche, NCI, Bethesda, MD). Immunoprecipitates were collected on protein A-coupled agarose beads (Affigel, Bio-Rad, Hercules, CA), rinsed three times in PBS containing 0.1% Triton X-100 plus 1 mM AEBSF, and then boiled for 10 min in 50 μl of 0.5% SDS and 1% β-mercaptoethanol. After cooling and mixing with 5.6 μl of 500 mM sodium citrate (pH 5.5), 20-μl aliquots were dispensed and incubated for 3 h at 37°C with or without addition of 50 U of endo H (New England Biolabs, Beverly, MA). Products of deglycosylation or mock deglycosylation reactions were then mixed with an equal volume of 2× SDS sample buffer (50 mM Tris-HCl, 2% SDS, 0.1 M dithiothreitol, and 10% glycerol), re-boiled for 5 min, and applied to 10% polyacrylamide gels for analysis by SDS-PAGE. Autoradiographs were then developed and analyzed using a BAS2000 PhosphorImaging system (Fuji Biomedical, Stamford, CT).

Titration of Ca^{++}-activated Luminescence

The Ca^{++}-activated luminescence function of chimeric and cytosolic aequorins was measured in crude cell lysates. Lysates were prepared by three cycles of freeze-thaw of $0.5–1 \times 10^7$ cells in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.1, and 0.5 mM each of *N*-(2-hydroxyethyl)-ethylenediamine-*N*, *N'*, *N'*-triacetic acid (HEDTA), ethylene glycol-*O*,*O'*-bis(2-aminoethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), and nitrilotriacetic acid (NTA) plus 0.1 mM AEBSF and 1 μg/ml leupeptin) followed by sonication with a model GE50 Daigger ultrasonic processor fitted with a 1/8" stepped microtip (10 times 1- to 2-s bursts on ice set at 50% maximal power). Luminescent holo-aequorin was made by mixing 20 μl 1 M K_2-tartrate, 4 μl 0.1 M reduced form glutathione (GSH), and 0.4 μl 1 mM coelenterazine with 0.376 ml of lysed cells followed by incubation on ice protected from light for 4–6 h. The rate of photon production of 2 μl aliquots was measured after mixing with 0.1 ml of Ca^{++} trigger solution in pre-rinsed 10×75 mm glass borosilicate tubes in a custom-built luminometer (see below, under measurement of luminescence). The rate of photon production was measured for 30–60 s and then 100 μl of a solution containing 10 mM HEPES (pH 7.1), 0.02 M $CaCl_2$ was mixed with the sample to saturate all remaining unquenched photoprotein with Ca^{++}. The fraction of total sample luminescence consumed per second was calculated as described below under measurement of luminescence. Ca^{++} trigger solutions were prepared by sequential dilution of K-HEPES chelator solution (50 mM K_2-tartrate, 10 mM HEPES, pH 7.1, and 0.5 mM each of EGTA, HEDTA, and NTA) with K-HEPES Ca^{++}-chelator solution (50 mM K_2-tartrate, 10 mM HEPES, pH 7.1, and 0.5 mM each of Ca^{++}-EGTA, Ca^{++}-HEDTA, and Ca^{++}-NTA). A Ca^{++}-chelator stock solution containing chelators plus equimolar Ca^{++} (5 mM each of Ca^{++}-EGTA, Ca^{++}-HEDTA, and Ca^{++}-NTA) was prepared by the pH-metric method as described (Tsien and Pozzan, 1989). The Ca^{++} ion concentration of Ca^{++}-chelator solutions was estimated by titration of fluo-3 fluorescence (2 μM pentapotassium salt of fluo-3, 488 nm excitation, 525 nm emission).

Preparation of Cells and Incubation with Chromophore

To prepare intact cell suspensions for luminescence measurements cultures were seeded in 6-well plates and confluent monolayers were incubated with coelenterazine (1–5 μM) in 0.7 ml of extracellular buffer (ECB, in mM: 140 NaCl, 20 KCl, 20 HEPES, pH 7.3–7.4, 5 glucose, 1 $MgCl_2$, 1 GSH, and 0.1 mg/ml BSA) supplemented with

0.35 mM each of EGTA, HEDTA, and NTA plus 0.15 mM each of Ca^{++}-EGTA, Ca^{++}-HEDTA, and Ca^{++}-NTA (final ionized [Ca^{++}] ~0.5–1 μM). Cells were incubated at RT or 4°C for 2–4 h. Following chromophore treatment cells were removed from the surface by gentle trituration with fresh buffer, centrifuged in 1.5 ml microcentrifuge tubes for 30 s at 1000 rpm in a Sorvall MC12V centrifuge and resuspended in 0.3 ml fresh media. Rinsed cells were held on ice for up to 1 h before luminescence measurements. Aliquots (0.05 ml) of chromophore-loaded cells were then placed in 10 × 75 mm round-bottom borosilicate glass tubes (pre-rinsed with 0.2 ml of ECB plus chelators), re-warmed by 2 min of incubation in a water-filled heat block set at 27°C, and placed in the luminometer without change of media. All measurements were carried out at RT. After identical treatment negligible luminescence activity was present in samples of control 293 cells that do not express aequorin polypeptide.

In other experiments luminescence measurements were performed with chromophore-loaded adherent 293 cultures grown in flat bottom 10 × 75 mm glass tubes. Cultures were seeded in 10 × 75 mm tubes at 10^5 cells/0.2 ml DMEM + 10% FCS (DMEM/FCS) 16–48 h before measurement. To load adherent cultures with chromophore DMEM/FCS was aspirated, cells were rinsed with 0.2 ml ECB and then 0.05 ml of the same buffer supplemented with 1 mM GSH, 1–5 μM coelenterazine, and Ca^{++}-chelators to give 0.5–1 μM ionized [Ca^{++}] was added for 2- to 4-h of incubation. For cells loaded with chromophore at 4°C samples were re-warmed for 2 min at 27°C and then were used for luminescence measurements performed at RT without exchange of media.

Permeabilized cell suspensions were prepared by 5 min of RT treatment of cultures in 10-cm plates with 4 ml intracellular buffer (ICB, in mM: 50 K_2-tartrate, 20 HEPES [pH 7.05], 10 glucose, 1 $MgSO_4$, 1 GSH, 0.1 mg/ml BSA) containing 50 μg/ml digitonin. Cells were then rinsed twice with 10 ml ICB and collected from the surface with 5 ml ICB, sedimented by centrifugation, and gently resuspended in ICB supplemented with 0.5 mM each of EGTA, HEDTA, and NTA plus 100 μM AEBSF and 1 μg/ml leupeptin. Permeabilized cell suspensions were incubated at 10^6-10^7 cells/ml with 2 μM coelenterazine for 2 h at RT or 4 h at 4°C. Luminescence recordings were performed with 0.05-ml portions of permeabilized cells in 10 × 75 mm round-bottom borosilicate glass tubes that were pre-rinsed with ICB + chelators.

Measurement of Luminescence

Detection of the cell responses was performed using a photon counting photomultiplier tube (PMT, Hamamatsu R464, quantum efficiency 22%, and 5–15 counts per second dark count at 25°C). The body of the luminometer is a custom built light-tight box connected to the PMT housing (Thorn-EMI Fact 50 MkII) via a shutter. An ellipsoidal mirror (Melles-Griot) was used to maximize the light collection by placing the cell containing tubes at the primary focus point and the photocathode at the secondary focus point. The signal out of the PMT was processed by a commercial photon counter (Thorn-EMI C-10) that provided convenient, independent, 50 ns wide TTL output pulses that were fed to a counter on a general purpose acquisition board (National Instruments NB MIO-16).

Recordings were performed at RT in 10 × 75 mm borosilicate glass tubes with 0.2–0.6 × 10^6 cells in 0.05 ml suspensions or with 0.1–0.3 × 10^6 adherent cells on flat bottom tubes in 0.05 ml of ECB. Photon production by the sample was monitored at 1-s intervals with data storage and manipulation performed by a LabView-operated virtual instrument. Additions of buffer containing test agents were made via hypodermic tubing that entered the sample tube through a light-tight seal at the top of the sample chamber.

To estimate Ca^{++} concentration from the photon signal it is necessary to calculate the fractional loss rate of aequorin luminescence, or the apparent rate constant of the luminescence reaction (Campbell *et al.*, 1981; Cobbold and Rink, 1987). The luminescence reaction of aequorin is first-order with respect to the amount of luminescent protein.

$$\text{photon signal intensity} = (dP/dt) = k_{app} \cdot L$$

where P is photon signal, k_{app} is the apparent rate constant that is dependent on the reaction's quantum yield and fractional saturation of photoprotein with Ca^{++}, and L is the amount of aequorin luminescence in the sample. Calculation of the apparent rate constant, k_{app}, for any time t during the measurement is performed by dividing the observed photon signal intensity at time t by the amount of luminescent photoprotein at time t (L_t).

$$k_{app} = \frac{(dP/dt)_t}{L_t}$$

Because aequorin's chemiluminescence is quenched completely following Ca^{++}-activated chromophore oxidation, the amount of luminescent photoprotein present in the sample declines continuously during the measurement. Therefore, to calculate k_{app} from the observed photon signal intensity the luminescent photoprotein remaining at each interval of the measurement must be calculated. The sample's total luminescence (L_{total}) is calculated retrospectively by integrating all the photons observed during the measurement. Because the photons are binned into intervals of approximately 1 s, this total is simply the summation over all the intervals (N).

$$L_{total} = \sum_{i=0}^{N} L_i$$

where L_i is the background-subtracted photon production during interval i. Determination of total sample luminescence requires cell lysis and saturation of the photoprotein with Ca^{++} after conclusion of the physiologic observations (achieved by measuring photon production for 10–30 s following the addition of 0.1% Triton X-100 and 10–100 mM $CaCl_2$). A running sum of luminescence detected up to interval x is subtracted from L_{total} to calculate the remaining luminescence activity ($L_{rem}(x)$) at each interval x, of the measurement.

$$L_{rsum}(x) = \sum_{i=0}^{x} L_i$$

$$L_{rem}(x) = L_{total} - L_{rsum}(x)$$

The apparent rate constant is thus equal to the luminescence rate detected at interval x divided by the remaining sample luminescence at time x.

$$k_{app} = \frac{(dP/dT)}{L} = \frac{L_i(x)}{L_{rem}(x)}$$

The apparent rate constant k_{app} is a function of Ca^{++} ion concentration. Calibration curves that relate luminescence rate of the aequorin chimeras to Ca^{++} concentration (see Figure 4) are used to convert the calculated k_{app} value to an estimated Ca^{++} concentration.

RESULTS

Construction and Expression of Chimeric Aequorins

Expression of aequorin at the GA or ER was accomplished by constructing chimeric ORFs in which the aequorin coding sequence was attached via its N-terminus to the long form of the human invariant chain (I_i), a resident protein of the ER, or to human galactosyltransferase (GT), a resident protein of the GA. Both GT and I_i are type II membrane proteins that possess cytosolic N-termini, single membrane-span-

ning domains, and intraluminal C-termini. The membrane topology of these GA and ER resident proteins is thus ideal for producing luminal aequorin residence because fusion of aequorin's N-terminus to other ORFs has been shown not to interfere significantly with luminescence activity (Rizzuto *et al.*, 1992, 1994). In contrast, the C-terminal proline residue of aequorin is essential for activity, and placing the ER retention signal KDEL at the C-terminus has been shown to greatly alter its luminescence activity (Kendall *et al.*, 1992).

Schematic representations of the chimeric aequorin proteins are displayed in Figure 1. ER-targeted aequorin (ERaeq) cDNA is composed of the entire ORF (232 amino acids) of the long form of I_i (Strubin *et al.*, 1986) fused in-frame with amino acids 2–196 of the aequorin ORF. In antigen-presenting cells short and long forms of I_i associate with the α and β subunits of class II MHC complexes in the ER. I_i subunits regulate assembly of class II heterodimers, transport of class II molecules to antigen-processing compartments, and binding of antigenic peptides to class II molecules. In the absence of expression of class II MHC α and β subunits, the long form of I_i (which possesses a double arginine ER-retention motif at its N-terminus) accumulates at the ER (Schutze *et al.*, 1994).

The GA-targeted aequorin chimera (GOLaeq) is comprised of the first 60 residues of GT fused to residues 2–196 of aequorin. This chimera exploits the known Golgi-retention signal that resides within amino acids 24–43 of the membrane-anchoring domain of GT (Aoki *et al.*, 1992), while avoiding production of a secreted form of GOLaeq by not including downstream GT sequences around Arg_{77}, a site of proteolytic cleavage that produces secreted GT. Thus, both chimeras encode polypeptides that combine the intact targeting signals of the N-termini and membrane spanning domains of GA or ER resident type II membrane proteins and the luminescence function of aequorin.

Steady state distribution of the chimeric aequorins was studied in 293 cells selected for stable expression of the targeted Ca^{++} reporters. In one group of experiments a polyclonal rabbit anti-aequorin serum was used for immunofluorescence staining of fixed cells. Representative fields of stained cells are shown in Figure 2. In cells expressing cytosolic aequorin (Button and Brownstein, 1993) (CYTOaeq) immunoreactivity was spread throughout the cell in a fairly uniform manner (Figure 2A). However, cells expressing ERaeq exhibited highly restricted immmunostaining distinctive for the ER membrane system, including dense staining of the nuclear envelope (Figure 2B). For purposes of comparison, the distribution of aequorin immunoreactivity in cells expressing GOLaeq is shown in Figure 2C. In these cells antibody staining also revealed a distinctive membrane structure consistent with that of the Golgi apparatus and markedly different from the distribution of ERaeq. Better definition of the extent to which ERaeq may spill over into the Golgi apparatus is provided by double staining of ERaeq-expressing cells with the aequorin antiserum (followed by CY3-conjugated secondary antibody, as described) in combination with an FITC-conjugated form of LCA (see Figure 2, D–F), which stains selectively the Golgi apparatus (Hsu *et al.*, 1992; Ridgway *et al.*, 1992). Stained preparations were observed with a laser scanning confocal microscope and reveal that there is little or no detectable crossover of the Golgi-selective FITC fluorescence and the CY3 signal that

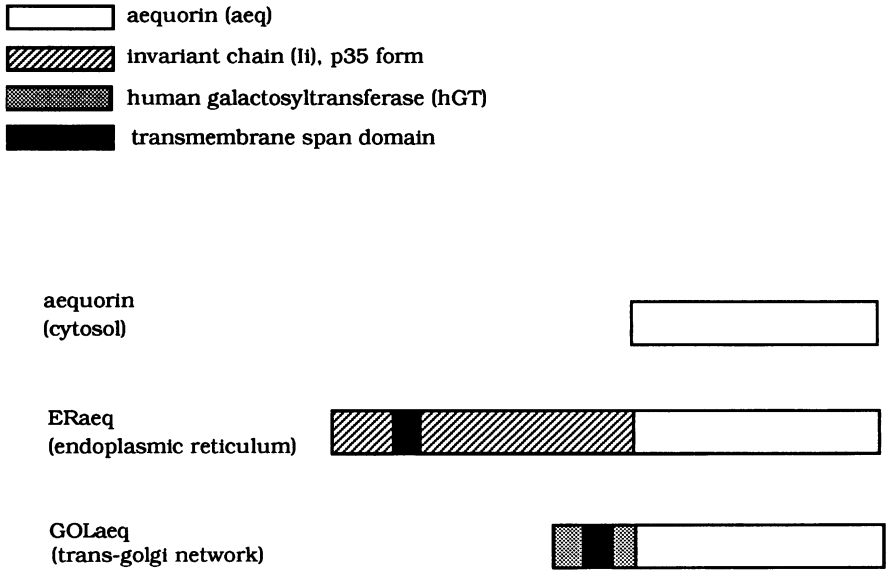

Figure 1. Schematic representations of chimeric aequorin photoproteins. The ORF for residues 2–196 of the aequorin polypeptide (represented by the open boxes) was fused with either the entire 232 residue ORF of the long form of human I_i (represented by the striped box) or with the coding sequence of residues 1–60 of human GT (stippled box). These chimeric proteins are expressed at the ER and GA, respectively, with the C-terminal aequorin portions located intraluminally.

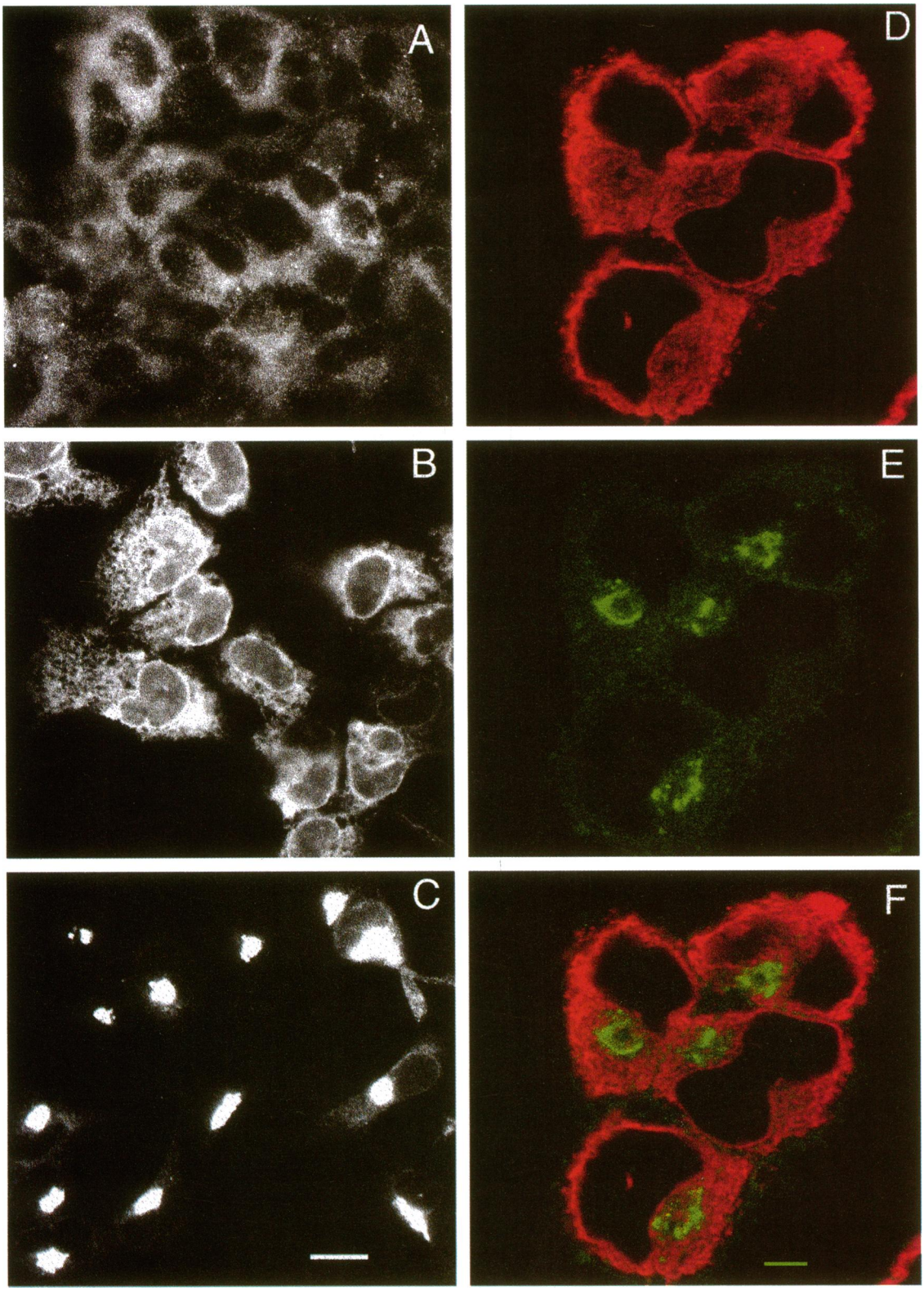

Figure 2. Immunohistochemical analysis of aequorin distribution in 293 cells. Cells expressing aequorin in the cytosol (A), ER (B), and *trans*-Golgi apparatus (C) were fixed and processed for antibody staining as described. Cells expressing aequorin in the cytosol display widespread and diffuse immunoreactivity that is excluded from the nucleus but cells expressing organelle-targeted aequorins show different

indicates the position of ERaeq. Thus, the double arginine ER retention signal present in the N-terminus of the ERaeq chimera works efficiently to retain the targeted Ca^{++}-reporter inside the ER membrane system. Furthermore, other double-fluorescence staining experiments were performed on cells that had first been incubated at 4°C under Ca^{++}-depleting conditions (identical to the procedure for chromophore loading and luminescence measurements; see below) and we found no discernible differences in the pattern of staining for either ERaeq or the Golgi-selective staining by LCA.

Sucrose density gradient fractionation of cell homogenates was also performed to verify that the targeted luminescence activities co-fractionate with their respective organelle marker enzyme activities. Consistent with the immunohistochemical findings, we found that luminescence of ERaeq was abundant in the same fractions enriched with mannosyltransferase activity (Figure 3A) and that GOLaeq luminescence activity co-fractionated with galactosyltransferase activity (Figure 3B). In three sub-cellular fractionation experiments performed with cells expressing ERaeq or GOLaeq, portions of cell homogenates were centrifuged at 100,00 × *g* and soluble luminescence activity in the supernatant was never found to exceed 2–3% of the total sample luminescence. On the other hand, greater than 98% of total sample luminescence was found in the 100,000 × *g* supernatant following centrifugation of homogenates of cells expressing CYTOaeq.

Finally, potential spillover of ERaeq out of the ER was examined by analysis of the sensitivity of ERaeq to deglycosylation by endo H. For proteins that receive N-linked glycan addition, passage through the Golgi apparatus results in trimming and addition of complex oligosaccharides that are resistant to cleavage by endo H. We performed pulse-chase experiments with ERaeq-expressing 293 cells to determine whether [^{35}S]methionine-labeled and immunoprecipitated ERaeq acquires this type of endo H–resistant oligosaccharide modification that indicates Golgi residence. Immunoprecipitates of both ERaeq (using the aequorin antiserum) or the heavy chain of class I of MHC, a protein expressed endogenously by the 293 cells (using a mAb W6/32; Barnstable *et al.*, 1978), were analyzed by SDS-PAGE following treatment with or without endo H (Figure 4). In Figure 4A, class

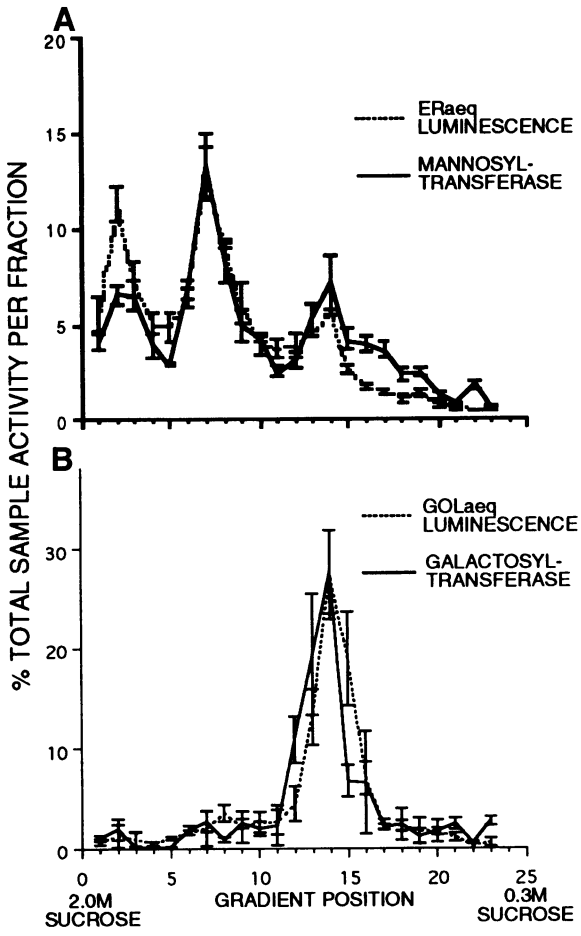

Figure 3. Co-fractionation on sucrose density gradients of chimeric aequorins and organellar marker enzymes. Post-nuclear supernatants prepared from homogenates of 293 cultures expressing the ERaeq chimera (A) or the GOLaeq chimera (B) were centrifuged on sucrose step gradients. Fractions collected from the gradients were assayed for abundance of luminescence activity and either the ER marker enzyme mannosyltransferase (A) or the GA marker enzyme galactosyltransferase (B).

I heavy chains immunoprecipitated by mAb W6/32 reveal that after only a 1-h chase interval the protein has already become resistant to deglycosylation by endo H and that by 8 h all labeled heavy chain is resistant to endo H. By comparison all of the immunoprecipitated ERaeq (Figure 4B) is found to be efficiently deglycosylated by endo H, even after an 8-h chase interval, consistent with effective retention of the targeted reporter within the ER. Cultures that had first been pulse-labeled and then incubated at 4°C under Ca^{++}-depleting conditions (identical to the procedure for chromophore loading and luminescence measurements; see below) were also found to contain only endo H–resistant forms of ERaeq (Figure 4, lanes labeled 4°C, nominal Ca^{++}).

(Figure 2 cont.) patterns of staining that are distinctive for the ER or GA. Bar in panel C, 30 μm (for panels A–C only). ERaeq-expressing cells double-labeled with aequorin antiserum and the Golgi-selective LCA (D–F). Confocal images of fixed and stained cells were obtained for both the indirect immunofluorescence labeling of ERaeq (D) and FITC-LCA (E). Double exposure image showing nonsuperimposition of the two labeled membrane systems (F). Bar in panel F, 10 μm (for panels D–F only).

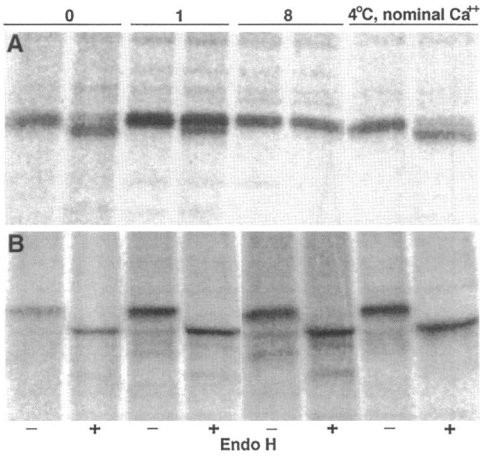

Figure 4. Sensitivity of ERaeq to deglycosylation by endo H. ERaeq-expressing 293 cultures (~2 × 10^6 cells) were pulsed with 0.1 mCi of [^{35}S]methionine for 20 min followed by chase intervals of 0, 1, or 8 h (indicated above gel lanes). After pulse-labeling other samples were held on ice for 2 h in fresh media containing 0.5–1 μM Ca^{++} before being re-warmed for a 10-min chase interval in the presence of 1.8 mM Ca^{++} (indicated above gel lanes as 4°C, nominal Ca^{++}). After chase intervals cell lysates were collected in 1 ml of ice-cold PBS containing 1% Triton X-100 and 1 mM AEBSF, vortexed, incubated on ice for 20 min, and centrifuged at 1300 × *g* for 10 min. Supernatants were incubated overnight on ice with either the aequorin antiserum or ascites fluid containing mAb W6/32 and immunoprecipitates were collected on protein A-conjugated agarose beads. Endo H treatment was performed at 37°C for 3 h and deglycosylation reaction products were analyzed by SDS-PAGE on 10% polyacrylamide gels. Samples run on the gel shown in panel A are from immunoprecipitation with mAb W6/32 (directed against class I heavy chains) and samples in panel B are from immunoprecipitation with aequorin antiserum. Although most of the labeled class I heavy chain molecules become insensitive to deglycosylation by endo H within 1 h (A), there is no detectable ERaeq that is insensitive to deglycosylation by endo H, even at the latest chase interval of 8 h (B).

Functional Characterization of Chimeric Aequorin

Because the structure of the aequorin polypeptide may be significantly altered in the context of organelle-targeted fusion proteins, we characterized the luminescence function of ERaeq by titration of Ca^{++}-activated luminescence. Sonicated lysates of cells expressing CYTOaeq and ERaeq were incubated with chromophore in the absence of Ca^{++}. Ca^{++}-activated luminescence was then determined for each aequorin variant by measuring the photon production rate in the presence of Ca^{++} from ~0.22 μM to 1 mM. Results from these experiments are displayed in Figure 5. As described in MATERIALS AND METHODS, the fractional loss rate or apparent rate constant (K_{app}) of aequorin luminescence was determined by dividing the instantaneous photon production rate by the corresponding remaining sample luminescence activity. In the absence of Ca^{++} K_{app} was ~0.4–1 × $10^{-5} \cdot s^{-1}$ for ERaeq and ~1–4 × $10^{-5} \cdot s^{-1}$ for CYTOaeq. At the lowest Ca^{++} concentration assayed (0.22 μM) the

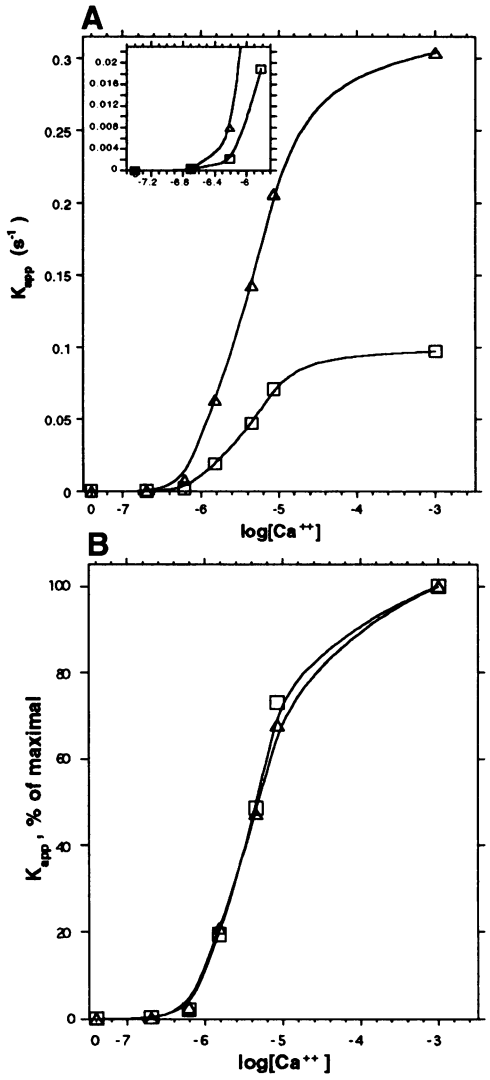

Figure 5. Titration of Ca^{++}-activated luminescence in lysates of 293 cells expressing cytosolic aequorin (triangles) or ERaeq (squares). Cell lysates were incubated ≥4 h with 1 μM coelenterazine in buffer containing 50 mM K_2-tartrate, 10 mM HEPES (pH 7.1), 1 mM GSH, and 0.5 mM each of EGTA, HEDTA, and NTA. Luminescence produced by 2-μl portions was measured at RT after injection of 100 μl of the same buffer containing 0–1 mM Ca^{++}. Inset graph shows the increase in luminescence rate at low Ca^{++} concentrations. In the absence of the Ca^{++} the K_{app} for cytosolic aequorin was 0.5–2 × $10^{-5} \cdot s^{-1}$ and at the lowest [Ca^{++}] tested (~220 nM) increased to ~1–3 × $10^{-4} \cdot s^{-1}$; for ERaeq, k_{app} in the absence of Ca^{++} was ~0.5 × $10^{-5} \cdot s^{-1}$ and increased by about 50-fold at 220 nM Ca^{++}. The concentration of Ca^{++} ion was estimated by titration of fluo-3 fluorescence in the same Ca^{++}-chelator buffer solutions.

K_{app} for both CYTOaeq and ERaeq increased to about 3 × $10^{-4} \cdot s^{-1}$ (ca. 10- and 50-fold increases, respectively, over the Ca^{++}-independent light production). Furthermore, with ionized Ca^{++} buffered at 1 μM the K_{app} of ERaeq light production increased ≥1000-fold to around 0.01·s^{-1} and the K_{app} of CYTOaeq changed

by a similar increment to about $0.04 \cdot s^{-1}$. Attachment of the membrane-spanning I_i to aequorin's N-terminus did not cause an increase in Ca^{++}-independent luminescence or any detectable change in the relationship between Ca^{++} concentration and photon production rate (Figure 5B). However, the maximal K_{app} observed during exposure of the aequorins to a saturating concentration of Ca^{++} (1 mM) was threefold less for ERaeq (Figure 5A), thus decreasing somewhat the Ca^{++}-activated increment in chemiluminescence signal for this reporter.

Aequorin's Ca^{++}-triggered luminescence is derived from the oxidized form of bound coelenterazine, the photoprotein's required chromophore. Therefore it is necessary to incubate cells expressing apo-aequorin with this co-factor before measurement of the Ca^{++}-reporting luminescence activity. It is not feasible to accumulate luminescence activity under conditions where coelenterazine binds aequorin in the presence of Ca^{++} because binding of chromophore occurs slowly (for cells expressing cytosolic aequorin 30–60 minutes are required for maximal accumulation of luminescence; Button and Brownstein, 1993; Yeong-An *et al.*, 1993) whereas Ca^{++}-triggered luminescence occurs on a sub-second time scale. Because the organelle studied contains high levels of Ca^{++} it was necessary to develop appropriate conditions for chromophore treatment of cells that express the ER-targeted Ca^{++} reporter. Our goal was to find conditions that temporarily reduce Ca^{++} levels in the ER. Accumulation of luminescence was therefore measured following chromophore treatment of intact 293 cells expressing targeted aequorins in the presence or absence of extracellular Ca^{++} at RT or 4°C.

In Figure 6A are results from experiments performed with cells expressing CYTOaeq. In these cultures luminescence accumulation is not diminished when coelenterazine treatment is performed at RT in the presence of Ca^{++} (i.e., nominal Ca^{++}, buffered with chelators at ≤ 1 μM) but is lowered in cells held at 4°C. This result is consistent with residence of aequorin in the cytosol where ambient Ca^{++} is normally maintained at low levels by temperature-sensitive Ca^{++}-ATPases at both the plasma and organelle membranes. In contrast, accumulation of luminescent ERaeq was greatly diminished under both nominal Ca^{++} and Ca^{++}-free conditions when the chromophore incubation was performed at RT (Figure 6B). When coelenterazine loading was performed in the complete absence of Ca^{++} about 60% less accumulation of luminescent photoprotein was observed with RT versus 4°C incubation. Furthermore, in the presence of nominal Ca^{++}, the production of luminescent ERaeq was reduced by 95% when cells were treated with chromophore at RT instead of 4°C. These findings presumably reflect incomplete loss of Ca^{++} from the ER stores when

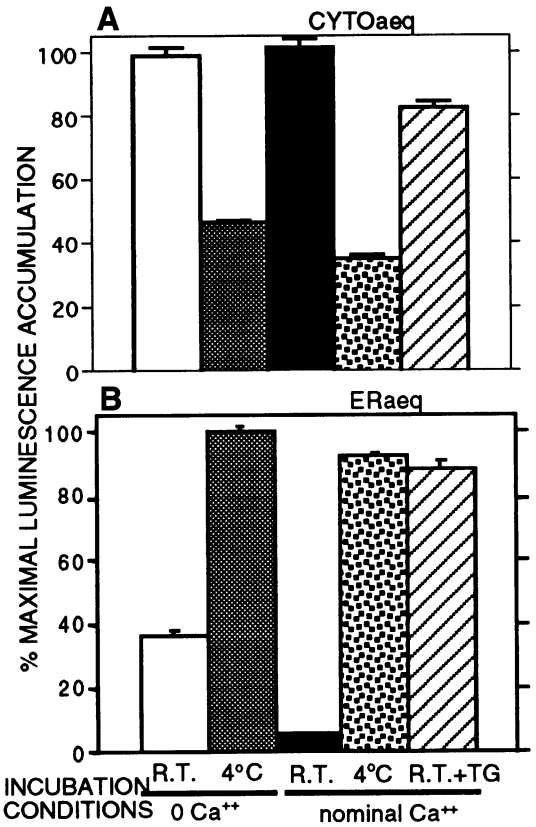

Figure 6. Accumulation of luminescence activity by intact cells expressing targeted aequorins. Suspensions of 293 cells ($0.4–1.0 \times 10^5$ cells/0.1 ml) were incubated at RT or on ice with chromophore in buffer containing chelators and no added Ca^{++} (0 Ca^{++}), or chelator plus Ca^{++} at 1 μM free ion concentration (nominal Ca^{++}), with or without 10 μM TG. Abundance of luminescence activity was measured after 2 h by addition of 100 μl 1 M $CaCl_2$, 10 mM Tris (pH 7.5), and 0.1% Triton X-100.

cells are held 2–4 h at RT (even though there is 0 Ca^{++} in the extracellular medium), whereas extensive depletion of Ca^{++} from intracellular organelles occurs when Ca^{++}-ATPase pump activity at the organelle is slowed at 4°C.

We also examined the effect of the Ca^{++}-ATPase inhibitor thapsigargin (TG) on the accumulation of luminescence in intact cells that express ERaeq. Because TG also depletes ER Ca^{++} stores due to its selective inhibition of Ca^{++}-ATPases of intracellular membranes (Thastrup *et al.*, 1990) we anticipated that accumulation of ERaeq luminescence would be enhanced if chromophore treatment were performed in the presence of TG. For 293 cells expressing ERaeq the presence of TG during chromophore incubation in nominal Ca^{++} at RT resulted in production of around 90% of the maximum luminescence accumulation, as compared with around 5% of maximal in the absence of TG (Figure 6, incubation condition RT + TG, nominal Ca^{++}).

ERaeq Reports Ca^{++} Changes inside the ER

Experiments on permeabilized cells were first performed to verify that the desired intraluminal orientation of the aequorin portion of the chimera had been achieved. These experiments also permitted us to measure changes in Ca^{++} levels in the ER during treatment with agents known to regulate Ca^{++} turnover in this organelle. For these measurements cells were first treated with 50 μg/ml digitonin for 5 min at RT to selectively permeabilize the plasma membrane and then incubated with 2 μM coelenterazine for 2–4 h in the absence of Ca^{++}. When chromophore treatment was performed at 4°C there was apparently extensive depletion of organelle Ca^{++}. Evidence for this is that addition of Ca^{++} and ATP to chromophore-loaded, permeabilized cells after re-warming resulted in a large luminescence signal that caused such rapid consumption of reporter luminescence activity as to preclude observation of steady-state concentration of Ca^{++} ion in the ER lumen ($[Ca_i^{++}]_{ER}$). For this reason chromophore treatment of permeabilized cells was performed in the absence of Ca^{++} at RT (in the presence of protease inhibitors). Despite lower luminescence accumulation under these conditions preparations contain sufficient activity for several minutes of observation and are apparently not excessively depleted of organelle Ca^{++}.

In the absence of ATP, addition of 0.3 μM Ca^{++} to chromophore loaded, permeabilized cells (Figure 7, A–C) resulted in an increase in K_{app} from background ($\leq 0.5 \times 10^{-5} \cdot s^{-1}$) to a rate approximately at the threshold for detection of Ca^{++}-triggered luminescence ($K_{app} \sim 1–3 \times 10^{-5} \cdot s^{-1}$). By comparison, the K_{app} for ERaeq in the presence of 0.3 μM Ca^{++} was measured in cell lysates to be $\sim 0.7 \times 10^{-3} \cdot s^{-1}$ (see Figure 5A), a value 20–70 times greater. This indicates that the ERaeq fusion protein is expressed with the aequorin portion of the chimera inside the organelle and not facing the cytosol where the presence of 0.3 μM Ca^{++} would activate production of a much larger luminescence signal.

Subsequent addition of 0.5 mM ATP to the permeabilized cells produced a very large increase in luminescence signal consistent with ATP-dependent uptake of Ca^{++} into the ER. The maximum K_{app} observed following addition of ATP was around $2 \times 10^{-2} \cdot s^{-1}$, indicating a maximum average $[Ca_i^{++}]_{ER}$ during ATP-dependent re-filling of about 2 μM (Figure 7A). After 4 min of re-equilibration with 0.3 μM Ca^{++} plus ATP $[Ca_i^{++}]_{ER}$ declined to a steady state level around 0.7 μM ($K_{app} \sim 2.8 \times 10^{-3} \cdot s^{-1}$). Addition of 10 μM IP_3 to the permeabilized preparation resulted in a rapid loss of luminescence signal (to $K_{app} \sim 0.7 \times 10^{-3} \cdot s^{-1}$ indicating average $[Ca_i^{++}]_{ER}$ of 0.45 μM) resulting from IP_3 stimulation of ER Ca^{++} release. Also shown in Figure 7A (dash-dot line) is the effect of 10 μM TG, which essentially abolishes the Ca^{++} and ATP-dependent increase in luminescence, consistent with the ability of TG to inhibit intracellular Ca^{++}-ATPase pumps. IP_3-mediated Ca^{++} release was also observed when IP_3 was added before $[Ca_i^{++}]_{ER}$ reached a steady-state plateau (at a time when average $[Ca_i^{++}]_{ER}$ was about 1 μM) and resulted in a lowering

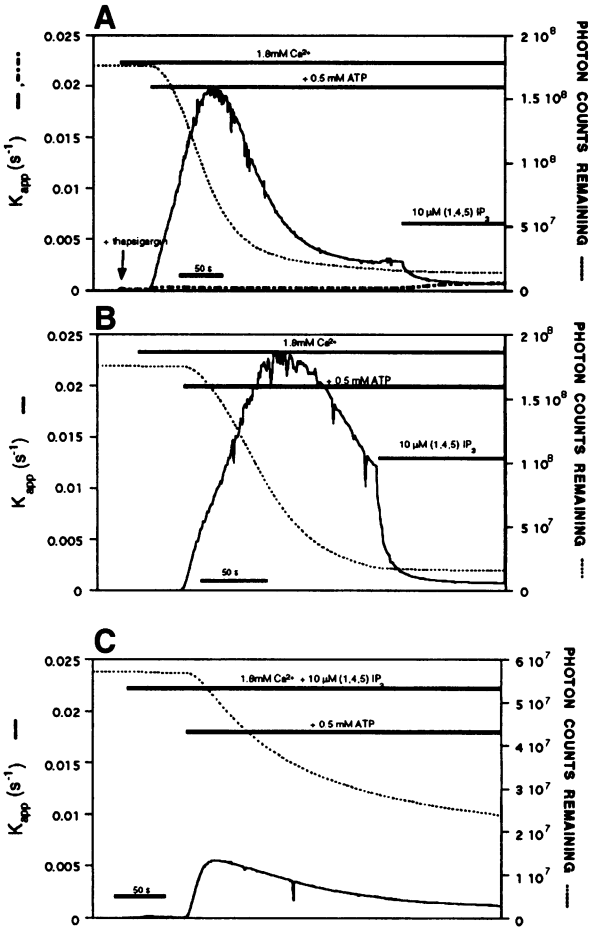

Figure 7. Luminescence recordings from permeabilized 293 cells that express ERaeq. Chromophore incubation was performed at RT in the absence of Ca^{++}, and 50-μl aliquots ($0.1–0.4 \times 10^6$ cells) were used in luminescence measurements. (A) ER Ca^{++} increases during recovery from Ca^{++} depletion in the presence of 300 nM Ca^{++} and 0.5 mM ATP (solid line) or in the presence of 300 nM Ca^{++}, 0.5 mM ATP, and 10 μM TG (dash-dot line). During refilling K_{app} reached a maximum more than 1000-fold over that of the depleted ER (peak $[Ca^{++}]$ estimated at 2 μM). By 5 min a plateau value was attained corresponding to 0.6–0.8 μM Ca^{++}. Subsequent addition of 10 μM IP_3 produced a reduction in intraluminal ER Ca^{++} to ≤ 0.5 μM. Ten micromolar TG almost completely inhibited Ca^{++} uptake (A, dash-dot line). The dotted line in panel A represents luminescence remaining (expressed in arbitrary photon count units) in the sample not treated with TG. (B) The addition of IP_3 to cells before ER Ca^{++} had declined to baseline also produced a rapid drop in Ca^{++} to essentially the same level. When IP_3 was added with Ca^{++}, an attenuated Ca^{++} rise occurred during refilling in the presence of ATP (C).

of the luminal $[Ca^{++}]$ to virtually the same level as with late addition of IP_3 (Figure 7B). Furthermore, the ATP-driven uptake of Ca^{++} at the ER was greatly reduced in the presence of IP_3 (Figure 7C). Inclusion of 10 μM IP_3 in the buffer during ATP-activated Ca^{++} accumulation by the ER resulted in a lower maximum $[Ca_i^{++}]_{ER}$ of 0.7–0.8 μM and slowed consumption of the sample luminescence. For the samples shown in Figure 7, A and B, repletion of Ca^{++} stores during 5 min of incubation with 0.5 μM Ca^{++} and 0.5 mM ATP resulted in consumption of greater than 90% of total sample luminescence, whereas in the presence of 10 μM IP_3 only about 65% of total signal was consumed over the same time.

Measurements of ER-specific Ca^{++} changes were also performed with intact cells. Both carbachol, an agonist of muscarinic receptors, and epinephrine, an agonist of adrenergic receptors, were found to reversibly decrease the ER-targeted luminescence signal in 293 cells that stably express ERaeq (Figure 8, B and C). Also shown is the carbachol-elicited transient increase in cytosolic Ca^{++} ($[Ca^{++}]_{CYT}$) reported by CYTOaeq (Figure 8A). Tracings of Figure 8 were obtained from monolayers of cells (1–4 $\times$ 10^5 cells per measurement) cultured in flat bottom 10 $\times$ 75 mm borosilicate glass tubes; qualitatively equivalent results were obtained with cell suspensions.

Cells expressing CYTOaeq were treated with chromophore at RT in ECB containing nominal Ca^{++} (~1 μM). Because the cells were treated with chromophore in low extracellular Ca^{++}, media Ca^{++} was restored to 1.8 mM at 30 s (Figure 8A), followed at 60 s by addition of 50 μM carbachol. Upon addition of extracellular Ca^{++} there was no change detected in the luminescence signal (K_{app} ~0.5 $\times$ 10^{-5}·s^{-1}), which reported $[Ca^{++}]_{CYT}$ of $\leq$0.2 μM. Not shown on the graph is the peak luminescence activity observed during the response to carbachol (K_{app} ~0.027·s^{-1}), which corresponds to a peak in average $[Ca^{++}]_{CYT}$ around 1 μM. After 90–120 s of carbachol treatment the cytosolic Ca^{++} level declined to an elevated plateau around 0.4 μM. Subsequent addition of the muscarinic receptor antagonist atropine reversed the effect of agonist and reduced the cytosolic aequorin signal to a K_{app} $\leq$6 $\times$ 10^{-5}·s^{-1}, a level near the limit for detection of Ca^{++}-activated luminescence ($[Ca^{++}]_{CYT}$ $\leq$0.2–0.3 μM).

ERaeq-expressing cells were treated with chromophore at 4°C in ECB containing nominal Ca^{++}. Before initiating luminescence measurements at RT, the samples were re-warmed by incubation for 2–5 min at 27°C. Representative tracings of ERaeq luminescence from intact cells are shown in Figure 8, B and C. Re-addition of Ca^{++} to the depleted, chromophore-loaded cultures produced a biphasic increase in the luminescence that was of smaller magnitude than that observed in permeabilized cells and consumed 30–

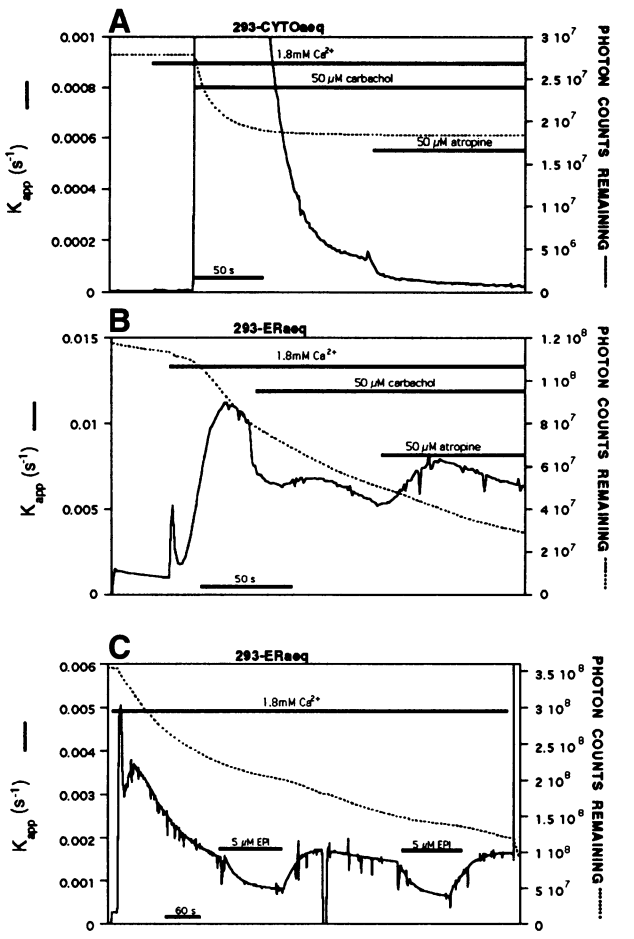

Figure 8. Luminescence recordings from intact cells expressing CYTOaeq (A) or ERaeq (B and C). Monolayers of cells in flat bottom 10 $\times$ 75 mm tubes were incubated with chromophore at RT (for cells expressing CYTOaeq) or at 4°C (for cells expressing ERaeq) in media containing nominal Ca^{++} (~0.5–1 μM). After re-warming for 2–5 min at 27°C the samples were used in luminescence measurements. (A) A representative tracing from cells expressing cytosolic reporter illustrates the large and rapid increase in cytosolic Ca^{++} that occurs upon treatment with carbachol (50 μM added at 60 s) and the subsequent reversal of the cytosolic transient upon addition of 10 μM atropine. (B) Low density cultures of cells expressing ER reporter display ER Ca^{++} changes during replenishment with Ca^{++} (added at the time indicated), during response to the muscarinic receptor agonist carbachol (50 μM), and after atropine blockade of agonist occupancy of receptor. (C) High density cultures of ERaeq-expressing cells repeatedly experienced decrease and recovery in $[Ca_i^{++}]_{ER}$ after treatment with and then wash-out of the adrenergic receptor agonist epinephrine.

50% of total sample activity within 1–3 min. Following an early and rapid spike in luminescence (K_{app} ~5 $\times$ 10^{-3}·s^{-1} or average $[Ca_i^{++}]_{ER}$ around 0.7–0.8 μM) ERaeq reported a second, longer-term elevation in signal that reached maximum 30–60 s after Ca^{++} addition at K_{app} ~3.5 to 10 $\times$ 10^{-3}·s^{-1} (average $[Ca_i^{++}]_{ER}$ around 0.7–1.4 μM). The trace in Figure 8B

is representative of those obtained from cells that were used in luminescence measurements within 16–18 h after seeding in glass tubes. In these cultures re-equilibration with Ca^{++} produced a greater maximum in average $[Ca_i^{++}]_{ER}$ and more rapid consumption of sample luminescence as compared with confluent cultures that were used in measurements around 48 h after seeding in glass tubes (representative trace, Figure 8C).

Changes in $[Ca_i^{++}]_{ER}$ during a response to carbachol are shown in Figure 8B. Restoration of extracellular Ca^{++} to 1.8 mM rapidly produced uptake of Ca^{++} at the ER and an increase in average $[Ca_i^{++}]_{ER}$ from ~0.3 μM under conditions of Ca^{++} depletion, to 1.3–1.4 μM. Subsequent treatment with 50 μM carbachol rapidly produced a reduction in the average $[Ca_i^{++}]_{ER}$ to ~0.7 μM, which was maintained during a 60-s exposure to agonist. Atropine (50 μM) blocked the effect of agonist and following a 10–15 s delay, the Ca^{++} level reported by ERaeq gradually recovered to an intermediate level. In cultures of higher cell densities changes in ERaeq luminescence were also observed during exposure to the adrenergic receptor agonist epinephrine (Figure 8C). Although these preparations yielded lower average $[Ca_i^{++}]_{ER}$ after re-equilibration with Ca^{++} receptor-activated signaling nonetheless repeatedly elicited decreases in ERaeq signal that were reversible upon washout of agonist.

Active aequorin photoprotein can be regenerated from the luminesced (i.e., Ca^{++}-bleached), blue fluorescent protein by incubation with coelenterazine in the presence of oxygen and reducing agent (Shimomura and Johnson, 1975). Therefore, because cell measurements were performed in the presence of coelenterazine ($\leq$5 μM) it was necessary to determine how much of the ERaeq luminescence we observed during measurement of changes in $[Ca_i^{++}]_{ER}$ was attributable to chromophore turnover. Cultures of ERaeq-expressing 293 cells were first depleted of Ca^{++} by incubation at 4°C in PBS supplemented with Ca^{++} chelator, lysed by sonication in buffer containing Ca^{++}-chelators, and then incubated overnight at 4°C in the presence of 1 μM coelenterazine. After removal of unbound chromophore by repeated washing of the lysate's membranes the abundance of ERaeq luminescence was determined by mixing small portions of the sample with 10 mM Ca^{++}. Photon production derived from re-binding of chromophore to Ca^{++}-bleached ERaeq was then measured by mixing fresh coelenterazine with replicate, Ca^{++}-bleached samples in the presence of 10 mM Ca^{++}.

Shown in Figure 9A is the time course of photon emission observed after addition of 10 mM Ca^{++} to washed membranes containing chromophore-loaded ERaeq (i.e., kinetics for production of the luminesced or Ca^{++}-bleached ERaeq). In Figure 9B are the luminescence signal intensities observed (in the presence of

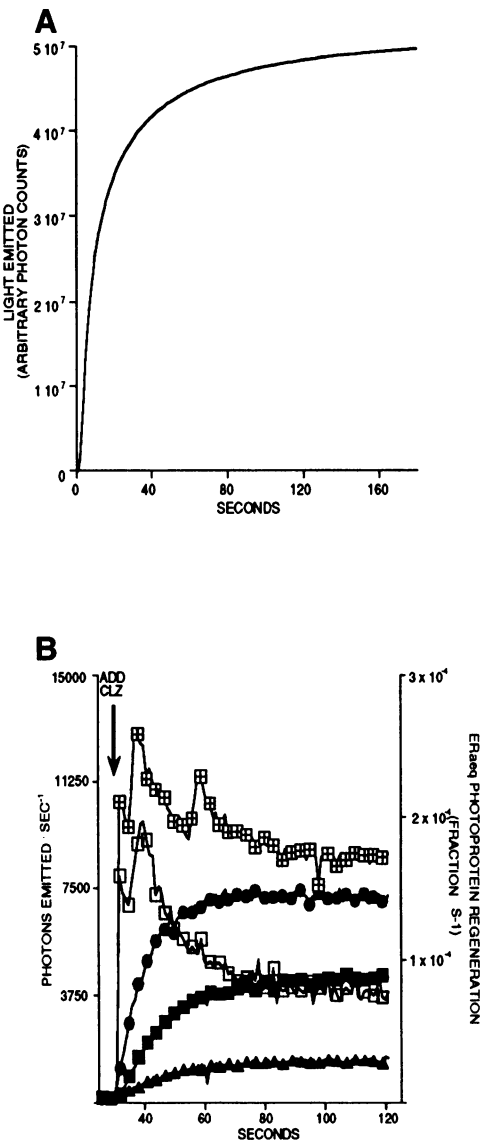

Figure 9. Time course of photon emission of ERaeq in 10 mM Ca^{++} (A) and magnitude of luminescence production when Ca^{++}-bleached ERaeq is regenerated during incubation with fresh coelenterazine (CLZ; B). ERaeq-expressing 293 cultures were used to prepare a crude membrane preparation that was incubated overnight at 4°C in chelator-containing buffer with 1 μM coelenterazine, washed extensively with fresh buffer to remove unbound chromophore, and then used for assay of luminescence abundance. (A) Luminescence produced by a representative 5-μl portion of the washed membranes upon mixing with 95 μl of a solution containing 10 mM Ca^{++}. After a 30- to 60-min treatment of coelenterazine-loaded and washed membranes with 10 mM Ca^{++}, luminescence was measured in replicate 5-μl aliquots during incubation in 100 μl of buffer containing 10 mM Ca^{++} in the presence of 2 (filled triangles), 10 (filled squares), or 50 μM (filled circles) fresh coelenterazine (B). Other bleached membranes were rinsed in fresh buffer containing 0 Ca^{++} and luminescence from re-binding of coelenterazine (10 μM) was assayed in the presence of 10 μM Ca^{++} or 1 mM Ca^{++} (B, open squares and crossed open squares, respectively).

10 mM Ca^{++}) after mixing fresh coelenterazine with replicate aliquots of ERaeq-containing membranes that had been in the presence of 10 mM Ca^{++} for 30–60 min. Luminescence intensity was found to be proportional to coelenterazine concentration: in the presence of 50 μM coelenterazine photon production equivalent to regeneration of ERaeq photoprotein at a rate of almost 1.5×10^{-4} s^{-1} was observed, whereas in the presence of 10 μM and 2 μM coelenterazine ERaeq regeneration occurred at rates of about 0.8×10^{-4} s^{-1} and 0.2×10^{-4} s^{-1}, respectively. Other chromophore-loaded lysate samples were bleached of chemiluminescence by incubation with Ca^{++} and then washed with buffer containing chelators and no added Ca^{++} before measuring the photon production derived from re-binding of 10 μM chromophore in the presence of 0.01 or 1 mM Ca^{++} (Figure 9B, open symbol traces). Although the regeneration rate was slightly higher in 1 mM Ca^{++} ($\sim 1.8 \times 10^{-4}$ verus 0.9×10^{-4} for 10 μM coelenterazine), it did not increase further in 0.01 mM Ca^{++}. Furthermore, in some experiments when cells were washed in fresh buffer following chromophore treatment a residual luminescence signal was still observed in Ca^{++}-repleted cells (data not shown). Thus, in measurements of intact or permeabilized ERaeq-expressing cells K_{app} was minimally around $10^{-3} \cdot s^{-1}$, with or without the presence of 1–5 μM coelenterazine. These results therefore reveal that regeneration of ERaeq luminescence from re-binding of fresh chromophore is unlikely to contribute significantly to luminescence signals observed during measurements such as those shown in Figures 7 and 8.

DISCUSSION

We have used a chimeric form of aequorin targeted to the ER lumen for measurement of ER Ca^{++} changes during physiologic responses of 293 cells. Staining of fixed cells with anti-aequorin serum and sucrose gradient fractionation of cell homogenates reveal that a fusion protein composed of I_i and aequorin (i.e., ERaeq) is concentrated in the ER. Fluorescence staining of both the Golgi apparatus and ERaeq in the same fixed cells reveals what appears to be essentially no overlap of the two fluorophores. In contrast, a second aequorin chimera in which GT and aequorin are fused is concentrated in a separate membrane system that has a structure consistent with that of the *trans*-Golgi apparatus and upon sedimentation on sucrose gradients is found to co-fractionate with the Golgi apparatus marker enzyme GT. Immunoprecipitation and endo H treatment of ERaeq from [^{35}S]methionine-labeled cultures shows that there is no detectable production of endo H–insensitive ERaeq, consistent with no accumulation of reporter within the Golgi apparatus. In permeabilized cells ERaeq reported ATP-dependent Ca^{++} uptake that could be blocked by TG and greatly attenuated by IP_3. ERaeq also reported IP_3 stimulation of Ca^{++} turnover inside the ER of permeabilized cells. Furthermore, we were able to record ER Ca^{++} changes in intact cells during cytosolic Ca^{++} transients elicited by the muscarinic receptor agonist carbachol and the adrenergic receptor agonist epinephrine. Taken together these observations strongly suggest that the ERaeq reporter is monitoring the luminal Ca^{++} concentration inside a functional compartment of the ER.

Our estimates of $[Ca_i^{++}]_{ER}$ around 1–2 μM are vastly lower than what is expected for the ER (see below) and apply only to a portion of the total ER as they are based on the chemiluminescence of ERaeq with wild-type affinity for Ca^{++} (i.e., EC_{50} for Ca^{++} activation of luminescence around 5 μM). In our experiments, after maximal accumulation of luminescent ERaeq is achieved (i.e., after production of holo-ERaeq by chromophore treatment under Ca-depleting conditions; see Figure 6) re-addition of Ca^{++} to depleted cells produces a large luminescence signal that yields a maximal K_{app} that decays spontaneously, even in the absence of stimuli (see Figure 7, A–C, and Figure 8, B and C, solid line traces). This signal observed during Ca^{++} re-equilibration therefore reflects the rapid and extensive consumption of Ca^{++} reporter in those regions of the ER experiencing restoration of $[Ca_i^{++}]_{ER}$ to the high levels expected (see Figure 7, A–C, and Figure 8, B and C, dotted line traces). During re-filling, the $[Ca_i^{++}]_{ER}$ presumably reaches a peak level of at least several hundred μM. Since the luminescence rate for ERaeq increases only marginally at Ca^{++} concentrations >50–100 μM (see Figure 5) it would not be possible to directly observe a peak of such magnitude using ERaeq. Furthermore, to the extent that the ER is heterogeneous with respect to $[Ca_i^{++}]_{ER}$ the luminescence report from ERaeq will reflect a composite of signals from independent ER microdomains that re-fill asynchronously with Ca^{++} to variable levels.

In studies by Kendall *et al.* (1994) aequorin chemiluminescence was used to monitor Ca^{++} uptake at the ER during re-warming of COS7 cells expressing a form of aequorin modified at its C-terminus by addition of the tetrapeptide ER-retention signal KDEL. The measurements of these investigators have provided estimates of $[Ca_i^{++}]_{ER}$ around 1 μM, in close agreement with our findings. However, ER Ca^{++} changes during agonist responses were not investigated. Although utility of the KDEL-tagged aequorin is limited by its high Ca^{++}-independent activity (it is known that loss of proline at aequorin's C-terminus alters the photoprotein's chemiluminescence activity; Nomura *et al.*, 1991; Watkins and Campbell, 1993), our strategy of fusing aequorin at its N-terminus with membrane-spanning resident proteins of the ER or GA has resulted in the construction of novel aequorin chimeras

that combine efficient targeting to either the ER or GA and robust chemiluminescence activity.

In permeabilized cells loaded with chromophore under Ca-depleting conditions, addition of Ca^{++} and ATP produces re-filling of the ER with Ca^{++} and within 2 min, 80% of the total sample luminescence was consumed (Figure 7A, broken line trace). The maximum K_{app} for ERaeq observed during re-equilibration of chromophore-loaded, permeabilized cells with Ca^{++} was on the order of $0.020–0.025 \cdot s^{-1}$ (see Figure 7, A and B, solid lines), a rate that is only 20–30% of maximal. Assuming that the ERaeq reporter is distributed widely throughout the ER membrane system, this relatively low average signal (corresponding to $[Ca_i^{++}]_{ER} \sim 1.5–2\ \mu M$) is consistent with considerable heterogeneity of $[Ca_i^{++}]_{ER}$ within the ER membrane system. Thus, during the few minutes of re-equilibration with Ca^{++} the vast majority of the ER network apparently experiences luminal Ca^{++} increases large enough to trigger the once-only luminescence of local resident ERaeq reporters, as >80% of sample luminescence is consumed. However, at any given time during the Ca^{++} repletion interval only a fraction of the entire ER membrane system contains sufficiently high Ca^{++} to support consumption of ERaeq at near-maximal rates (i.e., $K_{app} \sim 0.1 \cdot s^{-1}$) with the result that a relatively low average K_{app} is observed. The presence of 10 μM IP_3 during treatment with Ca^{++} and ATP decreased consumption of the sample's luminescence to only 40% of total over 2 min (see Figure 7C), indicating that a large fraction of the ER from which ERaeq is reporting functions as an agonist-sensitive Ca^{++} store. The rate of consumption for ERaeq reporter after re-addition of Ca^{++} to depleted cells was found to be slower for intact cells (see Figure 8, B and C, dotted lines) than for permeabilized cells (see Figure 7, A and B, dotted lines). This observation may simply reflect the function of the plasma membrane as a Ca^{++}-impermeable barrier in intact cells, could indicate partial loss of functional heterogeneity of ER microdomains in permeabilized cells, or may also relate to re-organization of organelle structures following incubation of intact cells at 4°C. Therefore, in our experiments luminescence signal is progressively removed from ER domains of high Ca^{++} while cells recover from Ca^{++} depletion. This in turn permits observation of Ca^{++} changes in ER domains containing low Ca^{++} through the monitoring of residual ERaeq signal.

The drop in ERaeq signal observed after addition of IP_3 to permeabilized cells, or with agonist-treated intact cells, attests to a functional role for ER microdomains that contain low levels of Ca. However, since these nonimaging measurements preclude observation of the spatial distribution of functional (i.e., nonbleached) reporter it remains obscure what molecular mechanisms may play a role in organization of the apparent compartmentation of ER Ca^{++} levels, or what cell functions are served by microdomains of variable Ca^{++} levels. Because the ERaeq probe is inherently limited to reporting over a finite range of $[Ca^{++}]$, the presence of a smooth continuum of ER Ca^{++} levels cannot be readily distinguished from a bimodal distribution of ER compartments into discrete regions of low versus high Ca^{++}. The electrochemical gradient available for producing rapid efflux of Ca^{++} from the ER is expected to be minimal in the case of domains containing $Ca^{++} \leq 1\ \mu M$. Consistent with this is the observation of a more rapid drop in ERaeq signal in agonist-treated cells as a function of ERaeq signal intensity. Addition of agonist to cells in which residual ERaeq signal indicated a Ca^{++} level of 1.4 μM resulted in a more rapid loss of ERaeq signal (Figure 8B, solid line), than for cells in which residual ERaeq signal indicated only 0.5 μM Ca^{++} before the agonist-induced drop (Figure 8C, solid line). Compartmentation of cytosol that produces regions with extremely low Ca^{++} is presumably required to achieve Ca^{++} release from neighboring ER with low Ca. Alternatively, loss of ERaeq signal from ER regions containing low Ca^{++} may result from intraluminal re-distribution of ion, rather than release into cytosol.

Predictions that $[Ca_i^{++}]_{ER}$ will fall in the range of 0.5–5 mM are based on extensive knowledge about both the function and molecular composition of the ER, which is well known to be an important source of agonist-sensitive Ca^{++} that undergoes rapid turnover. First, calsequestrin and calreticulin are examples of ER luminal proteins that possess high capacity for Ca^{++} binding (20–50 mol cation binding per mol protein) and low Ca^{++} affinity (K_d in the mM range). Such Ca^{++}-buffers are known to exist at high abundance inside the ER and are therefore well suited for storage and buffering of luminal ER Ca^{++} at concentrations around 1 mM (for reviews see Sitia and Meldolesi, 1992; Pozzan *et al.*, 1994). Furthermore, estimates of change in Ca^{++} saturation of calsequestrin during release of enough ER Ca^{++} to produce a muscle twitch suggest that modest decrements in the level of Ca^{++} binding to calsequestrin occur during cell activation. This argues that inside the ER lumen ionized Ca^{++} available for extremely rapid permeation of Ca^{++} release channels is minimally in the range of 10^{-4} M (Volpe and Simon, 1991). X-ray microprobe analyses of Ca^{++} mass in the ER also argue for $[Ca_i^{++}]_{ER}$ in the millimolar range (Somlyo *et al.*, 1980); however, the accuracy of such estimates is limited by incomplete knowledge of the concentration of Ca-buffering proteins in the ER. Finally, measurements of Ca^{++} changes inside intracellular membrane systems of rat gonadotropes (Tse *et al.*, 1994) and rabbit gastric gland cells (Hofer and Machen, 1993) have been performed with nonspecifically organelle-trapped mag-fura-2 or mag-indo-1 fluorescent dyes. In contrast to the ae-

quorin-based estimates reported here and those of Kendall *et al.* (1994), the measurements with trapped fluorescent dyes indicate average Ca^{++} levels of 30–200 μM. Reasons for such large discrepancies among methods may include cell type-dependent differences in volume or Ca^{++} capacity of intracellular organelles (for example, secretory vesicles or their precursors may be present in high abundance in secretory cell types; also, calsequestrin and calreticulin are not uniformally found at high abundance in all ER sub-compartments or in all cell types), interference by Mg^{++} (the presence of unaccounted Mg^{++} would lead to overestimation of $[Ca^{++}]$ by mag-fura-2 but underestimation of $[Ca^{++}]$ by aequorin), or overloading of fluorescent indicator into weakly buffered compartments of small volume.

The estimates of $[Ca_i^{++}]_{ER}$ of $\leq$1–2 μM that we have obtained with ERaeq are satisfactorily explained by consideration of the unique properties of aequorin's Ca-activated chemiluminescence. Ca-triggered photon emission by aequorin results in progressive consumption of the Ca-reporting luminescent activity because the chemiluminescent event can occur only once for each bound, nonoxidized coelenterazine molecule (for review, see Blinks *et al.*, 1976). For this reason it is necessary to convert the instantaneous photon production rate detected at the photomultiplier tube to a fractional loss rate (or the apparent rate constant K_{app}) to equate the magnitude of luminescence observed at any given time during the measurement to a corresponding Ca^{++} concentration (see "Measurement of Luminescence" in MATERIALS AND METHODS). Another consequence of the "once-only" chemiluminescence reaction that is extremely important for interpretation of results obtained with targeted forms of aequorin relates to the loss of signal from cell microdomains where high levels of Ca^{++} are found. Because Ca^{++} binding to aequorin produces photon emission only one time for each bound chromophore molecule, any ER compartment within which Ca^{++} rises to levels that produce high rates of ERaeq consumption will rapidly be depleted of reporter activity and eventually rendered nonobservable by measurement of photon production. Re-binding of fresh chromophore regenerates holo-ERaeq far more slowly than luminescence can be produced by Ca-activated consumption of preformed ERaeq (see Figure 9B).

The principal advantage of using organelle-targeted aequorins is that structural motifs of well-characterized organelle resident proteins can be exploited for highly specific placement of the Ca^{++} reporter. This feature permits selective monitoring of Ca^{++} changes in sub-cellular compartments of intact cells. Other workers have used similar approaches to measure nuclear-specific Ca^{++} changes using both targeted aequorins (Brini *et al.*, 1993; Badminton *et al.*, 1995) and with a Calcium Green dextran tagged with a nuclear localization sequence (Allbritton *et al.*, 1994). In addition, mitochondrial-specific Ca^{++} changes studied with a targeted aequorin have been found to occur even when agonists produce average cytosolic Ca^{++} increases that are lower than the K_m for mitochondrial Ca^{++} transport (Rizzuto *et al.*, 1993), and during depolarization-induced Ca^{++} entry (Rutter *et al.*, 1993). Such measurements with organelle-directed Ca^{++} reporters allow monitoring of cell functions that occur in sub-cellular domains that are not resolvable by conventional microscopy techniques. Based on the knowledge that the GA and ER membrane systems contain regions where luminal $[Ca^{++}]$ exceeds 3–10 μM, we are currently developing targeted aequorin probes with lower affinity for Ca^{++}. Meanwhile, the chimeric aequorin reporter described in this paper appears to be useful for studying those portions of the ER where Ca^{++} is present in low micromolar concentration.

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Note added in proof: During the review process for this paper another report (Montero, *et al.* (1995), EMBO J. *14*, 5467–5475) was published in which the use of a different ER-targeted chimeric aequorin was also described for direct monitoring of free Ca^{++} concentration in the ER lumen.

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