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Fluorescence polarization: a novel indicator of cardiomyocyte contraction☆

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Abstract

The changes measured in intracellular fluorescein fluorescence polarization (IFFP) are used as a new tool for tracing cytoplasmic effects during contractile cycles of cardiac myocytes (1–2-day-old rat hearts), in addition to the established Ca^{2+} monitoring and/or videometric methods of tracking cell-shortening. This novel method was found to be non-intrusive to the contraction cycles. The decay of the transient IFFP signal (from 0.220 ± 0.01 to 0.170 ± 0.013) seems to be closely related to the extended phase of contractile activation. This fact was further supported when Ca^{2+} exchanger inhibitor was introduced and significantly decreased (90%) the rate of beats of contraction and IFFP, but not the Ca^{2+} beat rate changes. This result suggests that the IFFP indicator is probably associated with the physiological activation, rather than with Ca^{2+} alterations. The IFFP measure monitors the average of effective changes in the micro-viscosity of the cytoplasm protein matrix, associated with cellular activation.

Keywords: Fluorescence polarization; Cardiac cells; Contraction rate; Amplitude

The ability to trace various physiological events during contraction cycles of single cardiomyocytes is greatly increased with the use of intracellular probes. These measurements might contribute to the fundamental understanding of cellular stimulation and activation. Thus, during contraction cycles of myocardial tissue, rapid changes within single cells can be measured and correlated, e.g., membrane potential, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), and intracellular pH, compared to shortening and tension generation [4,24]. All these physiological variables show a change during stimulation and activation responses in a large number of cell types. Processes linking early events following cell stimulation and activation involve conformational changes of the cytosolic enzymes and/or their regulatory proteins, and dynamic reorganization of their intracellular matrix [15]. Such structural changes might be easily monitored via fluorescence polarization (FP) emitted from a fluorescent molecule, hosted by the cellular media. Fluorescence polarization is considered to be one of the first functional cytometric parameters [23] and was initially suggested by Cerceks as an indicator for lymphocyte activation [23].

The FP is defined as the ratio $(FI_{II} - FI_{+})/(FI_{II} + FI_{+})$, where (II) and (+) correspondingly indicate the FIs measured parallel and perpendicular to the excitation field vector. The more the molecule rotational movement is restricted—the higher the FP value is and vice versa. The biophysical aspects of the FP of the cellular probe are based on the fact that the spectroscopic nature of a fluorescent solute is strongly dependent on the physical/chemical characteristics of the hosting media among which are viscosity, dielectric

☆ *Abbreviations:* BDM, 2,3-butanedione monoxime; DMSO, dimethyl sulfoxide; FDA, fluorescein diacetate; FI, fluorescence intensity; FP, fluorescence polarization; IFFP, intracellular fluorescein fluorescence polarization; PBS, phosphate-buffered saline; *R*, ratiometric.

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constants, polarity, pH, and temperature. This 'average influence' upon FP is fundamentally described by the Perrin formula [18]:

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{RT}{\eta V}\tau_{\mathrm{F}}\right),$$

where V is the molar volume of the spherical assumed probe, R is the gas constant, T the absolute temperature, and η is the viscosity of the solvent. $(RT/\eta V)^{-1}$ is defined as τ_{R}, the rotational correlation time of the probe. P_0 is the intrinsic polarization as measured in cases where $T/\eta \to 0$. Thus, as τ_{R} increases, FP decreases and vice versa. τ_{R} is the most dominant and common variable reflecting environmental changes such as temperature, viscosity, mobility, probe-binding, etc., as monitored by FP.

The cellular matrix is a heterogeneous and polyphasic media-solvent, in which those environmental features may differ between cellular micro-domains. It is therefore expected that fluorophor such as fluorescein molecules, embedded in living cells, might represent an ensemble of 'different fluorophores' that probe various cellular regions, of varying FP values. The steady-state intracellular fluorescein fluorescence polarization (IFFP) is therefore an intensity weighted average parameter [27] that can be expressed as:

$$\mathrm{IFFP}=\frac{\sum i_j P_j}{\sum i_j},$$

where i_j and P_j, respectively, are the local intensity and FP of a specific cellular domain noted by j. Therefore, IFFP is a sub-macroscopic measure rather than a microscopic one. This measurement of IFFP was found to be non-intrusive while monitoring lymphocyte stimulation [6–8,28]. This too has been verified in the present study by IFFP measurements following electrical stimulation of contraction cycles of single rat cardiac myocytes in culture, in the absence of spontaneous contractions.

Previous FP measurements in cells dealt with two states of fluorescent probe labeling: (a) non-covalent reversible binding of the soluble probe which is mainly incorporated into the cell cytoplasm [8,9,25], and (b) covalent binding of the probe to cytoplasm proteins or its hydrophobic embedding into membrane components, where it is confined in a micro-cone-like cage, which defines the "degree of the probe's orientation constraint" [2,3,14,16].

In the present study the entire intracellular cytoplasmic volume was loaded with fluorescein, utilizing the non-fluorescent ester, fluorescein diacetate (FDA). An accurate high resolution image analysis of FDA stained cells showed homogeneous loading over the entire cell for any given time point of the loading process, a fact which negates the possibility of compartmentalization.

FDA easily penetrates the cell since it is hydrophobic and electrically neutral. Intracellularly, it is enzymatically hydrolyzed to a hydrophilic fluorescein anion by a non-specific esterase. Hence, the rate of FDA influx is significantly greater than that of fluorescein efflux, yielding intracellular accumulation of the latter [19]. Low external FDA concentration and short labeling duration were used in order to ensure no FDA residue remained in the cytoplasm. This protocol enabled the obtaining of repeatable IFFP records during a sequence of cardiac contraction cycles.

Materials and methods

Cell culture. Cell culture of cardiac myocytes from rat hearts was prepared as previously described [11]. Briefly, rat hearts (1–2-day-old) were removed under sterile conditions and washed three times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced into small fragments and then agitated gently in a solution of proteolytic enzymes, RDB (Biological Institute, Ness-Ziona, Israel), which was prepared from a fig tree extract. RDB was diluted 1:100 in Ca^{2+} and Mg^{2+}-free PBS at 25 °C for a few 10 min cycles, as previously described [11]. Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Biological Industries, Beit Haemek, Israel) was added to supernatant suspensions containing dissociated cells and the mixture was then centrifuged at 300*g* for 5 min. The supernatant phase was discarded and the cells were resuspended. The suspension of the cells was diluted to 1×10^6 cells/ml and 1.5 ml was placed in 35 mm plastic culture dishes on collagen/gelatin-coated coverglasses. The cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Confluent monolayers exhibiting spontaneous contractions were developed in culture within 2 days. The growth medium was replaced after 24 h and then every 3 days.

Sample preparation for fluorescence measurements. For contraction and Ca^{2+} transient measurements by IFFP and indo-1, cells were grown on a 22 mm microscope coverslip, which was installed in a specially designed chamber containing connectors for electrodes. The chamber was then attached to a Zeiss inverted epifluorescence microscope stage equipped with a xenon lamp. The illuminated field was confined to approximately the cross-section of a single cardiac myocyte, which remained in the field during contraction. The above-mentioned effects were observed on individual cardiomyocyte contractions, as well as on a cardiomyocyte population. In the latter, the measurement field contains only a few cells, therefore negating any artifacts due to shifts in the measured cells or proteins.

Measurements of FP and indo-1 were carried out sequentially, but were time correlated to the fully controlled electrical stimulation pulses, which were used as a time reference frame. Therefore enabling the comparison of the different time dependencies of FP and indo-1, even though it was not simultaneously measured. This process could be carried out based on the fact that repeatability of measurements was tested on various samples from the same culture, and yielded high levels of repeatability.

Electrical stimulation. Stimulation was carried out at a frequency range of 0.1–1 Hz, with pulse duration and strength of ~1 ms, and ~40 V, respectively.

Cell loading. Loading procedures with FDA, or with indo-1/AM, were similar. They were carried out directly on the measurement chamber as follows: 100 μl staining solution of 2 μM marker (FDA: Sigma, St. Louis, MO F7378; indo-1/AM: Molecular Probes, I-1203), dissolved in modified Dulbecco's PBS [7] (PBS + 1.2 mM Ca^{2+} + 1.2 mM Mg, 280 mosmol), was introduced onto the cultured cells in the

chamber. Following loading at room temperature (5 min for FDA and 30 min for indo-1), the cells on the coverslip were meticulously rinsed three times with PBS in order to remove the extracellular marker.

Specific inhibition of Ca^{2+} beats was carried out by direct exposure of the measured cells to 800 μM 2,3-butanedione monoxime (BDM; Aldrich, Milwaukee, WI), dissolved in PBS.

Measurements. Intracellular free calcium concentrations, $[Ca^{2+}]_i$, were estimated from indo-1-AM fluorescence using the ratio method described by Grynkiewicz et al. [13]. IFFP measurements were carried out as previously described [12]. After incubation, the cells were rinsed twice with glucose enriched PBS and transferred to a chamber on the stage of Zeiss inverted epifluorescent microscope. Indo-1-AM was excited at 355 nm and the emitted light was then split by a dichroic mirror to two photomultipliers (Hamamatsu, Japan) with input filters at 405 and 495 nm. The fluorescence ratio of 405/495 nm, which is proportional to $[Ca^{2+}]_i$, was fed into a SAMPLE program written by Dr. Doron Kaplan from Biological Institute, Ness-Ziona, Israel. Calibration and analysis were performed at 24–25 °C. Under these conditions, the fluorescence ratio of indo-1-AM remained stable for 4–5 h. No compartmentalization of indo-1-AM could be observed when 4–5-day-old cultured cells were analyzed. For IFFP measurements, excitation was carried out at 470 ± 10 nm and FI was measured at 520 ± 10 nm. Under the experimental illumination doses used in the present study, photobleaching was undetectable.

Spontaneous contraction rates and amplitude measurements. The video technique used for contraction measurement was described previously [29]. Briefly, a culture dish containing adherent cells was attached to the stage of a phase interference inverted microscope. The observation of a selected cell area was recorded on an interlaced video camera at a final magnification of 800×. The brightness of the end border of the selected cell image, relative to that of the background, was enhanced by video subtraction, in other words, by mixing the positive (white) and negative (dark) video signals through an amplifier. The movement of the cell border was monitored at a rate of 400 Hz. The image length variation was then converted to voltage, filtered, and analyzed by a computer program. Contraction rates were calculated according to the number of data peaks in each measurement. The temperature and pH of the dishes were controlled throughout the experiment.

Results

IFFP and R monitoring of cardiac myocyte contraction cycle

Fig. 1 shows representative charts of IFFP(t) and $R(t)$, averaged five times for each time point and recorded along five sequential cardiac myocyte contraction cycles. The continuous line represents the $R(t)$ values that increase during 0.1 s and decrease to the steady state value after 1 s. The dashed line indicates the IFFP(t) values that decrease at the beginning of the contraction and recover to the steady state values after 1.5 s. IFFP decreases 30% (from about 0.220 ± 0.01 to 0.170 ± 0.013 at the extreme points) along the contraction cycle. Careful examination of a typical single contraction cycle shows the initial rate of change to be similar in both $R(t)$ and IFFP(t), while recovery rate of IFFP(t) is slower.

The IFFP(t) time–response curve in Fig. 1 indicates a transient increase in the rotational freedom of fluorescein molecules during the contraction cycle, while the total FI

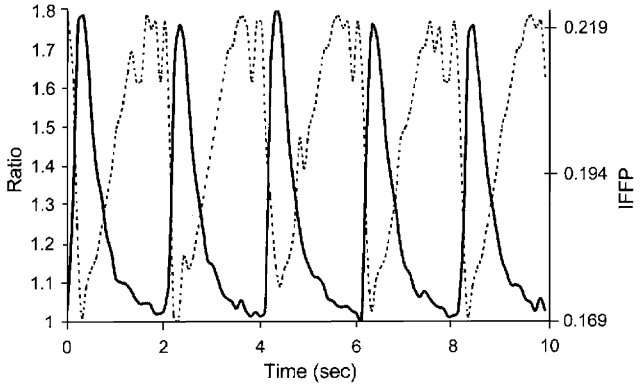

Fig. 1. A representative chart of averaged IFFP(t) (broken line) and of $[Ca^{2+}]_i$ ratiometric (solid line) measurements, recorded along five sequential individual cardiac myocyte contraction cycles. The characteristic standard deviation of IFFP and R for a given time point was 0.013 and 0.2, correspondingly.

remains constant. Reduction in IFFP may be related to an increase in either fluorescein dissociation and/or in protein flexibility at the probe-binding site. Fluorescein–protein complex formation is known to be associated with a decrease in the fluorescein quantum yield and consequently, in FI as well [22]. Since FI remains constant in our experiments, we conclude that there is no change in the degree of dissociation and therefore, the change is related to variations in flexibility at the binding site. In order to get an idea of the order of magnitude of this change, an effective viscosity at the binding site is considered. Average IFFP values are compared to those of fluorescein FP measured in glycerol–water solutions with a given viscosity [12]. This approach yields a cyclic change in the binding site effective viscosity from 18 cp at rest down to 8 cp during contraction.

Possible disturbance of fluorescein in the contraction/relaxation process

Intracellular markers might act as foreign agents that interfere with physiological processes [21,24]. In order to negate such a possibility when utilizing IFFP methodology to trace contraction/relaxation processes, the influence of FDA concentration upon the frequency and length of spontaneous contractions was examined. While installed in the measurement chamber, cells grown on a microscope coverslip were sequentially exposed to three staining solutions (2.4, 4.8, and 7.2 μM) of FDA in PBS, all having pH 7.4. The high concentration of FDA chosen was 7.2 μM. This choice was made as a result of a calculation of the Michaelis–Menten equation on an individual cell, for the measurement of physiologic conditions [26].

After 10 min loading with the chosen FDA concentration, cells were meticulously washed with PBS in order to terminate the loading process and remove extracellular fluorescein. For each of the staining solu-

tions, two parameters were measured using image analysis methods: (a) the average time interval between two sequential spontaneous contraction cycles, which yielded the average contraction frequency; and (b) the length of the contraction. In this protocol, the muscle contracts spontaneously without electric stimulation.

In addition, two control measures, the contraction length and the frequency, were performed in order to check for possible effects of FDA and the residual amount of dimethyl sulfoxide (DMSO). The first measurement was done by adding PBS, only. The second was made by adding the same amount of PBS solution containing DMSO as used with FDA.

Fig. 2 depicts contraction-length and frequency of five randomly selected individual myocyte (cell per figure) measurements. The data indicate that neither the contraction-length nor the frequency of each of the cells was disturbed by the intracellular fluorescein at the relevant concentration range. While the results indicate a heterogeneous contraction-length among cells, they show an almost homogeneous beat frequency compared to control measurements. The results show that 30 μl/100 ml PBS (0.03%) DMSO did not influence the length

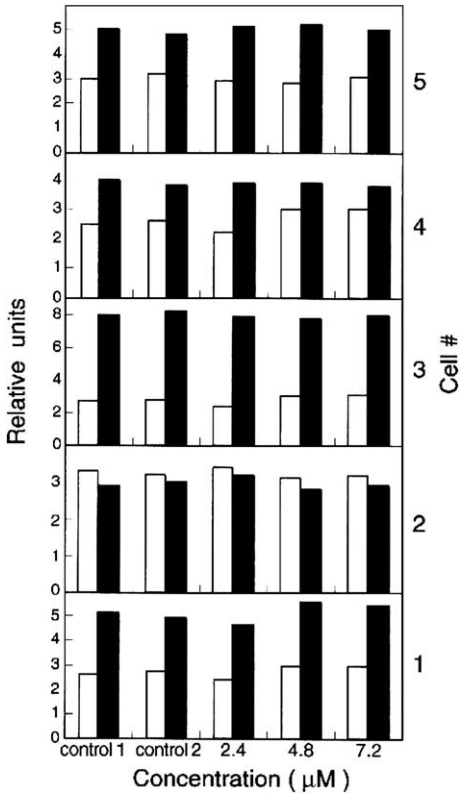

Fig. 2. Dependency of cardiac contraction rate (□) and amplitude (■) on dye concentration, measured on five (eight performed per cell) randomly selected representative cardiomyocytes. Each column presents an average buildup of 100 contraction cycle measurements. Controls are rate and amplitude measurements using PBS with (1) and without (2) DMSO (30 μl/100 ml PBS). Standard deviations were less than 4% and are omitted for brevity.

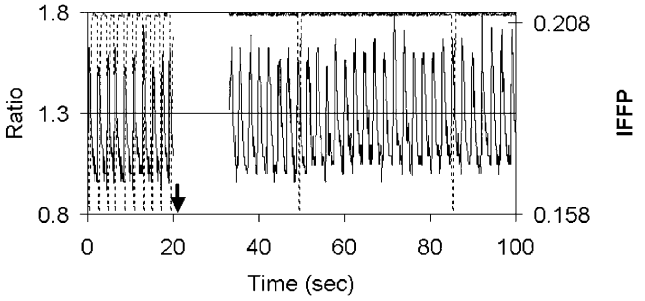

Fig. 3. A representative chart of averaged IFFP(t) (dashed line) and of $R(t)$ (solid line), before and after treatment of cells with 2,3-butanedione monoxime (BDM). The arrow indicates the time point of BDM addition.

or frequency of the contractions. Similar results were obtained for more than 50 contracting myocytes.

Possible association between IFFP, Ca^{2+}, and contraction beats

In order to determine whether alternations in IFFP occurring during cell contraction are Ca^{2+} dependent, cells were exposed to BDM. The latter is an effective inhibitor of skeletal muscle and cardiac muscle contraction [20].

BDM has been suggested to reduce force in cardiac muscle by exerting a direct effect on the contractile apparatus. At low concentrations of BDM (<1 mM), no effect on the Ca^{2+} exchanger was observed [1,10]. Hence, it was expected that BDM in low concentrations would fully or partially inhibit the contraction beat rate.

In the present study, 800 μM BDM in PBS was added directly to the spontaneously contracting cell culture during the measurement of R and IFFP, while simultaneously visually examining cell contraction. Charts of R and IFFP, before and after the addition of BDM, are presented in Fig. 3. As can be seen in this figure, 10 spontaneous cardiac myocyte R and IFFP cycles were recorded before and after exposure of the measured sample to 800 μM BDM. As can be seen in Fig. 3, while the R to IFFP beat frequency ratio is about one unit (0.5 Hz each) along the pre-exposure 20 s period, it dramatically increases by about one magnitude of order in the post-BDM exposure period. The R beat frequency remains unchanged while that of the IFFP beats decreased to approximately 0.03 Hz (dashed line, only 2 beats along about 60 s). The slow rate of the IFFP beats correlated and synchronized with that of the slow contraction rate, which was simultaneously examined visually.

Discussion

The main message of this study is the possible use of IFFP for monitoring and studying cardiomyocyte contraction, in addition to the established Ca^{2+} probes and/

or video recording of contractions. The time resolution of our measuring system enabled the analysis of the slower relaxation phase. In comparison to shortening analysis of video records, monitoring and analysis of IFFP(t) signals are more easily performed. Furthermore, as a potential parameter for physiological activation, the IFFP(t) method is also relevant to tension generation in an isometric contraction, which is poorly detected by video recording. As control measurements, [Ca^{2+}] and IFFP(t) were measured in KB-R7943 (3 μM) or BDM (2 mM) treated cardiomyocyte, where the Na^{+}–Ca^{2+} exchanger was inhibited. No contraction, beats of [Ca^{2+}], or IFFP was observed, following the induction of $[Ca^{2+}]_i$ increase, due to this treatment. Nevertheless, when significantly lower concentrations of BDM were used (800 μM), it was found that the profile and beat frequency of R remained unchanged, while the frequency of IFFP beats and beats of contraction, as were simultaneously examined visually, both decreased significantly, by about one order of magnitude (Fig. 3). This finding may therefore suggest that the IFFP indicator is probably associated with the physiological activation of contraction rather than directly with Ca^{2+} alternations. Similarly, the IFFP decrease in response to lymphocyte stimulation might monitor motile activation.

Cytoplasmic Ca^{2+} has been proposed as a key regulator of numerous cellular processes and probably plays several important roles in every cell type. However, measurement of the key variable, intracellular free calcium concentration, necessitates the use of fluorescent markers for free Ca^{2+}. Several ratiometric fluorescent indicators are currently in use (i.e., fura-2, indo-1, and fura-3), sharing similar binding sites which act as Ca^{2+} selective chelator ethylene glycol bis(β-aminoethyl ether)N [13].

Unfortunately, it is this unique chelation characteristic that interferes with the physiological process being investigated and monitored, by reducing and/or buffering free Ca^{2+} concentration. Spurgeon et al. [24] found that the loading of a cardiac myocyte with the Ca^{2+} indicator can significantly decrease the amplitude of the twitch and prolong its relaxation. These findings were found to be in agreement with those of others [17].

The intracellular fluorescent probe is flexible and easy to use. It allows simultaneous monitoring of various parameters, including FFP activation, calcium, and pH, while the result of a direct visualization shortens the course of action itself.

The IFFP(t) time–response curve indicates an alteration in the level of the rotational freedom of fluorescein molecules during the contraction cycle. Reduction in IFFP may be related to an increase in either fluorescein dissociation and/or in protein flexibility at the probe-binding site.

Nevertheless, the results obtained in this study with intracellular fluorescein in FDA-labeled cardiac myocytes show no indication of the possible influence of fluorescein or FDA concentrations on contraction length and frequency (Fig. 2), or other common detectable physiological features (data not shown). This finding may suggest that opposite to Indo-1, intracellular fluorescein, in concentrations used in this study, does not interfere with contraction.

The immediate implications of this study refer to our current studies of lymphocyte stimulation, where a persistent decrease in IFFP level is observed [8,9,25,30], which is quite similar to the transient decrease obtained in the present study during the contraction of a single cardiac myocyte. Furthermore, the cardiac myocyte contraction cycle model (as monitored by IFFP) was found to be not persistent but repeatable, wavelength independent (data not shown) and easy to perform.

An extensive investigation of this phenomenon is currently being carried out in order to explore its sources and mechanisms, and expand its possible uses. It is believed that some other acetoxymethyl-ester fluorescein derivatives (such as carboxyfluorescein diacetate (CFDA) and 2,7-bis-carboxyethyl-5 [18]—carboxyfluorescein acetoxymethyl-ester (BCECF/AM) might behave similarly.

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