Calcium Waves in Skinned Cardiac Myocytes Evoked by Two-Photon Excitation Photolysis of Caged Calcium

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Abstract: In rat ventricular myocytes chemically skinned with saponin, a local rise of $[Ca^{2+}]$ was achieved by two-photon excitation photolysis (TPP) of the caged Ca^{2+} compound 1-(2-nitro-4,5-dimethoxyphenyl)-*N,N,N',N'*-tetrakis-[(oxycarbonyl)methyl]-1,2-ethanediamine (DM-nitrophen). Confocal Ca^{2+} images, by use of fluo-3, were simultaneously collected. TPP of DM-nitrophen induced Ca^{2+} waves propagating over the myocyte, and the local rise of $[Ca^{2+}]$ at the site of photolysis sustained for 50–60 ms. These

TPP-induced Ca^{2+} events were completely suppressed by ryanodine (10 μ M), suggesting that Ca^{2+} release resulting from TPP of DM-nitrophen triggered regenerative Ca^{2+} release from the neighboring sarcoplasmic reticulum. The present techniques should be useful to investigate the interaction of elementary Ca^{2+} events, the process leading to global Ca^{2+} movements, in cardiac myocytes and other types of cells. [Japanese Journal of Physiology, 51, 127–132, 2001]

Key words: cardiac myocytes, two-photon excitation, caged calcium.

Recent advances in Ca^{2+} measurements, especially the use of confocal microscopy, revealed localized elementary Ca^{2+} events, such as Ca^{2+} sparks and quarks, in numerous types of cells, including nonexcitable cells (e.g., [1–8]). These elementary Ca^{2+} events are produced by activation of a single or a group of Ca^{2+} release channel(s) of the intracellular Ca^{2+} stores: ryanodine receptors and $InsP_3$ receptors. Under appropriate conditions, a local rise of intracellular $[Ca^{2+}]$ sequentially activates adjacent Ca^{2+} release channels (Ca^{2+} -induced Ca^{2+} release), resulting in Ca^{2+} waves to propagate over the whole cell [9].

Two-photon excitation is an optical technique recently applied in biology fields that can be used to photolyze caged compounds only at the focal point. Two-photon excitation photolysis (TPP) of caged

Ca^{2+} has been applied to induce global Ca^{2+} movements in isolated cardiac myocytes and neurons under whole-cell patch clamp conditions [10, 11]. In these whole-cell patch clamp experiments, however, the precise control of the intracellular environment (e.g., cytoplasmic $[Ca^{2+}]$) appeared difficult because of limited intracellular perfusion through the patch pipette.

In the present study, we applied TPP to produce a local rise of $[Ca^{2+}]$ in skinned (or permeabilized) cardiac myocytes in which various substances were in rapid equilibrium between extracellular fluid and cytoplasm [12]. Changes in $[Ca^{2+}]$ were simultaneously monitored by a fluorescent Ca^{2+} indicator fluo-3 with laser-scanning confocal detection (operating in ordinary one-photon excitation mode). By optimizing the composition of “internal solutions,” Ca^{2+} waves were

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Abbreviations: TPP, two-photon excitation photolysis; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; DM-nitrophen, 1-(2-nitro-4,5-dimethoxyphenyl)-*N,N,N',N'*-tetrakis[(oxycarbonyl)methyl]-1,2-ethanediamine; NP-EGTA, *o*-nitrophenyl-EGTA; MS, methanesulfonate; $InsP_3$, inositol trisphosphate; MOPS, 3-morpholinopropanesulfonic acid; $[Ca^{2+}]$, free Ca^{2+} concentration; $[Mg^{2+}]$, free Mg^{2+} concentration; SR, sarcoplasmic reticulum.

highly reproducible upon TPP of caged Ca^{2+} at any location of the myocytes.

Methods

Preparation of skinned cardiac myocytes.

Male Wistar rats weighing 300–400 g were anesthetized with sodium pentobarbital (100 mg/kg body weight, intraperitoneal). The animals were treated in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences published by the Physiological Society of Japan. The heart was quickly excised, and single ventricular myocytes were enzymatically isolated, as described previously [13]. After enzymatic digestion with 0.2 mg/ml collagenase (Collagenase S-1, Nitta Zeraichin, Tokyo, Japan) and 0.04 mg/ml protease (type XIV, Sigma, St. Louis, MO, USA), cells were stored in a low Ca^{2+} -Tyrode solution containing (mM) 135 NaCl, 5.4 KCl, 0.2 CaCl_2 , 1.0 MgCl_2 , 0.33 NaH_2PO_4 , 5.0 glucose, and 10 HEPES (pH 7.4) at 6°C until used. The myocytes were skinned with 100 $\mu\text{g}/\text{ml}$ saponin (Sigma) for 1 min in the relaxing solution containing (mM) 128 KMS, 4.57 MgMS_2 , 4.39 Na_2ATP , 1.0 EGTA, 20 MOPS, and pH 7.2. After a washout of saponin, skinned cells (length $\sim 100\ \mu\text{m}$, width $\sim 20\ \mu\text{m}$) were incubated in the photolysis solution and subsequently settled in an experimental chamber (30 μl in volume) on the stage of an inverted microscope (DM IRBE, Leica, Wetzlar, Germany). All experiments were carried out at 26–28°C.

Two-photon excitation photolysis and simultaneous Ca^{2+} measurements. A Ti:sapphire laser (Tunami 3960, Spectra Physics, Mountain View, CA, USA) was pumped by a diode-pumped continuous-wave visible laser (Millennia V, Spectra Physics). The pulsed laser system was operated at 710–715 nm (half-maximal width 53 fs, repetition rate 82 MHz and 340 mW). This pulsed laser light and visible laser light from a confocal unit (TCS NP, Leica) were introduced through a dichroic mirror (reflecting wavelength $> 660\ \text{nm}$) to a $63\times$ water-immersion objective lens (NA 1.20, PL APO $63\times 1.20\ \text{W CORR}$, Leica) and focused on the myocyte on the microscope stage. For TPP of DM-nitrophen [14], the pulsed laser light was delivered for 64 ms via a mechanical shutter (F77-4, Copal, Tokyo; F77 controller, Surugaseike, Shizuoka). For $[\text{Ca}^{2+}]$ measurements, the Ca^{2+} indicator fluo-3 was excited at 488 nm through a fiber optics using a 60 mW argon laser attenuated by 82%, and its emission was detected at 500–600 nm. Confocal x - y images were normally collected at 210-ms intervals. Line-scan x - t images were obtained by repetitive scanning along a horizontal line every 1.1 ms. In prelimi-

nary experiments using a model solution, a two-photon excitation of fluo-3 appeared to be negligible at 710–715 nm and the power levels used for TPP. When fluorescent beads of 0.1 μm diameter were excited by pulsed laser light (two-photon excitation in the same optical system), the width of the half-maximal intensity was 0.35 μm in xy -plane and 0.95 μm in z -direction. Data analyses were carried out by using NIH Image (ver. 1.62, NIH, Bethesda, MD, USA).

Solutions and chemicals. The photolysis solution contained (mM) 135 KMS, 2.0 MgMS_2 , 4.0 Na_2ATP , 0.06–0.075 CaMS_2 , 0.05 EGTA, 0.03 fluo-3, 0.2 DM-nitrophen, 20 MOPS, and pH 7.2. On one occasion, $[\text{Ca}^{2+}]$ of the photolysis solution containing 75 μM CaMS_2 was estimated to be 0.15 μM from fluo-3 fluorescence intensities measured after the addition of an excess amount (1 mM) of either EGTA ($F_{\min}$) or CaMS_2 ($F_{\max}$) and a dissociation constant value of 0.44 μM separately estimated for Ca^{2+} –fluo-3 reaction (not shown). There may, however, be some variation in $[\text{Ca}^{2+}]$ of the photolysis solution between experiments, depending on the amount of contaminant Ca^{2+} . In practice, the amount of added CaMS_2 was adjusted in each experiment to observe occasional spontaneous activity of skinned myocytes that was taken as an indication of significant loading of the sarcoplasmic reticulum (SR) with Ca^{2+} . The $[\text{Mg}^{2+}]$ in the presence of 150 nM free Ca^{2+} was calculated to be 83 μM under the assumption that the apparent dissociation constant for Mg^{2+} -ATP reaction was 100 μM (as assumed by Baylor and Hollingworth [15]) and the apparent dissociation constants of DM-nitrophen for Ca^{2+} and Mg^{2+} were, respectively, 5 nM and 2.5 μM [14]. During preparation and storage of the solution, care was taken to minimize exposure of DM-nitrophen to room light.

DM-nitrophen (tetrasodium salt) was purchased from Calbiochem (San Diego, CA, USA). fluo-3 (tetrapotassium salt) and NP-EGTA (tetrapotassium salt) from Molecular Probes (Eugene, OR, USA). EGTA (free acid), ryanodine, thapsigargin, and saponin from Sigma (St. Louis, MO, USA).

Results

We used a new combination of techniques: Two-photon excitation photolysis (TPP) of caged Ca^{2+} , and $[\text{Ca}^{2+}]$ was monitored with confocal imaging of fluo-3 fluorescence (Fig. 1). Figure 1A shows a transmission image of a skinned ventricular myocyte under exposure to the photolysis solution containing 75 μM CaMS_2 . Spontaneous Ca^{2+} waves and contraction were occasionally seen in this and many other cells, indicating that the SR was sufficiently loaded with Ca^{2+} . Figure 1, B–D, shows fluo-3 fluorescence im-

Fig. 1. (A) Transmitted light image of a skinned myocyte. (B–D) confocal images (x-y scan; 256×256 pixels, pixel size 0.155 μm) of fluo-3 fluorescence in F/F_0 unit, and (E–G) their surface plots obtained from the same location of the myocyte. (B, C) Consecutive images (210 ms apart) taken shortly after photolysis in the absence of ryanodine, and (D) image taken shortly after photolysis in the

presence of 10 μM ryanodine. (E–G) Surface plots corresponding to (B–D), respectively. For each fluorescence image, four preceding frames were averaged to yield F_0 of each pixel, and F/F_0 values were calculated pixel by pixel. Photobleaching during these frames was found to be negligible. Note that calibration bars (10 μm) of x-y dimensions are commonly applied to (A–D), but not to (E–G).

ages of the same cell in F/F_0 unit, fluorescence intensity values (F) normalized to the basal value (F_0), as an index of $[Ca^{2+}]$. In E–G, the surface plots corresponding to B–D, respectively, are shown. In the quiescent state between the events of spontaneous Ca^{2+} waves, the pulsed laser light of 710 nm delivered for 64 ms induced propagating Ca^{2+} waves (Fig. 1, B and C). An application of ryanodine (10 μM) completely

suppressed the Ca^{2+} wave, leaving a small rise of $[Ca^{2+}]$ localized only at the site of photolysis (Fig. 1D).

Ca^{2+} measurements of higher time resolution were carried out in the same myocyte with the line-scan mode (Fig. 2); laser scanning was repeated along a horizontal line shown in Fig. 2A at 1.1 ms interval. TPP of DM-nitrophen induced a Ca^{2+} wave propagat-

Fig. 2. (A) Transmitted light image of the same skinned myocyte as shown in Fig. 1. (B, C) Confocal line-scan images of F/F_0 (x - t scan; 512×512 pixels, pixel size $0.078 \mu\text{m} \times 1.1 \text{ ms}$) taken along a white line shown in (A) during photolysis. Line-scan images taken in the absence (B) and in the presence of $10 \mu\text{M}$ ryanodine (C) consist of a series of 512 lines scanned at 1.1 ms interval (from top to bottom). For each line-scan image, two preceding frames were averaged to yield F_0 of each pixel, and F/F_0 values

were calculated pixel by pixel. Photobleaching during these frames was found to be negligible. The horizontal calibration bar indicates $10 \mu\text{m}$ in (A–C), and the vertical bar corresponds to $10 \mu\text{m}$ in (A) and 140 ms in (B, C). (D) Time courses of F/F_0 at the site of TPP. Traces shown in red and blue were obtained from (B) and (C), respectively, by averaging 64 horizontal pixels ($=5 \mu\text{m}$), indicated underneath by arrows. The time axes of the two traces have been aligned by the timing of TPP, indicated by a horizontal bar.

ing toward both sides with the velocity of approximately $60 \mu\text{m/s}$ (Fig. 2B). A time course of local $[\text{Ca}^{2+}]$ changes at the site of TPP shows that the increase in $[\text{Ca}^{2+}]$ was sustained for 50–60 ms after ces-

sation of the pulse (red line in Fig. 2D). After an application of $10 \mu\text{M}$ ryanodine, no Ca^{2+} wave was seen (Fig. 2C), and the local $[\text{Ca}^{2+}]$ at the site of TPP quickly returned toward the basal level following ter-

mination of the photolysis pulse (blue line in Fig. 2D). Essentially similar results were obtained in four other myocytes. In a separate experiment, we also examined the effect of 10 μ M thapsigargin, an inhibitor of the SR Ca^{2+} pump. The TPP-induced Ca^{2+} waves were also effectively inhibited by this agent. Overall, these results suggest that we were able to trigger propagating Ca^{2+} waves by sequential Ca^{2+} -induced Ca^{2+} release from neighboring ryanodine receptors.

Although the experiments shown in Figs. 1 and 2 used a relatively low concentration of DM-nitrophen (0.2 mM), preliminary TPP experiments were performed in the presence of a higher concentration of DM-nitrophen. In several trials with 1 mM DM-nitrophen and the addition of various concentrations of CaMgS_2 , TPP caused only a local rise of $[\text{Ca}^{2+}]$ at the focal point with no propagating Ca^{2+} waves. The reason of this absence of Ca^{2+} waves was not further investigated in the present study.

Discussion

In saponin-skinned ventricular myocytes, propagating Ca^{2+} waves are highly reproducible upon TPP of DM-nitrophen. Local photolysis of caged Ca^{2+} appears to be a useful tool to study the interactions between adjacent ryanodine receptors (or groups of receptors) with no intervention of Ca^{2+} influx from the extracellular space, i.e., L- and T-type Ca^{2+} channels and the Na^+ - Ca^{2+} exchanger. The use of skinned cells has the advantage over intact cells that the intracellular environment is precisely controlled, and hydrophilic compounds can be easily applied to alter properties of Ca^{2+} release.

In previous TPP studies in intact cells, 1 mM DM-nitrophen was introduced into the cytoplasm through a patch pipette [10, 11], and global Ca^{2+} movements were successfully induced by TPP. In the present study, on the contrary, we were unable to observe Ca^{2+} waves upon TPP of the same concentration (1 mM) of DM-nitrophen as used in the previous studies. This apparent discrepancy may be due to limited intracellular dialysis under a whole-cell patch clamp that could result in significantly lower concentrations of DM-nitrophen in the cytoplasm than in the patch pipette. In skinned myocytes, high concentrations of the caged Ca^{2+} compound, DM-nitrophen might reduce Ca^{2+} diffusion to adjacent Ca^{2+} releasing sites because of its Ca^{2+} buffering effect. Alternatively, the discrepancy may be attributed to the difference in "intracellular" ionic conditions used. For example, $[\text{Mg}^{2+}]$ is known to affect Ca^{2+} -induced Ca^{2+} release; the internal solutions used in the earlier studies did not contain Mg^{2+} , whereas a small amount of Mg^{2+}

was always included in the present study.

Since DM-nitrophen, a photosensitive derivative of EDTA, binds Mg^{2+} with an affinity only 500 times lower than that for Ca^{2+} , it was required to set $[\text{Mg}^{2+}]$ of the photolysis solution quite low (83 μ M) in the present study. Even at this low level of $[\text{Mg}^{2+}]$, it is calculated that the slightly greater fraction of DM-nitrophen molecules are Mg^{2+} -bound (52%) before photolysis, in comparison with its Ca^{2+} -bound form (47%). To produce Ca^{2+} waves by TPP in the presence of normal cytoplasmic $[\text{Mg}^{2+}]$ (0.4–0.9 mM; e.g., [16, 17]), it is obviously preferable to use an EGTA-based caged Ca^{2+} compound. We therefore tested TPP of NP-EGTA [18], but trials with high concentrations of NP-EGTA (1.0–2.2 mM) were not successful (unpublished observation). This could be explained by (i) the same reason for the case of high concentrations of DM-nitrophen, i.e., Ca^{2+} buffering effect, and/or (ii) its low extinction coefficient causing insufficient Ca^{2+} release from the caged compound upon TPP [19]. A newly synthesized caged Ca^{2+} compound, dimethoxynitrophenyl-EGTA-4 [19], which is not commercially available at present, may solve the problem related to Mg^{2+} .

In conclusion, to investigate how elementary Ca^{2+} events produce global Ca^{2+} movements, we developed a new combination of techniques: two-photon excitation photolysis of caged Ca^{2+} in skinned cardiac myocytes. Ca^{2+} waves were highly reproducible with TPP of caged Ca^{2+} , and possible modulators for Ca^{2+} release and uptake could be easily examined. Furthermore, it is suggested that "intracellular" ionic conditions, including the concentration of caged Ca^{2+} compound itself, should be carefully optimized.

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