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Confocal Fluorescence Lifetime Imaging of Free Calcium in Single Cells

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Ca^{2+} concentrations in biological cells are widely studied with fluorescent probes. The probes have a high selectivity for free calcium and exhibit marked changes in their photophysical properties upon binding. The differences in the fluorescent lifetime of the probes can now be used as a contrast mechanism for imaging purposes. This technique can be further exploited for the quantitative determination of ion concentrations within the cells. We describe the use of a fast fluorescence lifetime imaging method in combination with a standard confocal laser scanning microscope for the determination of Ca^{2+} concentrations in single rat cardiac myocytes using the intensity probe CalciumGreen.

KEY WORDS: Confocal microscopy; fluorescence lifetime imaging; calcium green; Ca^{2+} imaging.

INTRODUCTION

Calcium plays an important role in controlling numerous biological processes. Free Ca^{2+} concentrations in biological cells are widely studied with fluorescent probes. The probes have a high selectivity for free calcium and exhibit marked changes in their photophysical properties upon binding. In particular, changes in the fluorescence intensity (intensity probes) or spectral shift (ratio probes) upon binding to Ca^{2+} are monitored. The main drawback of intensity probes is that the fluorescence intensity is affected by both the probe concentration and the free Ca^{2+} concentration. Consequently, a quantitative determination of Ca^{2+} distributions requires the probes to be partitioned homogeneously in the sample.

Conventional quantitative determinations of Ca^{2+} concentration with ratio probes overcome the dependence on local probe concentration by exploiting ratiometric procedures using excitation or detection at two wavelengths [1,2]. However, this calls, in general, for excitation in the UV region, which is cumbersome for work with living cells. In addition, quantification of the ratiometric procedures relies on involved *in vivo* calibrations of the observed spectral shifts.

The advent of fluorescence lifetime imaging techniques [3–6] opens new horizons for the quantitative determination of ion concentrations, in general, and of Ca^{2+} using intensity probes, in particular. The fluorescence lifetime imaged is determined by factors such as the chemical environment of a fluorescent molecule and thus provides valuable information about its ion binding states. Importantly, since the lifetime is independent of the fluorescence intensity, such measurements have wide-ranging applications to samples in which the probes have an inhomogeneous distribution. An additional advantage of the lifetime imaging technique is that

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the images are not compromised by photobleaching and absorption effects.

We describe here a fast fluorescence lifetime imaging method implemented on a standard confocal laser scanning microscope. The potential of the method is illustrated with the imaging of Ca^{2+} concentrations in single rat cardiac myocytes using the intensity probe CalciumGreen.

MATERIALS AND METHODS

The Confocal Fluorescence Lifetime Microscope

The fast fluorescence lifetime determination method employed by us combines pulsed excitation of the sample with fast time-gated detection. A commercially available confocal laser scanning microscope (CLSM) [7] was modified to produce short light pulses by the insertion of a fast Electro Optical Modulator (EOM) between the laser and the CLSM (see Fig. 1). The EOM (Quantum Technology 27-50) serves as an optical chopper for the standard low-power continuous-wave argon ion laser, producing light pulses of 488-nm light with a width of about 1 ns and a variable repetition frequency of up to 25 MHz.

The detection channel was also modified to accommodate the time-gated technique. A fast (0.8-ns-rise time) red-sensitive photomultiplier tube (selected Hamamatsu R1894) was used for photon counting purposes together with a fast discriminator having a 3-ns-pulse pair resolution (Phillips Scientific 6908). The signals were fed to two home-built time-gated counter circuits which were synchronized with the pulse generator driving the EOM. All the time-critical electronics were built using emitter-coupled logic (ECL) electronics. This detection circuit can be used at (continuous) count rates up to $25 \cdot 10^6$ cps without serious pileup effects.

The emitted fluorescence was collected in two time windows at different delay times with respect to the incoming excitation pulse (see Fig. 2). The ratio of the signals in the two windows, the window ratio, is now a measure of the fluorescence lifetime τ:

$$\tau = \frac{\Delta t}{\log(S_A/S_B)}$$

where Δt is the time offset between the two windows and S_A and S_B are the signal intensities in window A and B, respectively. This expression is exact for a monoexponential fluorescence intensity decay and yields an effective lifetime for the case of multiexponential decays.

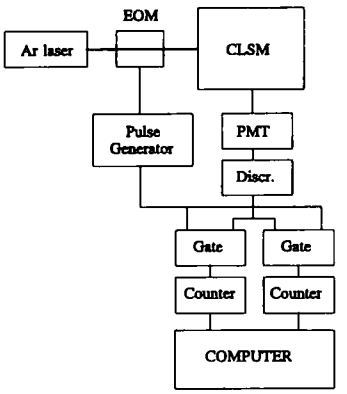

Fig. 1. The modified conventional confocal microscope. The sample is excited by brief light pulses produced by a CW Argon-ion laser and an electrooptical chopper. The fluorescence signal is accumulated in two time-gated counters.

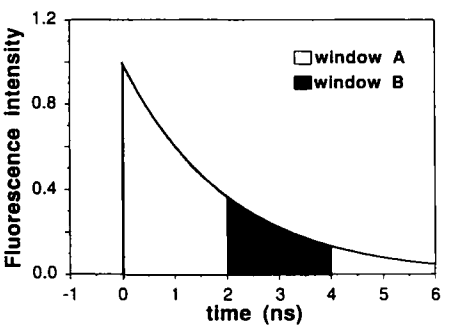

Fig. 2. In the rapid lifetime determination method the lifetime can be derived from the ratio of the accumulated intensities in windows A and B.

Moreover, it is valid only for very short excitation pulses and an ideal response of the detector system. In practice, the nonideal response is overcome by calibrating the instrument.

The experiments described in this study were carried out using the following window settings: The first window started 0.5 ns after the maximum of the excitation pulse and had a width of 1.9 ns. The second window started immediately after the trailing edge of the first and had the same width. It is important to note that in the time-gated detection method described above, the fluorescence emission is recorded in both windows for every excitation laser pulse, so that the window ratio is not affected by photobleaching effects.

Calibration

The lifetime response of the microscope was calibrated using a series of CalciumGreen (Molecular Probes,

Eugene, OR) containing calcium buffers at pH 7.5. The concentration of CalciumGreen in the buffers was 3.23 μ*M*, at which the samples exhibited an optical density of about 0.1.

The calcium buffers were prepared by mixing two buffers at different ratios: one containing KCl (150 m*M*), ATP (5 m*M*), EGTA (10 m*M*), Hepes (20 m*M*) at pH7.5 and the other containing KCl (150 m*M*), ATP (5 m*M*), Ca-EGTA (10 m*M*), Hepes (20 m*M*) at pH7.5. The free ionic calcium concentration in the calibration buffers ranged from 3 n*M* to 1 m*M*.

Cell Preparation

The experiments on single cardiac myocytes of rats were performed 4 or 5 days after the isolation of the cells. Prior to staining, the cells were carefully washed with a buffer solution containing NaCl (130 m*M*), KCl (4.7 m*M*), $CaCl_2$ (1.3 m*M*), NaH_2PO_4 (0.44 m*M*), $MgCl_2$ (1.1 m*M*), $NaHCO_3$ (20 m*M*), glucose (0.2%, w/v), and Hepes (10 m*M*), pH 7.4. Next the cells were incubated with 980 μl of the buffer and 20 μl of a 1 mg/ml DMSO solution of CalciumGreen/AM. After an incubation period of 60 min the cells were washed again with the buffer solution. The imaging experiments were carried out immediately after the staining procedure. The imaging experiments as well as the calibration of the microscope response were all carried out at room temperature.

RESULTS AND DISCUSSION

The results of the calibration procedure are summarized in Fig. 3, where the window ratio is given as a function of *p*Ca ($-^{10}\log[Ca^{2+}]$). It can be clearly seen that CalciumGreen is useful for fluorescence lifetime imaging in the *p*Ca range from 8.5 to 6.5 (Ca^{2+} concentration of 3–300 n*M*). This Ca^{2+} concentration range is particularly interesting for biological applications.

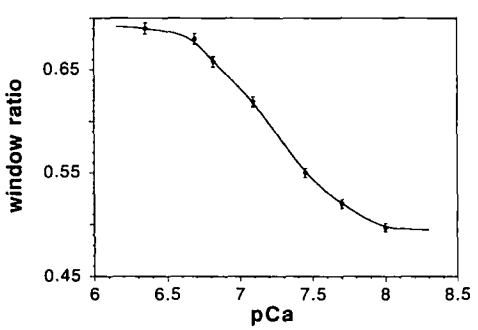

Fig. 3. The window ratio of CalciumGreen as a function of *p*Ca.

The results shown in Fig. 3 can be explained by assuming the existence of two states of the probe, one bound to Ca^{2+} and one free. Each state is characterized by its own distinct fluorescence lifetime. Consequently, the effective lifetime, obtained from the window ratio, is a weighted average of the lifetimes of the two states. The weighing factor depends on the relative concentrations of the free and bound probes, which in turn is determined by the Ca^{2+} concentration. The correctness of this description has been confirmed by standard time-correlated single-photon counting measurements, which indeed yielded a biexponential decay behavior. These will be described elsewhere.

At high Ca^{2+} concentrations (*p*Ca < 6) almost all the probes are bound to Ca^{2+}, so that the window ratio is determined by the fluorescence lifetime of the bound probe. In contrast, at low Ca^{2+} concentrations (*p*Ca $>$ 8.5), the window ratio is dominated by the fluorescence lifetime of the free probe. In the intermediate concentration regime the window ratio is the weighted average of the contributions from both the free and the bound probes.

A conventional fluorescence intensity image of a cardiac myocyte stained with CalciumGreen is shown in Fig. 4A. The fluorescence intensity in the nucleus of the cell is significantly higher than that in the cytoplasm, suggesting a higher Ca^{2+} concentration in the nucleus. Figure 4B shows the fluorescence lifetime image of the same monocyte. Here the gray-value is a measure of the window ratio and, thus, of the *p*Ca. It is estimated that the Ca^{2+} concentration amounts to 20 n*M* ± 8% in the nucleus and to 18 n*M* ± 18% in the cytoplasm. The errors in the calcium concentration were obtained from the gradient of the calibration curve (Fig. 3) and the local signal-to-noise ratio in the image. The latter was determined from the standard deviation as determined over 16 pixels.

The Ca^{2+} concentrations determined from the image are at the low end of the concentration of comparable myocytes determined using ratio probes. The ratio of the Ca^{2+} concentrations in the nucleus and the cytoplasm determined by lifetime imaging is significantly lower than the intensity ratio in the conventional intensity image. We ascribe this to a preferential partition of CalciumGreen into the nucleus. Finally, we want to note that the Ca^{2+} concentrations found by us are comparable to the values reported by Berlin and Konishi [8] using the ratio probe Fura-2.

The results presented here show that a conventional CSLM can be modified in a straightforward way for flu-

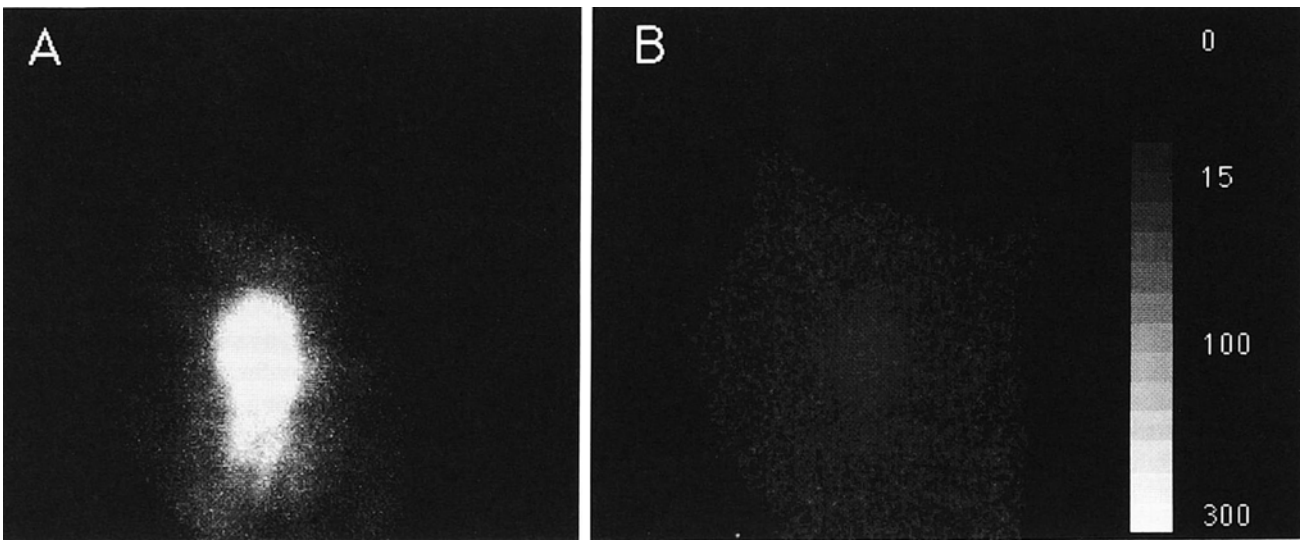

Fig. 4. (A) The fluorescence intensity image of a CalciumGreen-stained rat cardiac myocyte. (B) The fluorescence lifetime image of the same cell.

orescence lifetime imaging. This opens the way for the quantitative imaging of ion concentrations using intensity probes such as CalciumGreen and obviates the need for ratio probes with absorption bands in the UV. We note that the usefulness of CalciumGreen as a fluorescence lifetime probe was examined before by Lakowicz *et al.* [9].

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