Original Article

Aequorin as Intracellular Ca^{2+} Indicator Incorporated in Follicular Lymphoma Cells by Hypoosmotic Shock Treatment

(intracellular Ca^{2+} indicator / aequorin / follicular lymphoma / hypoosmotic shock treatment / cell viability)

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Abstract. Natural proteins can be used in measuring intracellular Ca^{2+} concentration. As one of the Ca^{2+}-regulated photoproteins, aequorin has several advantages in comparison to widely used Ca^{2+} fluorescence indicators (e.g., fura-2, indo-1 and fluo-3), including high dynamic range and resistance to motion artefacts. However, incorporation of aequorin into cells remains a challenge. Hypoosmotic shock treatment was optimized and used as a method for loading aequorin into the cytoplasm of follicular lymphoma cells. Measurement of aequorin luminescence in the cells was performed using a luminometer with a sensitive photomultiplier tube and the luminescence intensity was recalculated into intracellular $[Ca^{2+}]$. The value of $(0.85 \pm 0.52) \cdot 10^{-6}$ M was found. We show that the optimized method of incorporation was effective for loading aequorin into follicular lymphoma cells *in vitro*. The cell viability remains high immediately after the procedure. This method can also be used for measuring intracellular Ca^{2+} concentration in other types of non-adherent cells.

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Abbreviations: GFP – green fluorescent protein, HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HOST – hypoosmotic shock treatment.

Introduction

Intracellular $[Ca^{2+}]$ in B lymphocytes has been studied in the past decade with a focus on the details of cellular processes in leukaemia and lymphoma cell signalling (Feske, 2007). Numerous techniques for analysing cellular or subcellular $[Ca^{2+}]$ have been established. Each method has its advantages when monitoring one or more types of cells or cellular processes.

Fluorescent probes (fura-2, indo-1, fluo-3, fluo-4) are widely used synthetic indicators of intracellular Ca^{2+} concentration in B lymphocytes. Calcium-regulated photoproteins have been used to monitor intracellular Ca^{2+} for more than 30 years (Vysotski et al., 2006). Aequorin, a 21.4 kDa bioluminescent protein that has been studied extensively since its discovery nearly 50 years ago (Shimomura et al., 1962; Shimomura, 1995), has numerous advantages over other indicators, in particular high signal-to-noise ratio, high dynamic range and resistance to motion artefacts. Because of its molecular weight, it is difficult to load aequorin into cells, while on the other hand, its diffusion to other cell compartments is very limited.

Aequorin cannot spontaneously penetrate into cells through the lipid membrane as can acetoxymethyl esters of fluo-4 or indo-1. Aequorin can be loaded into cells by the methods of microinjection, diffusion from patch-clamp pipette, and lipotransfer delivery or hypoosmotic shock treatment (HOST). Transfection with vectors of aequorin is greatly advantageous, because functional aequorin can be regenerated by subsequent treatment with coelenterazine. Microinjection has been used especially for neuronal cells (Meech, 1981), diffusion from patch clamp for neuronal and muscle cells, and lipotransfer delivery (Hallett and Campbell, 1982; Barber et al., 1996) as well as centrifugation (Borle et al., 1986) for several types of cells. HOST has been used espe-

cially for measurements in kidney and liver cells (Borle and Snowdowne, 1982; McNeil et al., 1984; Snowdowne and Borle, 1984). Aequorin can also be targeted specifically to individual compartments of the cell, such as the endoplasmic reticulum (Alvarez and Montero, 2002) or mitochondria (Núñez et al., 2010). Pozzan and Rizzuto (2008) discussed the problems concerned with targeting and summarized the advantages and drawbacks of using recombinant aequorin.

When cells are exposed to a hypoosmotic solution, the plasma membrane becomes permeable and the indicator can enter the cells. Although a short period of HOST exposure induces only low incorporation of aequorin into the cells, long-lasting HOST may cause loss of cells' functional integrity and change in their metabolism. A compromise should thus be found between attaining a sufficient level of aequorin incorporation and retaining the viability of B cells.

After aequorin's incorporation into cells, its luminescence is detected, then quantified, and this value is recalculated to $[Ca^{2+}]$. The method described by Allen et al. (1977) can be used for the recalculation. This method is based on the relationship between $[Ca^{2+}]$ and the L/L_{max} ratio, where L is the actual value of luminescence and L_{max} is the maximum value at aequorin discharge at a saturating Ca^{2+} concentration (see Material and Methods and Fig. 3 for details).

Aequorin mutants are currently under investigation with the aim of improving aequorin's bioluminescence properties (Rowe et al., 2008; Dikici et al., 2009). These studies use a two-pronged approach: altering the structure of coelenterazine and mutating the amino acid sequence of the apoprotein itself. As a result, photoproteins with different emission wavelengths are obtained. This allows their use in multi-analyte experiments and in multimodal imaging applications (Rowe et al., 2009).

Material and Methods

Loading aequorin into cells

WSU-NHL follicular lymphoma cells (Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were washed with Ca^{2+}-free Hank's buffer. Prior to use, the buffer was purified by Chelex 100 column (Bio-Rad Laboratories, Hercules, CA). Cell suspension in the quantity of 30 µl was added to 200 µl of hypoosmotic solution (redistilled water, 2 mM HEPES buffer, pH 7.1) and aequorin (Friday Harbor Laboratories, Friday Harbor, WA) at final concentration 0.1 mg ml^{-1} and the mixture was mixed gently. Next, 230 µl of hyperosmotic solution (NaCl 272 mM, KCl 10 mM, glucose 11 mM, HEPES 2 mM, pH 7.2, purified by Chelex 100 column) was added. The time between addition of hypoosmotic and hyperosmotic solutions varied from 10 s to 10 min. All reagents used in our work were purchased from Sigma-Aldrich, St. Luis, MO unless otherwise specified.

Measuring cell viability

The viability of cells was estimated using a Bűrker chamber before the loading procedure and 3, 6, 9, 12, 15, and 18 h thereafter. The live/dead cell viability kit (Invitrogen, a brand of Life Technologies, Grand Island, NY) was used and viability was expressed as the ratio of the absolute count of viable cells in the sample before and after the loading procedure.

Measurement of intracellular $[Ca^{2+}]$

After the loading procedure, cells were incubated for 10 min at 4 °C. The cell suspension was then transferred to a glass cuvette and inserted into a luminometer cuvette holder (Fig. 1). Injection of 100 µl of 0.1 M $CaCl_2$ solution followed. High Ca^{2+} concentration discharged extracellular aequorin. After this procedure, intracellular aequorin was the only remaining light source in the cuvette and, as depicted in Fig. 2, its L_1 luminescence signal was measured using a model R6095, 28 mm head-on type photomultiplier tube for 300–600 nm (Hamamatsu Photonics, Hamamatsu City, Japan). In order to measure the amount of intracellular aequorin, lysis buffer with 1% Triton X-100 was added into the cuvette, the cell membranes were immediately destroyed, and aequorin was exposed to a saturating concentration of Ca^{2+}. Peak luminescence signal L_{max} was measured. When all intracellular aequorin was consumed, the background luminescence signal (L_2) was determined. Measurements were repeated 20 times.

The method of Allen et al. (1977) was used to calculate $[Ca^{2+}]$ from the recorded luminescence signals. The $(L_1\text{-}L_2)/L_{max}$ ratio was calculated and a dose-response curve for aequorin at 1 mM Mg^{2+} concentration (Fig. 3, curve 2) was used to determine the $[Ca^{2+}]$ value.

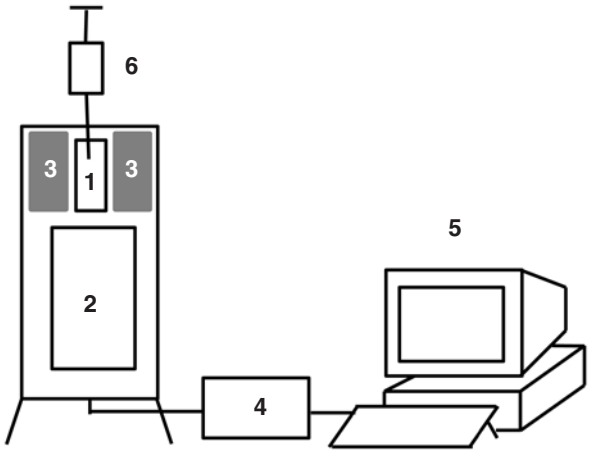

Fig. 1. Schematic diagram of the lab-made apparatus for luminescence measurement
(**1**) Glass cuvette with sample, (**2**) photomultiplier tube, (**3**) cuvette holder, (**4**) analogue/digital transmitter, (**5**) personal computer, (**6**) syringe

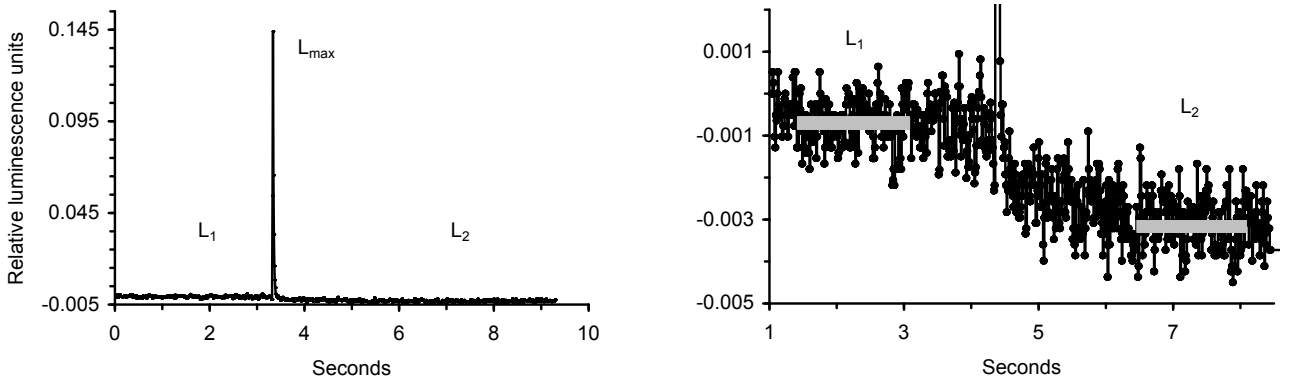

Fig. 2. Luminescence intensity of intracellular aequorin measured before and after cell lysis
Typical pattern of the cells after 2 min of hypoosmotic shock treatment is shown. L_1 – steady-state luminescence value; L_{max} – peak luminescence value after perforating the cells with Triton X-100 solution; L_2 – background signal. L_1 and L_2 values were computed as average signal value in the time intervals of 1.5–3.0 s and 6.5–8.0 s, respectively.

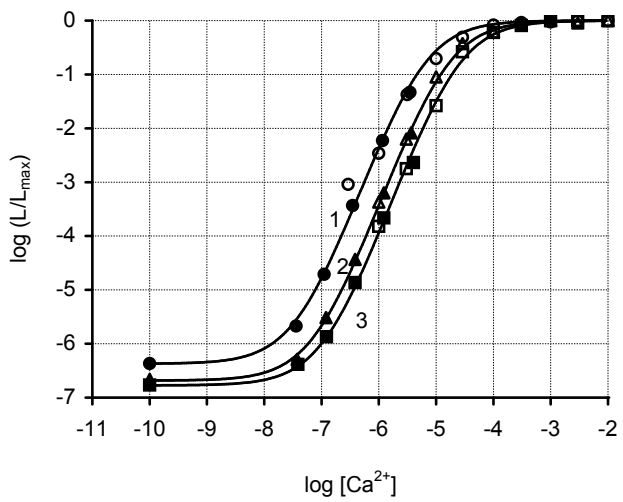

Fig. 3. Relationship between luminescence emission and Ca^{2+} concentration presented as a double logarithmic plot of relative luminescence intensity *vs.* Ca^{2+} concentration
Experimental data were obtained at various Mg^{2+} concentrations: curve (1) at 0 mM Mg^{2+}, (**2**) 1 mM Mg^{2+}, (**3**) 3 mM Mg^{2+}. Filled symbols represent calcium standard buffers (Klabusay and Blinks, 1996); empty symbols denote diluted calcium standards. The curves were calculated according to the nonlinear two-state model involving three Ca^{2+} binding sites in the aequorin molecule (Allen et al., 1977).

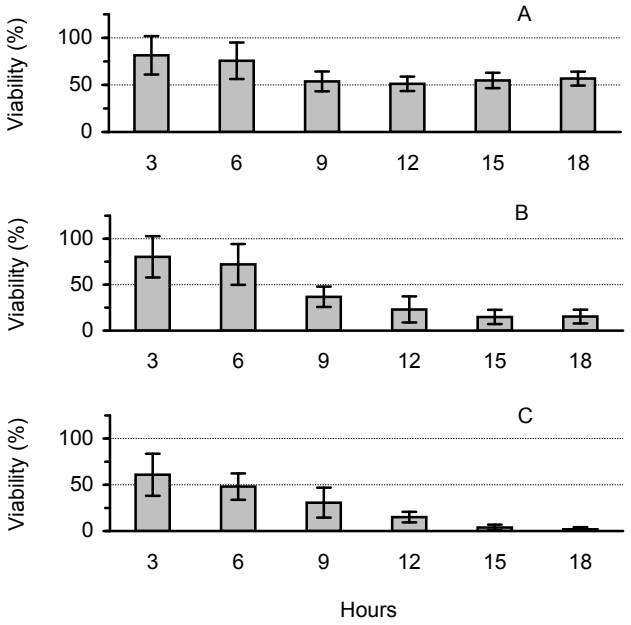

Fig. 4. Viability of WSU-NHL cells after treatment with hypoosmotic solution
Percentage of viability is shown relative to time after hypoosmotic shock treatment. Treatment times: (A) 2 min, (B) 4 min, (C) 6 min.

Green fluorescent protein loading

A comparison as to the effectiveness of phosphate-buffered saline and the hypoosmotic solution in incorporating macromolecules with molecular weights similar to that of aequorin into the cells was made using green fluorescent protein (GFP, purchased from Friday Harbor Laboratories, Friday Harbor, WA). GFP has molecular weight of 28 kDa versus aequorin's 21.4 kDa and a similar isoelectric point value. The presence of GFP in the cytoplasm was analysed by measuring its fluorescence signal using a model BD FACSVantage flow cytometer (BD Biosciences, San Jose, CA).

Results

While short-term hypoosmotic shock treatment (HOST) in the range of 10 to 30 s resulted in high viability of WSU-NHL cells, measurement of aequorin luminescence showed that almost no aequorin was thereby loaded into the cells. A treatment for 10 min, on the other hand, caused low cell viability. Therefore, treatment times of 2, 4, and 6 min were tested precisely. The results, obtained 3 h up to 18 h after termination of HOST, are shown in Fig. 4.

The results indicate that 2 min is the best period for HOST, as the cells did not lose their integrity while the viability and level of aequorin incorporation were sufficient (L_1 and L_{max} were well measurable). The value of

$(0.85 \pm 0.52)\cdot 10^{-6}$ M $[Ca^{2+}]$ was found by 20 times repeated measurement with WSU-NHL cells after 2 min HOST time.

The effectiveness of the HOST method for incorporating molecules larger than 20 kDa into cells was supported by flow-cytometry measurement of GFP fluorescence. Cells from hypoosmotic solution were more GFP positive. The median fluorescence intensity was 2.96 for control cells and 4.04 for cells loaded with GFP using the hypoosmotic solution.

Discussion

The importance of intracellular $[Ca^{2+}]$ in living cells has been known for more than 100 years. At the very beginning, intracellular $[Ca^{2+}]$ was measured only indirectly (using ionophores) or by complicated methods (e.g., using glass microelectrodes). The spectrum of cells suited to these methods was limited. Since synthesizing the first generation of intracellular trappable Ca^{2+} fluorescence indicators in 1980 (quin-2, fura-2), Ca^{2+} has emerged as a key second messenger in cells. The second generation of fluorescence indicators was represented by fluo-3, fluo-4, and indo-1.

During the 1980s and 1990s, aequorin was used as an alternative $[Ca^{2+}]$-sensitive probe. Many techniques for incorporating aequorin were developed, including, for example, direct injection and electrophoretic and hypoosmotic methods. However, the use of aequorin was limited, especially for robust cells like those of muscle, neural and ovarian tissues. Several experiments were based on parallel incorporation of aequorin and one of the fluorescence indicators (Johnson et al., 1985; Brini et al., 1995). Following great interest and growth in transfection methods during the 1990s, aequorin has been used for measurement in smaller cells (HeLa cells and others) and also in compartments within cells. The optimized HOST method for incorporating aequorin into B cells presented in this paper enriches the portfolio of basic methods for intracellular $[Ca^{2+}]$ measurement.

Main advantages and disadvantages of the HOST loading method

The precise location of aequorin in the cells after loading by HOST has not been well described in the literature to date, but experimental data show that aequorin should not diffuse into high-$[Ca^{2+}]$ compartments (endoplasmic reticulum, tubular systems and mitochondria) (Kendall et al., 1992). This is a great advantage of aequorin, because fluo-4 and other, similar low-molecular-weight probes diffuse from the cytoplasm into internal subcellular compartments.

In using aequorin, we also cannot rule out the possibility that elevated $[Ca^{2+}]$ exists in a small fraction of dying cells and that this elevated $[Ca^{2+}]$ results in aequorin's discharge and artificial increase of measured $[Ca^{2+}]$ (Johnson et al., 1985). On the contrary, however, our results show that this possible systematic error in quantification of $[Ca^{2+}]$ was probably minimal in our lymphoma cells, because more than 90 % of cells were viable at the time of measurement.

The question could be raised whether $[Ca^{2+}]$ homeostasis could be altered by osmoregulation during the loading period. It must be emphasized that there is no free Ca^{2+} present in the extracellular media after washing the cells with Ca^{2+}-free Hank's buffer. The presence of Ca^{2+} in extracellular media would cause rapid consumption of aequorin as well, which must be avoided. It has been demonstrated that during the procedure of osmotic restitution the plasma membrane slowly reseals and becomes impermeable to aequorin (Snowdowne and Borle, 1984). Also, as demonstrated in this paper, no change was shown in $[Ca^{2+}]$ fluxes between compartments; therefore, it could be assumed that the HOST technique does not grossly alter calcium homeostasis (Snowdowne and Borle, 1984). The used procedure of aequorin incorporation into cells and measurement of intracellular calcium is described and critically evaluated in the review published by Takahashi et al. (1999).

Another important property of any $[Ca^{2+}]$ probe is the molecule's influence on the viability and metabolism of the treated cells. Aequorin is a natural protein, and no physiological reaction induced by its presence in mammalian cells was observed or has been reported elsewhere. There is also no artificial buffering of intracellular $[Ca^{2+}]$. This is another advantage compared to fura-2, quin-2, and other fluorescent dyes.

Accuracy of $[Ca^{2+}]$ measurement using aequorin and HOST

There are only a few published studies using aequorin for determination of intracellular $[Ca^{2+}]$. A $[Ca^{2+}]$ level of 0.5–0.9 μM has been reported in platelets, depending upon composition of the surrounding medium (Ware et al., 1988). The results obtained using aequorin (Ware et al., 1989) were comparable to those from a study (Merrit et al., 1990) in which platelets were loaded with fluo-3 and intracellular $[Ca^{2+}]$ of 0.2 μM was estimated.

The value of $(0.85 \pm 0.52)\cdot 10^{-6}$ M intracellular $[Ca^{2+}]$ found in our work is comparable to 0.1 μM determined in B cells by using indo-1 (Hauschildt et al., 1991). In lymphocytes, a level of approximately 0.1 μM of intracellular $[Ca^{2+}]$ has been reported (Radošević et al., 1995).

We do not know the precise $[Mg^{2+}]$ in our B cells. We assume B cells to have the average intracellular $[Mg^{2+}]$ of 0.95 mM (Rink et al., 1982). For the dose-response curve that was measured *in vitro*, it was therefore assumed that $[Mg^{2+}]$ is 1 mM. The importance of this problem is discussed in the literature (Ware et al., 1988).

In previously published reports of experiments involving non-B cells and non-blood cells and using the HOST method of aequorin incorporation, the majority of measurements were performed in liver and kidney cells. The HOST method was applied to measure $[Ca^{2+}]$ in cultured kidney cells and the findings were 0.06 μM

(Snowdowne and Borle, 1984) and 0.07 μM (Snowdowne et al., 1985). In monkey kidney cells, the 0.057 μM Ca^{2+} concentration was determined (Borle and Snowdowne, 1982).

Although our experiments were performed on a time scale of just 1–10 min, other experiments using aequorin have demonstrated the possibility for continual precise measurement up to 90 min after aequorin loading as, for example, in kidney cells (Borle and Snowdowne, 1982). This points to an advantage of aequorin, because precise experiments over long time periods are impossible when using fluo-4 or indo-1 probes.

Future of aequorin and the HOST method

While B cells were treated in this work using aequorin and the HOST method, it can be anticipated that this method may be used for intracellular [Ca^{2+}] measurements also in other types of non-adherent blood cells. The size of the cells is not important. In contrast to some other methods, using HOST to introduce aequorin into small mammalian cells is not problematic. Based on previous data, the HOST procedure is believed not to have an impact on calcium ion homeostasis.

Intracellular calcium measurement using aequorin is often based mainly on quantification of steady-state intracellular [Ca^{2+}] and relative quantification of [Ca^{2+}] changes in the cytosol or subcellular compartments. High-resolution spatiotemporal analysis of calcium transit in subcellular and sub-cytosol areas could be performed in the future.

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