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Research Note

Determination of Intracellular Calcium Concentration of Individual Host Cells Infected with *Trypanosoma cruzi* Using Microfluorimetry in Conjunction with Digital Image Processing

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Chagas' disease, caused by *Trypanosoma cruzi*, is of major importance in cardiac diseases in vast areas of Central and South America. Different approaches through morphological or immunological methods have been undertaken in order to understand the processes of pathogenesis elicited by *T. cruzi* infection of host cells. However, the cellular and biochemical bases of the pathogenic processes have not yet been clarified. The pathogen *T. cruzi* multiplies discontinuously in vertebrate hosts (Brener 1973, de Souza 1984). The nondividing trypomastigote form, which has a flagellum for locomotion, circulates in the bloodstream before penetrating host cells. On the other hand, the amastigote form, which has no free flagellum, parasitizes the cytoplasm of host cells, divides repeatedly by binary fission, and thus may trigger malfunctioning in the host cells, leading to pathogenesis in Chagas' disease.

Calcium ion (Ca^{2+}) plays key roles in the regulation of cellular functions, relaying chemical messages that are received at the surface of the plasma membrane to the biochemical machinery inside the cell (Campbell, 1983; Carafoli and Penniston, 1985). In typical mammalian cells, the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is about 100 nanomolar, a very low level equivalent to 1/10,000 the concentration in the blood. It has been reported that the infection by *T. cruzi* alters calcium homeostasis in human endothelial cells under basal and stimulated conditions (Morris *et al.*, 1988). They used, however, a population of cultured host cells, with highly variable infection rates from 30 to 75%, for the determination of Ca^{2+} concentration. In contrast, this study has been aimed at the quantitative determination of the $[Ca^{2+}]_i$ in individual normal host cells and cells infected with *T. cruzi*, employing fura-2 as a Ca^{2+}-specific probe, in connection with microfluorimetry and digital image processing (Grynkiewicz *et al.*, 1985; Poenie *et al.*, 1986; Iida *et al.*, 1990).

Recently, we have succeeded in establishing an *in vitro* culture system of the host HeLa cells infected with the Tulahuene strain of *T. cruzi* and in determining quantitatively the time course of growth of amastigotes and trypomastigotes (Nakajima-Shimada *et al.*, manuscript submitted). This *in vitro* system was used for the present study with some modifications. Four to five 15-mm round coverslips coated with poly-L-lysine were placed in 60-mm dishes. Exponentially growing HeLa cells harvested from the subcultures were inoculated into the dishes with an inoculum size of $2–3\times10^4$ cells per dish, and incubated for 2 days at 37°C in 5% CO_2 in air. The

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HeLa cells in a 60-mm dish were then infected with *T. cruzi* trypomastigotes ($5–7\times10^4$ parasites) obtained from the medium of the preceding subcultures (Nakajima-Shimada *et al.*, manuscript submitted). Three to five days after the infection, the cells were washed with phosphate-buffered saline (PBS) and then incubated at 37°C for 45 min in 5 ml of PBS containing 1.8 mM $CaCl_2$ and 1 μM fura-2-AM (1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester, Dojindo Laboratories, Kumamoto, Japan). Various mammalian cells reportedly incorporate fura-2-AM and convert it into fura-2 by endogenous esterase activity. We have confirmed the conversion from fura-2-AM to fura-2 in HeLa cells by additions of 1 μM ionomycin and 10 mM $MnCl_2$ that induce an immediate breaching of the fluorescence in the cells. A coverslip with fura-2-AM-loaded cells was washed twice with PBS containing 1.8 mM $CaCl_2$, set on a slide glass invertedly and sealed with nail varnish.

Measurements of $[Ca^{2+}]_i$ in HeLa cells were carried out at room temperature according to the procedure of Iida *et al.* (1990). Briefly, the specimens were observed under a Nikon Microphot-FX microscope with an integral fluorimeter system generating two synchronized light beams of selected wavelengths (340 and 380 nm). The time of excitation for one frame at each wavelength was 1/30 sec. The interval between the excitations was approximately 1.2 sec. A filter through which passes light of wavelength less than 500 nm was used to measure the intensity of emitted fluorescence. Two images of fluorescence of fura-2 excited at 340 and 380 nm were acquired by a SIT camera (C2400-08H; Hamamatsu Photonics, Hamamatsu) and relayed into a television monitor and an image processor of Hamamatsu Photonics ARUGUS-100. From the ratio of fluorescence intensities obtained through the image processor, the $[Ca^{2+}]_i$ was calculated using the equation described by Grynkiewicz *et al.* (1985). Pseudocolor images were printed by using a color video printer (GZ-P11; Sharp Ltd., Osaka). Since it is known that fura-2 gradually becomes sequestered into organellar compartments (Williams *et al.*, 1985), we have done the measurements for fluorescence at room temperature and within 15 min, during which time the cells retain an appropriate cytosolic dye concentration in these experiments. Cellular autofluorescence was not detectable at the excitation light levels and camera gain setting used.

Figure 1 shows two pairs, one for control and another for *T. cruzi*-infected HeLa cells, of phase contrast pictures and computer-processed images of the same cells. Control HeLa cell had an evenly distributed deep blue color and a $[Ca^{2+}]_i$ calculated to be 104 nM. In contrast, the infected HeLa cell exhibited a differential color distribution, *i.e.*, a higher level of $[Ca^{2+}]_i$ in the cytoplasm than in the nucleus, giving an average $[Ca^{2+}]_i$ value of 283 nM. Since the fluorescence of fura-2 was not demonstrated in intracellular amastigotes, these concentrations are directly associated with the cytosolic $[Ca^{2+}]_i$ of host cells.

Figure 2 shows the calculated $[Ca^{2+}]_i$ in individual host cells. Uninfected HeLa cells showed the $[Ca^{2+}]_i$ value of 115±9 nM (mean±S.E.), obtained from 50 separate determinations, which was comparable to that measured in various mammalian cells (Campbell, 1983). The $[Ca^{2+}]_i$ value was markedly elevated in infected cells that harbored 5 to 15 amastigotes, 304±25 nM, the value being a 2.6-fold higher concentration than the control value. Student's *t*-test clearly indicates a significant difference in $[Ca^{2+}]_i$ between uninfected and infected cells ($p<0.01$).

There are several possibilities of mechanisms for the increase in $[Ca^{2+}]_i$ in infected HeLa cells: (1) the Ca^{2+} influx by Ca^{2+} channel in the plasma membrane of host cells increases, (2) the internal Ca^{2+} stores, such as mitochondria and/or endoplasmic reticulum, are deprived of regulatory properties for controlling cytosolic Ca^{2+} concentrations, and (3) the Ca^{2+} efflux by Ca^{2+} pump does not function effectively. Experimental analysis for these possibilities is now in progress in our laboratory. Whatever the mechanism is, an alteration in intracellular Ca^{2+} mobilization should be physically or chemically associated with the presence of proliferating amastigotes. For further understanding of mechanisms of calcium homeostasis in the host cells infected with *T. cruzi*, the method developed in this study is useful for monitoring $[Ca^{2+}]_i$ in individual cells.

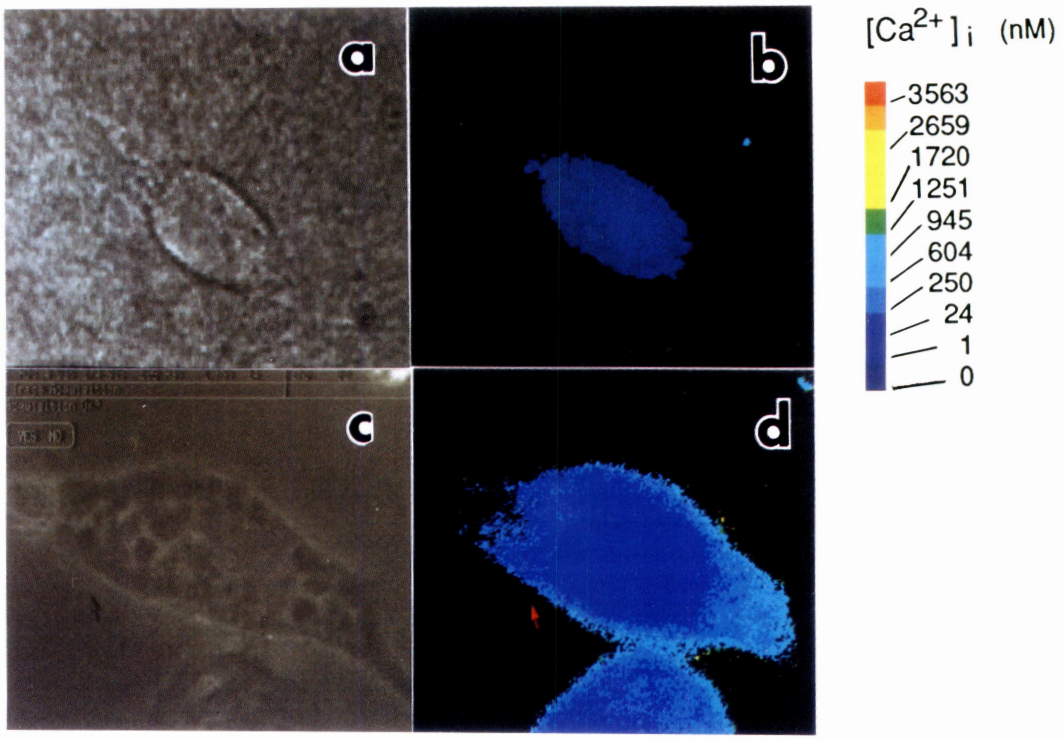

Fig. 1 Fluorescence ratio imaging for the determination of $[Ca^{2+}]_i$ in *T. cruzi*-infected HeLa cell loaded with fura-2. Fluorescence ratios are coordinated with pseudocolor hues and calibrated in terms of nanomolar Ca^{2+}. The color scale gives an approximate calibration for $[Ca^{2+}]_i$ in nanomolar. Left: phase contrast pictures of the control (a) and infected (c) HeLa cells. Right: fluorescent images of the control (b) and infected (d) HeLa cells of the same fields as (a) and (c), respectively. Note that the infected HeLa cell was enlarged, due to the presence of about 15 amastigotes.

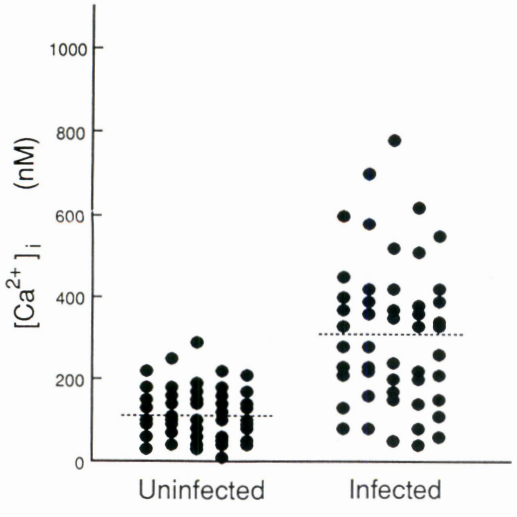

Fig. 2 Measurements of $[Ca^{2+}]_i$ in individual *T. cruzi*-infected HeLa cells. Fifty uninfected and fifty infected cells were analyzed for the $[Ca^{2+}]_i$, and each value is plotted as a closed circle. Broken lines indicate the mean values of $[Ca^{2+}]_i$.

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