Microscopic Detection of Thermogenesis in a Single HeLa Cell

Madoka Suzuki,* Vadim Tseeb,† Kotaro Oyama,† and Shin'ichi Ishiwata*†
*Consolidated Research Institute for Advanced Science and Medical Care, and †Department of Physics, Faculty of Science and Engineering, Waseda University, Tokyo, Japan

ABSTRACT We report here the technique for detection and measurement of the temperature changes in single cells using a recently devised microthermometer (a glass micropipette filled with the thermosensitive fluorescent dye Europium (III) thenoyltrifluoroacetonate trihydrate). We found that the heat production in a single HeLa cell occurred with some time delay after the ionomycin-induced Ca^{2+} influx from the extracellular space. The time delay inversely depended on extracellular [Ca^{2+}], and the increase in temperature was suppressed when Ca^{2+}-ATPases were blocked by thapsigargin. These observations strongly suggest that the enzymatic activity of Ca^{2+}-ATPases in endoplasmic reticulum leads to the heat production. This study has therefore paved the way for studying the thermogenesis at the single-cell level.

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Madoka Suzuki and Vadim Tseeb contributed equally to this work.

Address reprint requests and inquiries to Shin'ichi Ishiwata, E-mail: ishiwata@waseda.jp.

In a long history of the physiological studies on thermogenesis, the thermodynamic parameters have mainly been examined for different parts of the body, tissues or organs, as a whole (1–4). Although several groups have succeeded in imaging the heat production in single cells by the direct incorporation of fluorescent dyes into each cell to detect temperature changes, these studies had obvious weak points: the fluorescence intensity was also sensitive to either the fluidity of the plasma membrane (5) or the pH value of solution (6). Thus, to overcome the technical problems in the study of thermogenesis in single cells, there was a need to devise a new tool to measure the local temperature in an aqueous solution, having high spatial resolution without interfering with any environmental parameters. For this purpose, we recently devised a microthermometer (7).

The directional flow of various kinds of ions in living cells driven by electrochemical potentials and energy-consuming pumping processes is hypothesized to result in the heat production (8,9). Steep temperature gradients in cells have recently attracted strong interest from cell biologists, especially concerning the effects of local intracellular thermogenesis on the rates of chemical reactions, the rate of the diffusion process (e.g., the transmitter-receptor mismatch in the brain (10)), the speed of exocytosis (11), and so on. One of the most interesting targets in this respect is the thermogenesis coupled with the changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{in}$), because up to 3% of the total energy production by a cell is estimated to be used even in the resting conditions, simply to maintain large [Ca^{2+}] gradients between the cytoplasm and the lumens of endoplasmic/sarcoplasmic reticulum, as well as against the extracellular space (12,13). Heat is produced by the initiation of calcium pumping because the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) utilizes only a part of the energy of ATP hydrolysis to pump Ca^{2+}, whereas the rest is dissipated in the form of heat ranging from 10 to 30 kcal/mol hydrolyzed ATP (14).

Although the integrated thermogenesis in a large number of cells studied by microcalorimetry has been reported (15), the sensitivity of this method is not sufficient to measure heat production in single living cells. Therefore, to reveal how the cellular heat production correlates with $[Ca^{2+}]_{in}$ and other important cellular parameters, the measurements should be performed on the level of individual cells.

In this work we present a simple approach to measure the real-time thermogenesis in a single HeLa cell with simultaneously monitoring $[Ca^{2+}]_{in}$ regulated by ionomycin, which is a powerful ionophore making cellular and intracellular membranes highly permeable to Ca^{2+} (16). In all experiments, two microthermometers were used: one was gently pressing the cell to ensure good contact with the cell membrane, and the other, separated at least 20 μm from the cell, served as a reference thermometer (Figs. 1 and 2).

Ionomycin (20 μl of 0.2 mM solution) was added from one side of the dish (ϕ35 mm) containing 2 ml of the medium, to the final concentration 2 μM. The addition of this small volume did not result in the temperature change of the medium or the mechanical noise. The dish was kept still throughout the measurement. Although the HeLa cells survive in the presence of ionomycin better than other types of cells (16), we found that 10 min is the longest time the HeLa cells could survive in our conditions.

Next, we recorded three fluorescence signals: from Fluo-4 loaded into the cell to monitor $[Ca^{2+}]_{in}$, and from Europium (III) thenoyltrifluoroacetonate trihydrate (Eu-TTA) dissolved in DMSO, in two microthermometers, to monitor the

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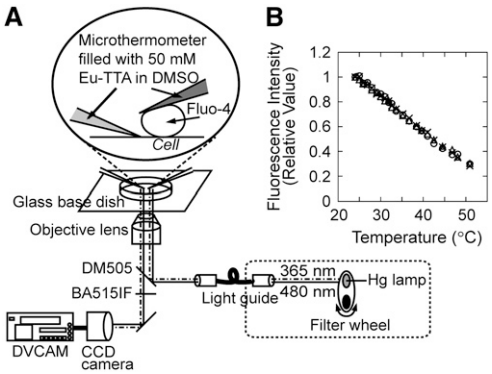

FIGURE 1 Schematic illustration of the setup (*A*) and the obtained temperature versus fluorescence intensity relationship (*B*). (*A*) See Supplementary Material for details. (*B*) Fluorescence intensity was measured in several regions of the microthermometer: the tip (*circles*), the central part (*triangles*), and the root (*crosses*).

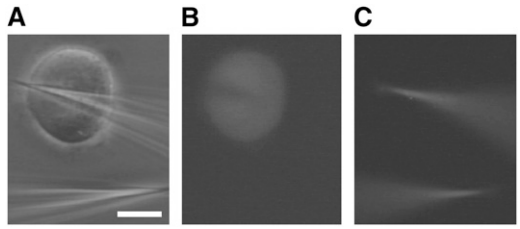

FIGURE 2 (*A*) Phase-contrast image of a cell and the two pipettes. (*Upper*) Measurement pipette. (*Lower*) Reference pipette. (*B* and *C*) Fluorescence images of Fluo-4 and Eu-TTA, respectively. Scale bar, 10 μm.

temperature. These two fluorescent dyes are compatible because their excitation spectra are well separated and the emission spectra are in the region of the wavelengths longer than 515 nm (cf. Fig. 1 and Supplementary Material). The temperature change was estimated with the use of the slope of the calibration curve, −0.0274/°C (Fig. 1 *B*), which indicates strong dependence of the Eu-TTA fluorescence on temperature compared with other dyes (e.g., −0.018/°C for the rhodamine fluorescence; (17)).

Fig. 3 shows the recordings of $[Ca^{2+}]_{in}$ made simultaneously with the thermogenesis detected in a single HeLa cell upon application of ionomycin, at two different extracellular $[Ca^{2+}]$, 5 mM (Fig. 3 *A*) and 1 mM (Fig. 3 *B*). As the filter wheel and the charge-coupled device camera were not synchronized, the signals from Fluo-4 and Eu-TTA were separated by subsequent analysis with the use of a self-written macro in Microsoft Excel. The recordings made over the long period of observation include photobleaching caused by excitation light. However, because the degree of photobleaching was small for 50 mM Eu-TTA (Fig. 3, *Aa* and *Ab*), it was well approximated by a single exponential and could therefore be removed from the raw traces.

When ionomycin reached the cell surface, the increment of $[Ca^{2+}]_{in}$ was observed (Fig. 3, *Ac* and *Ba*). As a result, the fluorescence intensity of Eu-TTA in a pipette contacting the cell decreased with some time delay (Fig. 3, *Ad* and *Bb*). No detectable change in the fluorescence intensity, however, occurred in the reference pipette (Fig. 3, *Ae* and *Bc*). The value of temperature change varied between the measurements

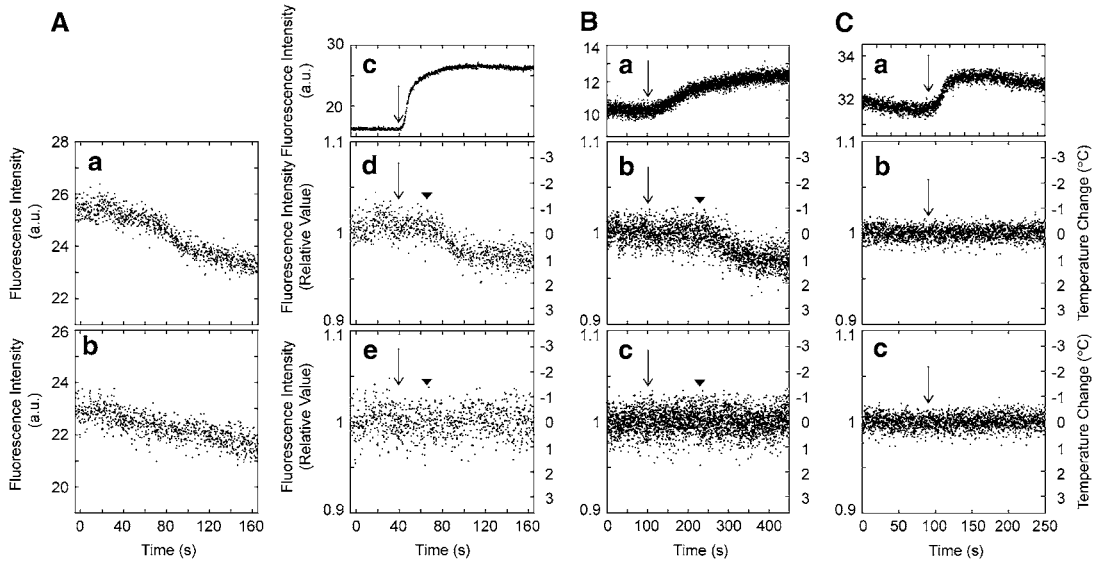

FIGURE 3 Time courses of the fluorescence intensity at different extracellular $[Ca^{2+}]$. Ionomycin was added to the petri dish at time 0 in the presence of 5 mM Ca^{2+} (*A*), 1 mM Ca^{2+} (*B*), and 2 mM Ca^{2+} with 2 μM thapsigargin after the pretreatment with 2 mM Ca^{2+} and 2 μM thapsigargin for 1 h (*C*). A part of the experiment in panel *A* is presented as Supplementary Movie 1. Each dot represents the average value in the region of interest at the tip of the pipette in a single video frame. (*Aa*, *Ad*, *Bb*, and *Cb*) The fluorescence intensity of Eu-TTA in the pipette contacting the cell. (*Ab*, *Ae*, *Bc*, and *Cc*) Fluorescence intensity of Eu-TTA in the reference pipette. (*Ac*, *Ba*, and *Ca*) The fluorescence intensity of Fluo-4. The arrows show the moment at which Fluo-4 signal increased, and the arrowheads indicate the moment at which the initiation of the positive thermogenesis was detected.

depending on the area of the contact between the pipette and the cell. The largest observed temperature increase was ~1°C.

The rate of the $[Ca^{2+}]_{in}$ increase was faster at higher extracellular $[Ca^{2+}]$ (Fig. 3, *Ac* and *Ba*), due to a larger gradient of $[Ca^{2+}]$ between the outside and the inside of the cell. The positive thermogenesis always followed the increase in $[Ca^{2+}]_{in}$ and, in addition, occurred earlier in the presence of 5 mM Ca^{2+} than with 1 mM (Fig. 3, *Ac*, *Ad*, *Ba*, and *Bb*). The values of the time delay (*s*, mean ± SE (*N*)) were 28 ± 5 (5) for 5 mM Ca^{2+} and 126 ± 12 (3) for 1 mM.

What is the heat source for the thermogenesis observed here? It is reasonable to consider that SERCA pumping up Ca^{2+} into endoplasmic reticulum (ER) is involved in the heat production (14). The dependence of the time delay on the extracellular $[Ca^{2+}]$ (Table 1) would then be understandable, because SERCA will have to start operating to keep constant $[Ca^{2+}]_{in}$ after $[Ca^{2+}]_{in}$ exceeds a threshold level due to the influx of extracellular Ca^{2+}. To confirm this consideration, we next examined the effect of thapsigargin, an inhibitor of SERCA for ER (18). In these experiments, cells were first incubated with 2 μM thapsigargin for 1 h in the presence of 2 mM Ca^{2+}, and then 2 μM ionomycin was applied together with 2 μM thapsigargin and 2 mM Ca^{2+}. As expected, we observed the influx of Ca^{2+} upon the application of ionomycin, but no temperature change was detected (Fig. 3 *C*).

In summary, we demonstrated that the coupling between the real-time thermogenesis and the SERCA's activity could be detected in single HeLa cells. We can therefore conclude that SERCA plays a key role in the cascade of cellular heat production. The next targets will be the quantitative determination of the temperature distribution inside the cell, the identification of the source(s) of the thermogenesis, and the elucidation of their physiological roles. The thermodynamic parameters for the cell, e.g., the thermal conductivity of the cytoplasm containing high concentrations of proteins, and of the inner structures with phospholipid bilayers, which must be different from the homogeneous medium, should also be clarified in future studies.

Finally, we stress that using a microthermometer is potentially a highly powerful technique for studying local thermogenesis in tissue experiments because it allows one to easily penetrate the tissue, such as a brain slice, with the simultaneous use of electronic, chemical, and optical setups to monitor other physiological parameters.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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