Use of aequorin-based indicators for monitoring Ca^{2+} in acidic organelles☆

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ABSTRACT

Over the last years, there is accumulating evidence that acidic organelles can accumulate and release Ca^{2+} upon cell activation. Hence, reliable recording of Ca^{2+} dynamics in these compartments is essential for understanding the physiopathological aspects of acidic organelles. Genetically encoded Ca^{2+} indicators (GECIs) are valuable tools to monitor Ca^{2+} in specific locations, although their use in acidic compartments is challenging due to the pH sensitivity of most available fluorescent GECIs. By contrast, bioluminescent GECIs have a combination of features (marginal pH sensitivity, low background, no phototoxicity, no photobleaching, high dynamic range and tunable affinity) that render them advantageous to achieve an enhanced signal-to-noise ratio in acidic compartments. This article reviews the use of bioluminescent aequorin-based GECIs targeted to acidic compartments. A need for more measurements in highly acidic compartments is identified.

1. Introduction

Ca^{2+} is a key intracellular second messenger that controls a plethora of physiological functions, ranging from exocytosis and muscle contraction to control of transcription and proliferation [1].The Ca^{2+} signal is organized into spatially distinct domains, allowing localized regulation of different processes. Over the last years, there is accumulating evidence that acidic organelles are bona fide intracellular Ca^{2+} stores, able to generate Ca^{2+} signals and to respond to them [2,3]. Acidic compartments in animal cells include large organelles like the Golgi apparatus or small ones like endosomes (early, recycling and late), lysosomes and secretory vesicles. In plant cells, there are also acidic compartments like vacuoles, thylakoid lumen of chloroplasts and apoplasts. Hence, reliable Ca^{2+} recording is essential for dissecting the contribution of a particular acidic organelle to the overall Ca^{2+} signal, and for gaining insight into specific organelle function.

Pioneering work assessing Ca^{2+} dynamics in acidic organelles used chemical fluorescent Ca^{2+} indicators commercially available (such as Fura-dextran, Rhod dextran or calcium orange-5N). These studies provided valuable insights on the physiology of these organelles [4]. Later, the advent of genetically encoded Ca^{2+} indicators (GECIs) facilitated the specific localization of the probe, being the Golgi apparatus, the first acidic organelle reported as a Ca^{2+} store [5]. In general, there are two classes of GECIs: fluorescent and luminescent. The fluorescent GECIs are the most widely used, but most of them are pH sensitive and lose their fluorescence at a pH lower than their neutral pK$_a$ (~6), compromising use in acidic organelles. In general, citric or yellow fluorescent proteins are less sensitive to pH than green fluorescent proteins (GFPs), and they have been fused to calmodulin to generate more acidic-resistant GECIs for the Golgi apparatus (YC or D1) [6].

The bioluminescent GECIs are mostly based on the photoprotein aequorin. The use of bioluminescence offers several advantages that can be especially useful to monitor acidic organelles. Here, we review the use of aequorin-bioluminescent probes to study Ca^{2+} signalling in acidic organelles and summarize technical approaches that have been employed to genetically target the probe into these organelles.

2. Aequorin-based photoproteins: generalities

The photoprotein aequorin is a monomeric 22 kDa, Ca^{2+}-binding protein of 189 amino acid residues originally extracted from the *Aequorea victoria* jellyfish. It consists of two components: the apoprotein (apoaequorin) and the chromophore, consisting of the luciferin coelenterazine (a substitute imidazopyrazinone) and molecular oxygen. Its full structure was originally determined by X-ray crystallography [7] (Fig. 1A). It contains four helix-loop-helix "EF-hand" domains (I to IV), homologous to the Ca^{2+} binding sites of calmodulin, of which three (I, III and IV) are functional and can bind Ca^{2+}. They are arranged into two pairs to form the globular molecule. The chromophore is composed of oxygens bound to the C-2 carbon of coelenterazine in the form of peroxide or hydroperoxide. The binding site of the chromophore to apoprotein is a hydrophobic core pocket located in the central part of the protein. When Ca^{2+} binds to aequorin, an intramolecular reaction occurs in which coelenterazine is oxidized by the bound oxygen to coelenteramide and CO_2, yielding blue light at maximum wavelength of 470 nm (Fig. 1B). Apoaequorin can be regenerated into active aequorin by incubation with coelenterazine, oxygen and a thiol agent, although this reaction is extremely slow and, in practice, the light emission is irreversible in experiments in vivo [8]. The luminescence reaction exhibits an absolute requirement for the C-terminal proline, that does not permit to be fused, deleted or substituted [9].

Aequorin was the first proteinaceous probe used to monitor intracellular levels of Ca^{2+}, when injected into excitable cells to follow the $[Ca^{2+}]_C$ transients associated with action potentials [10]. After the cloning and expression of its cDNA was reported in 1985 [11,12] recombinant aequorins were extensively used to monitor Ca^{2+} transients in living cells, in organelles and in whole organisms [13]. The use of aequorin bioluminescence offers several advantages: 1) It does not require excitation, thus avoiding cytotoxic effects caused by irradiation. 2) It displays a high signal-to-noise ratio, typically in the range of 100–1000, in comparison with 5–10 for fluorescent sensors. This is due to the combination of a very low background (mammalian cells do not contain bioluminescent proteins) and a high dynamic range. 3) The affinity of aequorin for Ca^{2+} can be tailored to match the expected Ca^{2+} concentration in a particular compartment. This is achieved by pairing different aequorin variants with various synthetic coelenterazines (see Section 3), resulting in the coverage of the $[Ca^{2+}]$ range from 10^{-8} to 5×10^{-3} M. 4) Aequorin is marginally sensitive to pH variations in the range 7.2–6.0 pH (see Section 4). 5) Aequorin can be specifically targeted to a particular subcellular location by fusing its cDNA to a minimal targeting sequence. 6) Calibration can be easily performed by calculating the Luminescence/total Luminescence ratio. Experimentally, this is performed by lysing the cells at the end of each experiment and collecting the light emitted by the remaining aequorin triggered by saturating Ca^{2+}. This signal is calibrated into $[Ca^{2+}]$ values, using the calibration curves previously reported [14].

In jellyfish, the excited energy of the luminescent substrate coelenterazine bound to aequorin is efficiently transferred intermolecularly to the acceptor *Aequorea* Green Fluorescent Protein (GFP) by a Bioluminescence Resonance Energy Transfer (BRET) mechanism. Based on this natural intermolecular BRET, fusions of GFP to aequorin (GFP-aequorin, dubbed GA) have been developed [15]. They display a shifted luminescence spectrum of green light (maximum at 503 instead of 470 nm; GA and GAP in Fig. 1B) and have several advantages like a higher stability, an increased luminescence yield (~10 fold) and the possibility to observe the GFP fluorescence as a readout of the expression level and the subcellular localization of apoaequorin in living cells. By replacing of the GFP gene with other spectral fluorescent variants, the palette of BRET-based complexes has been expanded. Among these are red aequorins, chimeras with Venus, mRFP, Tomato or mOrange (Redquorin in Fig. 1B; [16]). These fusions have also been targeted to subcellular organelles, hence increasing the number of applications, including simultaneous monitoring of Ca^{2+} concentrations in various organelles [17,18].

3. Choosing the appropriate affinity

The original measurements with aequorin were carried out using wild type aequorin reconstituted with native coelenterazine. This system is adequate for measuring Ca^{2+} in the cytosol; however, it gets saturated and rapidly consumed in compartments of high Ca^{2+} such as the endoplasmic reticulum (ER) or other Ca^{2+} stores. In order to lower aequorin affinity to these high levels of Ca^{2+}, an aequorin mutant was engineered that contains the D119A substitution in the third EF-hand domain [19]. Other mutants carrying various mutations in other EF-hand have also been generated (see below). Further decreases in the affinity for Ca^{2+} can be achieved by using synthetic analogues of coelenterazines like *n* coelenterazine [20]. Thus, by combining different forms of aequorins (wild type or mutated variants) with different coelenterazines (native, *n* or others) $[Ca^{2+}]$ can be measured in a wide range, from 10^{-8} to 10^{-3} M (systems 1, 2 and 3 in Fig. 2). Like for any GECI, choosing an aequorin/coelenterazine system with the appropriate affinity for a particular subcellular domain is crucial to obtain reliable measurements. For example, cytosolic or nuclear Ca^{2+} require a high affinity system like the one comprising wild type aequorin /native coelenterazine (system 1)
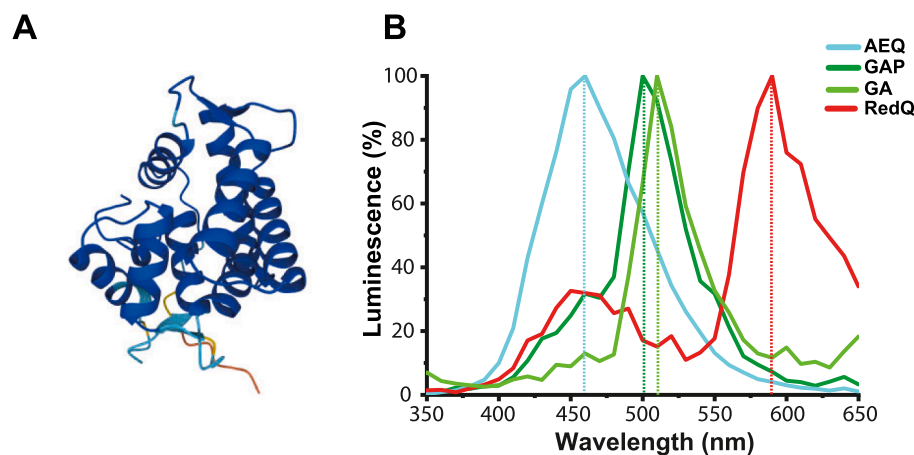
Fig. 1. Structure of aequorin and bioluminescence spectra of selected aequorin variants. A. 3D structure of wild type aequorin taken from Uniprot (https://www.uniprot.org/uniprotkb/Q6J4T7/entry; [54]). B. Wild type aequorin (AEQ, blue), GFP-aequorin (GA, light green), GAP (dark green) and Redquorin (RedQ, red) carrying the mutations N26D and Q168R, all reconstituted with native coelenterazine. Protein extracts were obtained from *E.coli* for AEQ, GA and GAP, and from transfected HEK cells for RedQ. Proteins were reconstituted 2 h with native coelenterazine (2 μM). Protein (40–300 μg) were placed in each well of a 96-well plate in a buffer containing 145 mM KCl, 5 mM NaCl, 1 mM MgCl$_2$, 0.1 mM EGTA and 20 mM Na-HEPES (pH 7.2). Luminescence spectra were performed in a Cytation 5 multimode plate reader (BioTek). 25 μL of 1 mM CaCl$_2$ (final $[Ca^{2+}]$ was 0.1 mM) was injected, and data were collected between 300 and 700 nm range each 10 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
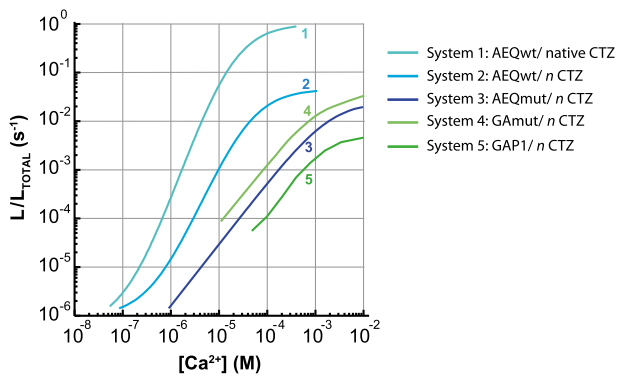
Fig. 2. Calibration curves of various aequorin systems used to measured Ca^{2+} in acidic organelles. (1) Wild-type aequorin (AEQ) reconstituted with native coelenterazine (CTZ); (2) wild-type AEQ with *n* coelenterazine; (3) low Ca^{2+} affinity D119A mutated aequorin (^{D119A}AEQ) and *n* coelentarazine; (4) GFP-^{D119A}AEQ (also named GAmut) with *n* coelenterazine; (5) GAP1 (GFP-$^{D117A, D119A, D163A}$AEQ) reconstituted with *n* coelenterazine. All calibrations were performed at 22 °C. Ordinate units are L (counts per second)/L_{TOTAL} (total counts) (s^{-1}). Data are taken from [14,17,20,22].

that allows measurements at around 10^{-6} M. However, at [Ca^{2+}] such as 5 μM, the consumption rate of this aequorin is around 1 % per second, leading to consumption of most aequorin within few minutes. So, for mitochondrial Ca^{2+} recordings, wild type aequorin/n coelenterazine (system 2) would be more appropriate for the range of 2–20 μM. Measuring [Ca^{2+}] within the lumen of high Ca^{2+} content requires the lowest affinity system, a combination of mutated aequorin/ n coelenterazine (system 3). This system allows reliable estimations in the range of 20–1000 μM and permits monitoring of dynamics in the lumen of the ER, the Golgi apparatus, and overloaded mitochondria. Importantly, the fusion GFP-aequorin does not significantly modify the affinity of aequorin for Ca^{2+} (compare system 4 and 3, in Fig. 2). Most reports on Ca^{2+} in acidic compartments of animal cells have been performed using low affinity aequorin versions (see Section 5 and Fig. 3).

The low affinity aequorin (systems 3 and 4, Fig. 2) has been instrumental for getting insights into the high Ca^{2+} (or overloaded) organelles in a broad variety of cell types and physiological or pathophysiological conditions [21]. However, at resting luminal [Ca^{2+}] this indicator gets consumed within a few minutes and is not appropriate for longer experiments. In order to overcome this limitation, lower affinity aequorins have been generated. GAP1 is a variant of GAP that contains three

substitutions (D119A, D117A and D163A) in the III and IV EF-hands of aequorin. This apoaequorin mutant combined with the *n* coelenterazine reduced the affinity of GAmut for Ca^{2+} by one order of magnitude (system 5 in Fig. 2) and this highly improved the time course (up to 1 h) of the experiment and, importantly, the accuracy of the quantification. Thus, GAP1 has recorded intraluminal Ca^{2+} in the 100–2000 μM range in the ER or Golgi apparatus, as expected for these organelles [22]. Another low affinity aequorin mutant (N28A/D119A) has proven effective in the ER and mitochondria and it has the advantage of not requiring depletion of the ER of Ca^{2+} prior to the reconstitution of aequorin [23].

4. The issue of the pH

A defining feature of the organelles of the secretory and endocytic pathways is their acidic nature. V-ATPase is the essential driver of organellar acidification, and it is generally assumed that the luminal acidity is achieved by the combined actions of vacuole H$^+$–ATPases and ion exchangers such as Cl$^-$/H$^+$, Na$^+$/H$^+$ or K$^+$/H$^+$. The luminal pH is not homogeneous throughout the secretory or endocytic pathways (Fig. 3): whereas the pH of the ER is near neutral, similar to that of the cytosol, downstream compartments become progressively more acidic. This acidification is important for proper post-translational processing, sorting and transport of newly synthesized proteins. Thus, the cis-Golgi is significantly more acidic (pH ~ 6.7) than the ER and the acidification increases in subsequent cisternae of the Golgi complex, reaching pH ~ 6.0 in the trans-Golgi network. Finally, the pH of secretory vesicles has been reported to be as low as 5.2–5.5. On the other hand, the organelles along the endocytic pathway drop from early endosome (pH 6.5) to late endosome (pH 6.0) and, finally, reaches its maximal acidity in the lysosome (pH 4.5–5.0).

The main caveat of Ca^{2+} indicators targeted to acidic environments is their functionality at these low pHs. This is a major problem for most of the fluorescent indicators, whose fluorescence is, in general, very sensitive to pH. Pioneering work showed that the light yield of the Ca^{2+}-triggered aequorin reaction was insensitive to pH between pH 6.0 and pH 8.5 [24,25]. Although the properties of aequorin at more acidic pH are not well described, this has prompted the use of aequorin to measure Ca^{2+} in acidic compartments.

5. Use of aequorin-based probes to monitor Ca^{2+} in acidic organelles

Experimentally, specific targeting is accomplished by attaching an
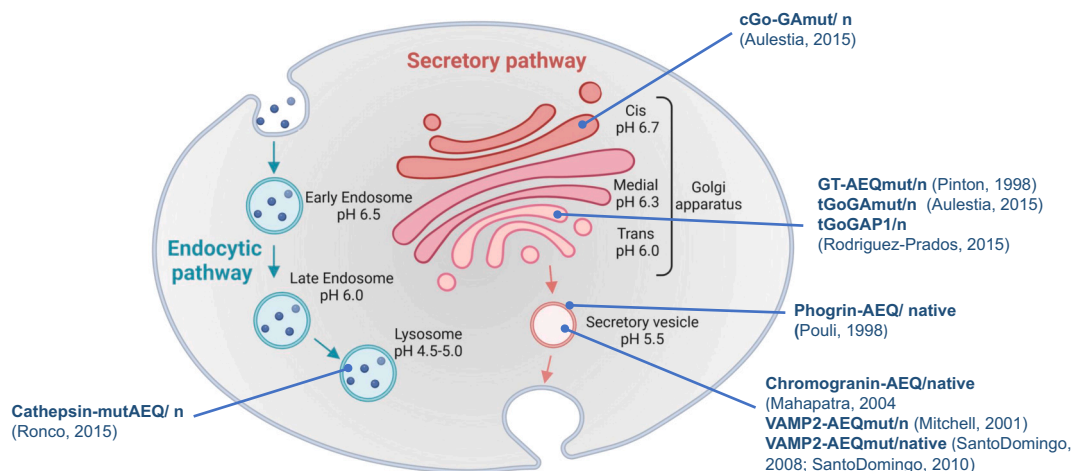
Fig. 3. Aequorin-based probes used for Ca^{2+} measurements in acidic organelles in animal cells. pH gradually drops along both secretory pathway (pink organelles) and endocytic pathway (blue organelles). AEQ, aequorin; AEQmut, D119A aequorin; cGo, cis-Golgi; tGo, trans-Golgi network (TGN); n, *n* coelenterazine; native, native coelenterazine. Modified from [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organelle-specific signal sequence or a targeting peptide derived from resident proteins to either the N- or the C-terminus of the sensor. However, due to the absolute requirement for the Proline residue at the C-terminus of the aequorin protein for bioluminescence activity, all targeting signals must be placed in the N-terminus [9]. Aequorin has the merit to be the first GECI to be targeted to a subcellular location namely the mitochondrial matrix [26] and the ER [19]. Since then, aequorin has been successfully used for detecting Ca^{2+} dynamics in several organelles with neutral pH, including nucleus, plasma membrane and peroxisome. The reader is referred to reviews covering targeting aequorin to organelles with more details [13,27–29].

Acidic organelles are believed to be important for Ca^{2+} signalling since they can accumulate high Ca^{2+} levels. Due to their acidic nature, it is challenging to monitor Ca^{2+} signals with fluorescence Ca^{2+} indicators, both chemical or proteinaceous ones, because most of them are pH sensitive and their fluorescence is quenched at acidic pHs. By contrast, aequorin can still be functional at acidic pHs and this is probably the reason why it has been often chosen as a Ca^{2+} sensor for acidic organelles. In fact, aequorin was the first GECI targeted to the lumen of the Golgi apparatus, and this was instrumental to demonstrate the high Ca^{2+} content in this organelle (see Golgi apparatus section). Additionally, other relevant features are a larger dynamic range and a steeper Ca^{2+} dependence compared with those of fluorescent Ca^{2+} indicators. A further reason is the extremely low background of luminescence in cells, which permit the detection of a specific signal even at very low protein expression, resulting in an excellent signal to noise ratio. This is particularly relevant for small organelles, such as most acidic ones, where the overall level of expression of the Ca^{2+} sensor is not as high as in larger organelles.

Examples of Ca^{2+} measurements in acidic organelles by luminescence include Golgi, secretory vesicle, vacuoles, the thylakoid lumen and the apoplast of plant cells and lysosomes (Fig. 3). We will next discuss in more detail monitoring of Ca^{2+} in these compartments, ordered by increasing acidity.

5.1. Golgi apparatus

The studies on Ca^{2+} signalling in this organelle have been dominated by Ca^{2+} measurements with aequorin. A pioneering study employed a chimeric aequorin consisting of a fragment of sialyltransferase fused to mutated aequorin [5]. The fusion polypeptide was retained in the Golgi apparatus due to the 17 amino acid membrane-spanning domain of sialyltransferase, a resident protein of the lumen of the *trans*-Golgi network (TGN). In this and follow-up studies aequorin was instrumental to establish the Golgi apparatus as a bona fide Ca^{2+} store and to characterize the roles of secretory pathway Ca^{2+} ATPase 1 (SPCA1) [30] and sarco-endoplasmic reticulum Ca^{2+}- ATPase (SERCA) [31] in accumulation of Ca^{2+} in this organelle. Although the Golgi apparatus is a heterogeneous organelle composed of three main subcompartments (*cis*-, *medial*, and *trans*-Golgi), most of the original studies considered it as a homogeneous compartment. This, together with a potential mislocalization of the probe due to its over-expression may account for conflicting results on functional expression of IP3Rs or SERCA in the Golgi apparatus [6]. In order to address the issue of heterogeneity within this organelle, aequorin was targeted to the *trans*- and *cis/medial* Golgi Ca^{2+} by fusing it to targeting sequences derived from the galactosyltransferase and acetylglucosaminyltransferase, respectively [32]. This study showed that both *cis*- and *trans*-Golgi accumulate Ca^{2+} to high concentrations (200–300 μM), through SERCA and SPCA1, respectively.

In contrast to most of the GECIs for which the Ca^{2+}-responsive element consists of calmodulin or troponin, the GAP family of Ca^{2+} sensors are based on the photoprotein apoaequorin. This makes GAPs a unique dual GECI, able to operate either as a fluorescent or as a bioluminescent sensor, depending on whether it is reconstituted with the cofactor coelenterazine. The fluorescent indicator is suitable for imaging, as binding of Ca^{2+} renders GFP to be dependent on Ca^{2+}. The low

affinity GAP member, GAP1, has been successfully used to monitor $[Ca^{2+}]$ in the lumen of the ER in various cell types and organisms, including in transgenic contexts, both in mice and flies. GAP1 has been used as a Ca^{2+} sensor for the Golgi apparatus, by fusing mutated aequorin to a fragment of galactosyltransferase I to generate goGAP1 (Fig. 4A) [33]. The correct localization of the goGAP1 protein in the Golgi cisternae was assessed by immunostaining using an antibody against GFP visualized by electron microscopy (Fig. 4B) [34]. Expression analysis of goGAP1 protein in a stably expressing HeLa cell line revealed a characteristic juxtanuclear punctate-staining pattern that colocalized with the trans-Golgi network (TGN) marker, TGN46 (Fig. 4C). goGAP1 operating as a fluorescent Ca^{2+} indicator can be visualized by recording the fluorescence at 470 and at 405 nm (Fig. 4D). $[Ca^{2+}]_{GO}$ changes upon stimulation with IP3-producing agonists (ATP + histamine), caused a reproducible and reversible decrease of the F470/F405 ratio (Fig. 4D, 4E). The same cell line, expressing the same goGAP1 indicator can be used in the luminescence mode following incubation with *n* coelenterazine (Fig. 4F) [22]. The resting $[Ca^{2+}]_{GO}$ reached was ~400 μM, similar to results obtained by a different probe (GoGA) previously used. A maximal stimulation with a mixture of ATP/Carbachol triggered a rapid Ca^{2+} release of ~70 % of the Golgi apparatus calcium content. The signal recovered immediately to the level of resting Ca^{2+} upon washing out of the stimuli. Further stimulations provoked reproducible Ca^{2+} releases (Fig. 4F).

Since GoGAP1 expressed in the Golgi apparatus can report both, fluorescent (Fig. 4D) and luminescent (Fig. 4F) Ca^{2+} signals, this allows a direct comparison of the two methods. The luminescent data are more robust than the fluorescent ones due to a higher-signal-to-noise ratio (~500 versus ~10), combined with a higher luminescent dynamic range. Additionally, the responses to consecutive applications of the same stimuli are more reproducible when using luminescence, due to the lack of excitation of the sample, which avoids quenching of the signal. Finally, calibration of luminescence is relatively straightforward, and it is performed for each experiment, and renders more accurate results. The main drawback, when applying the luminescent method in the Golgi apparatus, a high Ca^{2+} content organelle, is the requirement for depletion of Ca^{2+} prior to reconstitution of aequorin with its cofactor. This manoeuvre might not be tolerated by all cell types. Finally, the luminescent signal reflects the average of all the expressing cells in the well, and therefore, it does not report the variations among cells which might be relevant in some cases.

The acidic character of Golgi apparatus has also been confirmed in plants, where pH values of 6.3 and 6.8 have been reported for TGN and *cis*-Golgi, respectively [35]. By using a wild type aequorin targeted to Golgi apparatus of *Arabidopsis,* and reconstituted with native coelenterazine (system 1 in Fig. 2), a resting $[Ca^{2+}]_{GO}$ of ~0.70 μM was reported, a much lower value than its counterpart in mammalian cells [36]. Moreover, Ca^{2+} transients were detected in response to several abiotic stimuli, such as cold shock, mechanical stimulation, and hyperosmotic stress, whereas auxin induced a slow decrease of Golgi luminal Ca^{2+}. Further studies are required to clarify if this organelle has a high Ca^{2+} content in plants.

5.2. Secretory vesicles

A first attempt to measure $[Ca^{2+}]$ in the vicinity of a secretory vesicle (pH ~ 5.5) by luminescence was conducted by the group of Rutter, who expressed aequorin fused to phogrin, a membrane granule protein [37]. This indirect approach measured microdomains of $[Ca^{2+}]$ 10 fold higher than bulk cytosolic $[Ca^{2+}]$ detected upon stimulation of the insulinoma B-cell line MIN6 and adrenal phaechromocytoma PC12 cells. Later, the same laboratory developed another aequorin chimera based on vesicle associated membrane protein (VAMP2/synaptobrevin) fused to low affinity (mutated D119) aequorin and used it to report intravesicular Ca^{2+} concentration ($[Ca^{2+}]_{SV}$) in neuroendocrine cells [38]. In MIN 6 cells, the measured $[Ca^{2+}]_{SV}$ was in the range of 50 μM, one order of
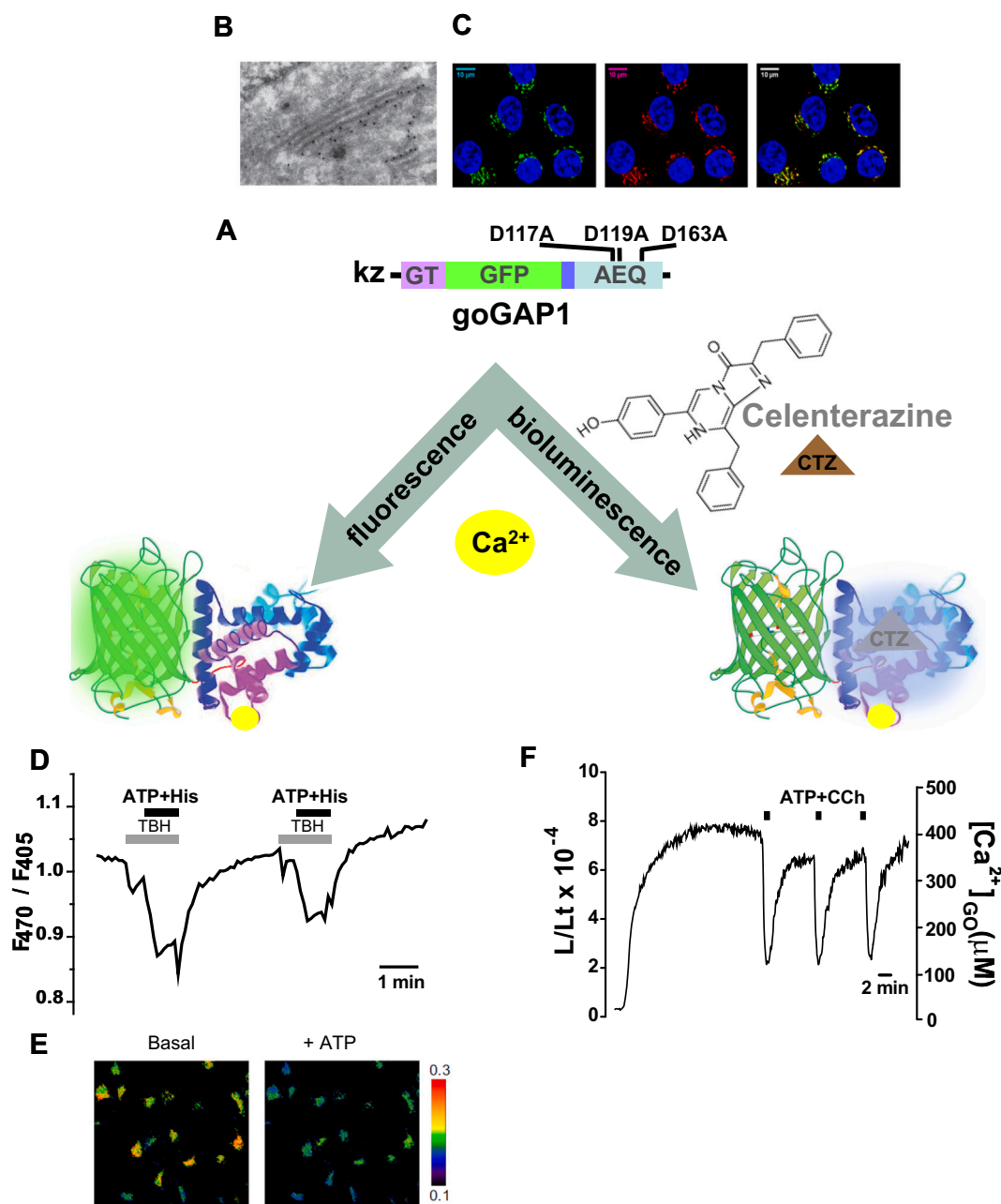
Fig. 4. Monitoring Ca^{2+} in Golgi apparatus by fluorescence and luminescence. A. Domain structure of goGAP1 construct targeted to the trans-Golgi network apparatus. kz, Kozac sequence; GT, galactosyltransferase I. B. Immunogold-EM of GAP1 in stably transfected goGAP1 HeLa cells fixed and labelled using an anti-GFP antibody. C. Localization of goGAP1 in the trans-Golgi network. Confocal fluorescence images of same cell line as in (B) expressing endogenous goGAP1 (green fluorescence) colocalized with antibody against TGN46 (red fluorescence), a marker of TGN. Nuclei were stained with DAPI (blue fluorescence). D-F. Performance of GAP1 as fluorescent probe (left) or luminescent (right). For luminescence emission goGAP1 must be reconstituted with n coelenterazine (CTZ). In contrast, coelenterazine is not required nor does it modify fluorescence emission. D. Representative response of fluorescent goGAP (expressed as F470/F403 ratio) to stimulation with ATP + histamine (100 μM each) in a goGAP1-expressing HeLa cell. Buffer contained 1 mM Ca^{2+} and 10 μM TBH as indicated. E. Fluorescent (ratio) image of the microscope field taken at rest (Basal) or during stimulation (+ATP). Most of the cells responded to the stimuli with a decrease of the Ratio F470/F405. Figure modified of [33]. F. goGAP1-HeLa cells were incubated with TBH (10 μM) for 10 min in Ca^{2+}-free medium and then with n coelenterazine for 1 h. Representative response to stimulation with ATP + carbachol (100 μM each) in 1 mM $CaCl_2$. Figure modified from [22].

magnitude higher than within the cytosol and lower than in the ER, and it was released by the second messengers ryanodine, cADPR and NAADP, but not by IP3 [39]. In parallel to Ca^{2+}, the intravesicular pH was determined using a fusion of VAMP2 and a mutated pH-sensitive GFP (pHluorin) resulting in a pH value of 6.3 [40]. The same VAMP2-Aequorin chimera was targeted to the secretory granules of other neuroendocrine cell types, e.g. PC12 cells, insulin-secreting INS-1 cells and chromaffin cells [41–43]. In these studies, aequorin was reconstituted with native coelenterazine (equivalent to system 2 in Fig. 2), more appropiated than the n analogue for the expected Ca^{2+} in the secretory vesicle, and use lanthanun to quench the light emission of aequorin present on the plasma membrane (~20 % of the total). With these two technical improvements it was shown that intravesicular Ca^{2+} was taken up through SERCA-type Ca^{2+} pumps reaching values around 20–40 μM in the three cell types studied. Intravesicular Ca^{2+} was released by ATP, and the second messengers IP3 and caffeine, but not by

NAADP or cADPR. Thus, different cell types could exhibit a different palette of receptors in their secretory vesicles.

An abundant Ca^{2+} binding protein in the secretory granules is the low affinity Ca^{2+}-binding protein chromogranin (A and B). In fact, a fusion of chromogranin A –aequorin was generated to use it as a Ca^{2+} indicator in the vesicular lumen of PC12 cells [44]. In this work aequorin luminescence is calibrated at pH 5.5 by comparing the L/Lmax values obtained at this pH with those at pH 7.0, being the difference around 3.3 fold lower at acidic pH. In spite of this calibration, measurements revealed a resting $[Ca^{2+}]_{SV}$ of 1.4 μM that increased up to 8.0 μM upon cell stimulation with exocytotic stimuli like ATP or nicotine. In general, this study found values one order lower than that found in other studies, probably due to the use of a wild type aequorin reconstituted with native coelenterazine, a combination (system 1 in Fig. 2) with too high affinity for the expected Ca^{2+} in the secretory vesicle.

5.3. Plant acidic organelles

Aequorin is probably the most frequently used GECI in plants, in which it has permitted non-invasive monitoring of free Ca^{2+} levels in different cell types and model species. The reader is referred to an excellent recent review on aequorin in plants [45]. In addition to the Golgi apparatus described above, aequorin-based sensors have been targeted to several other acidic compartments, namely the thylakoid lumen, the vacuole, and the apoplast.

The thylakoid lumen, a part of the cloroplast, displays a moderate acidic pH (between 5.8 and 6.5), that can drop to pH ~5 under certain physiological conditions [46]. The first study measuring Ca^{2+} in this subcompartment employed a high affinity probe comprised of the fusion between YFP and aequorin, and reconstituted with native coelenterazine (system 1 in Fig. 2) in *Arabidopsis thaliana*. Although no calibration curve at acidic pH was provided, the resting luminal $[Ca^{2+}]$ measured in the thylakoid was 0.5 μM, a 3- to 5- fold higher concentration than that found in the chloroplast stroma. Additionally, oxidative and salt stresses provoked intrathylakoid Ca^{2+} transients [47].

The vacuole is the largest organelle, 100-fold bigger than the ER, occupying ~80 % of the cell volume. It is the major Ca^{2+}-storing compartment, capable to accumulate $[Ca^{2+}]$ in the range of 0.2 to 1–5 mM [48]. With a pH of 5.2, the vacuole is often likened to the animal lysosome [49]. Due to these combined features, monitoring intra-vacuolar Ca^{2+} represents a formidable undertaking and it has only been indirectly addressed by targeting an aequorin probe to the cytosolic face of the vacuolar membrane [50]. This pioneering study showed a Ca^{2+} transient of ~2 μM in response to cold shock. This value was not significantly different to that recorded within the bulk cytosol, probably due to the choice of a high affinity system (aequorin and wild type coelenterazine) to monitor a Ca^{2+} microdomain.

The apoplast is the space outside the plasma membrane and it is another main source of Ca^{2+} in plant cells. Upon cell stimulation Ca^{2+} enters from the apoplast into the cytosol, and extracellular chelators like EGTA or BAPTA reduced the cytosolic Ca^{2+} signal. Like in the vacuole, the low pH values reported (between 5.3 and 6.7), combined with the high stored $[Ca^{2+}]$ (from 0.3 to 1 mM) have limited the number of studies in this compartment. An exception is the work employing an aequorin (both wild type and low affinity) fused to the chitinase signal sequence to deliver the probe to the apoplastic space of *Arabidopsis* [51]. This study measured Ca^{2+} and pH signals in vivo, comparing both responses in apoplast (pH 6.3) and cytosol (pH 7.2) to osmotic and saline stresses. In both compartments differential responses were observed although no Ca^{2+} calibration curve at acidic pH was provided.

5.4. Lysosomes

The lysosome is the most acidic organelle (pH 4.5) and it contains high levels of Ca^{2+} (~500 μM) [4]. A probe comprising a fragment of the lysosomal protease cathepsin D fused to the N-terminus of the mutated (D119A) aequorin has been developed to target it to lysosomes [52]. This study reported that aequorin was functional at extreme acidic pH (up to pH 3.8) although a calibration at acidic pH was not provided. With the targeted probe, the authors showed a resting lysosomal $[Ca^{2+}]$ of ~200 μM, similar to the value measured in parallel in the ER. SERCA inhibitor blocked lysosomal Ca^{2+} refilling and other treatments that mobilize acidic Ca^{2+} stores (GPN, Bafilomycin A1 and nigericin) affected both Ca^{2+} refilling of the lysosome and the ER in a similar way suggesting a cross-talk between ER and lysosome to mutually control their refilling. This probe was also used in a further study exploring the contribution of lysosomes and the transcription factor TFEB in the capacitative Ca^{2+} entry [53].

5.5. Concluding remarks

In sum, the photoprotein aequorin is a versatile probe that can be advantageous to monitor Ca^{2+} in acidic organelles due to a combination of features: marginal sensitivity to acidic pH (in the mildly acidic range); easy targeting; high signal-to-noise ratio; high dynamic range; easy affinity tuning; and an uncomplicated calibration. It has also some disadvantages like the requirement to deplete the target store of Ca^{2+} prior to aequorin reconstitution, or its limited capacity for performing cell imaging, especially in high Ca^{2+} compartments. In order to obtain reliable results, it is important to carefully choose the adequate aequorin affinity system. Examples of acidic organelles recorded by targeted aequorin include Golgi apparatus and secretory vesicles where resting luminal $[Ca^{2+}]$ are reported to be ~200–400 μM and ~50 μM, respectively, values distinct from those found in the ER. Moreover, both Ca^{2+} stores are mobilizable by IP3-producing stimuli. Other stimuli (NAADP, cADPR, ryanodine, or caffeine) can also evoke Ca^{2+} release from secretory vesicles, although the precise response might be cell type/tissue specific. Further studies are required to allow valuable results in e. g. highly dynamic compartments with extreme low pH values. These should include accurate monitoring of the signal corrected for the luminal pH and calibrated into $[Ca^{2+}]$.

CRediT authorship contribution statement

M.T. Alonso performed writing – original draft and conceptualization; P Torres-Vidal, B Calvo, C Rodriguez, A Delrio-Lorenzo and J Rojo-Ruiz performed investigation, visualization and review and editing; J Garcia-Sancho and S. Patel performed conceptualization and review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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