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Original article

Simultaneous measurement of cytosolic and mitochondrial calcium levels: Observations in *TRPC1*-silenced hepatocellular carcinoma cells

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ABSTRACT

Introduction: The measurement of intracellular Ca^{2+}, cytosolic or stored in organelles, i.e., mitochondria, gave valuable data for numerous areas of research. In case of tumor cells, mitochondrial Ca^{2+} levels play essential roles in apoptosis along with endoplasmic reticulum (ER) Ca^{2+}. In this study, we describe a Ca^{2+} monitoring system that allows studying both adherent cells and tissues and discuss data obtained from hepatocellular carcinoma cells and rat thoracic aorta by using this system. **Methods:** For this purpose, two apparatus, one for adherent cells and the other for intact rat aorta, were designed and produced. With this system, changes in cytosolic Ca^{2+} levels following store-operated calcium (SOC) entry induced by sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) blockers were recorded in different hepatocellular carcinoma cells. Furthermore, cytosolic and mitochondrial Ca^{2+} levels were simultaneously measured in *TRPC1*-silenced Huh7 hepatocellular carcinoma cells. In addition, the effects of trifluoromethylphenylimidazole (TRIM) on cyclopiazonic acid (CPA)-, serotonin (5-HT)-, and phenylephrine (PE)-induced changes in isometric force and cytosolic Ca^{2+} levels were determined simultaneously in rat thoracic aorta. The effects of aging on PE-induced responses were also investigated. **Results:** After SOC entry activation, cytosolic Ca^{2+} levels were increased, as expected in all hepatocellular carcinoma cells. Mitochondrial Ca^{2+} levels following CPA-induced ER depletion were significantly ($p < .05$) diminished in *TRPC1*-silenced Huh7 cells. In addition, TRIM partially inhibited both 5-HT-induced contractions and cytosolic Ca^{2+} levels without affecting CPA and PE responses. PE-induced contractions and cytosolic Ca^{2+} levels were similar in aorta from young and old (3 and 22 months, respectively) rats. **Discussion:** We confirmed that the system provides valuable data about intracellular Ca^{2+} dynamics by allowing simultaneous measurements and sequential addition of compounds in adherent cells. The decrease in mitochondrial Ca^{2+} loading following CPA-induced ER depletion in *TRPC1*-silenced Huh7 cells suggests a possible role of TRPC1 in hepatocellular carcinoma cell apoptosis. The system also enables the simultaneous measurement of isometric force and cytosolic Ca^{2+} levels and promotes understanding vascular physiology and disease.

1. Introduction

Intracellular Ca^{2+} has diverse functions both in physiological and pathological processes in various cell types (Berridge, 2001). The spectrofluorometric measurement of intracellular Ca^{2+} is widely used since development of fluorescent dyes (Takahashi, Camacho, Lechleiter, & Herman, 1999). Among several fluorescent Ca^{2+} indicators, with different affinities for Ca^{2+}, fura-2, a ratiometric dye (Grynkiewicz, Poenie, & Tsien, 1985), and rhod-2, a rhodamine-based indicator (Minta, Kao, & Tsien, 1989), are used for the measurement of cytosolic and mitochondrial Ca^{2+} levels, respectively. Flow cytometry that allows the measurement of calcium on single-cells or isolated cell populations with different phenotypic characteristics (June, Abe, & Rabinovitch, 2001; June & Moore, 2004) was first used in 1988 by Davies *et al.* to determine thrombin-induced changes in intracellular Ca^{2+} in subpopulations of platelets (Davies, Drotts, Weil, & Simons, 1988). The delay in detection after addition of compounds precluding the determination of rapid initial changes in Ca^{2+} levels via flow cytometry (Takahashi et al., 1999) has been recently overcome with development of new generation flow cytometers (Jones et al., 2014).

Contraction of vascular smooth muscle largely depends on the elevation of cytosolic Ca^{2+} (Karaki et al., 1997). Store-operated Ca^{2+} (SOC) entry also regulates vascular tone (Leung, Yung, Yao, Laher, & Huang, 2008; Tosun, Paul, & Rapoport, 1998). SOC entry that participate in Ca^{2+} homeostasis (Putney, 1986) can be activated by endoplasmic reticulum (ER) depletion either by inositol 1,4,5-trisphosphate producing agonists or ER Ca^{2+} ATPase (SERCA) inhibitors such as thapsigargin (Tg) and cyclopiazonic acid (CPA).

Ca^{2+} homeostasis and SOC entry deteriorate in cancer cells. It is well known that mitochondrial Ca^{2+} overload results in apoptosis through mitochondrial swelling and release of mitochondrial apoptotic factors.

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However, in a recent study, it was shown that following mitochondrial Ca^{2+} overload-induced apoptotic stimuli, mitochondrial Ca^{2+} levels were decreased along with the apoptotic response (Giorgi et al., 2012).

ER Ca^{2+} overload following SERCA overexpression may also be associated with apoptosis in COS cells (Ma, Mann, Lee, & Gallinghouse, 1999). An increase in ER Ca^{2+} levels results in mitochondrial Ca^{2+} overload and promotes apoptosis (Rizzuto et al., 2003). The anti-apoptotic effect of Bcl-2 oncogene is also associated with reduction of ER Ca^{2+} levels (Pinton et al., 2001). It was also suggested that releasable ER calcium levels rather than ER Ca^{2+} load regulates apoptosis (Rizzuto et al., 2003). In addition, ER depletion and SOC entry promoted apoptosis in LNCaP cells (Wertz & Dixit, 2000). The role of transient receptor potential canonical 1, TRPC1, a purported regulator of SOC entry (Selli, Erac, Kosova, & Tosun, 2009), in the regulation of apoptosis is still controversial, as TRPC1 promotes anti-apoptotic effects on epidermal skin cells (Pani et al., 2006) while sensitizing intestinal epithelial cells to apoptosis (Marasa et al., 2006).

Based on these data, the purpose of our study was to improve traditional spectrofluorimetric Ca^{2+} measurement methodology with the addition of self-designed apparatus that enables sequential drug additions during monitoring of Ca^{2+} levels in adherent cells and the simultaneous measurement of changes in Ca^{2+} levels as well as isometric force in isolated rat thoracic aorta. By using our monitoring system, we observed a significant decrease in mitochondrial Ca^{2+} load following CPA-induced ER depletion in *TRPC1*-silenced Huh7 cells.

2. Methods

2.1. Ca^{2+} monitoring system

A high-speed multi-wavelength (300–600 nm excitations) spectrofluorometer (PTI QM-8/2005, Photon Technology International NJ, USA) was used. Two apparatus that fit into spectrofluorometer cuvette, one to monitor changes in Ca^{2+} levels in adherent cells and the other for the measurement of isometric force simultaneously with cytosolic Ca^{2+} levels in intact tissues were designed and manufactured (Fig. 1A, B). Polyoxymethylene lids of the cuvette also hold a perfusion manifold. A peristaltic pump is attached to the system for continuous application of agents and solutions for washout.

The system was calibrated by using increasing Ca^{2+} concentrations (0–1.350 μM, Calcium Calibration Buffer Kit, Molecular Probes). The emission intensity at 510 nm increased proportional to elevations in Ca^{2+} concentrations, as expected. Furthermore, there was no change in fluorescent intensity at 360 nm (isosbestic point of fura-2) confirming the proper response of the dye (Fig. 1C).

2.2. Measurement of cytosolic Ca^{2+} levels in adherent cells

Cells were grown on 12 mm diameter round glass coverslips in 24-well plates to 50–80% confluence. Then, they were washed with a HEPES-buffered saline (HBS; NaCl, 135; KCl 5.9; $MgCl_2$, 1.2; $CaCl_2$, 1.5; $NaHCO_3$, 5; glucose, 11.5; HEPES, 11.6; in mM; pH: 7.3) solution containing 1% bovine serum albumin (BSA–HBS).

For the measurement of cytosolic Ca^{2+} levels, Mahlavu, Hep3B, and Huh7 cells were incubated with 5 μM fura-2/AM and 0.02% pluronic F127 in BSA–HBS solution for 60 min at room temperature and in the dark. After the incubation period, the coverslip carrying the fura-2-loaded cells were washed twice with BSA–HBS solution for 15 min and were mounted into the polymethyl methacrylate cuvette via the holder facing the excitation and emission paths at 45% angle (Fig. 1A). Cells were excited at 340 and 380 nm wavelengths, and the emission intensities at 510 nm monitored. CPA (10 μM) and Tg (1 μM) were used to activate SOC entry. Cells were taken into Ca^{2+}-free solution then CPA/Tg was applied, following a Ca^{2+} transient due to depletion of stores. Ca^{2+} (1.5 mM) was added to determine SOCE. Background fluorescence was determined at the end of the experiment by quenching the fura-2 fluorescence with $MnCl_2$ (5 mM) in the presence of 10 μM ionomycin in Ca^{2+}-free solution containing 2 mM EGTA (Tosun et al., 1998). Cytosolic Ca^{2+} levels were expressed as ratio of fluorescence intensities [Ratio (340/380)].

For the simultaneous measurement of cytosolic and mitochondrial Ca^{2+} levels, Huh7 cells were sequentially loaded with rhod-2/AM and fura-2/AM in neostigmine-containing BSA–HBS solution, to inhibit breakdown of dyes by extracellular esterases. Cells were first loaded with 5 μM rhod-2/AM for 30 min at room temperature, as lower temperatures increase mitochondrial loading of Rhod-2 (Nieminen, Saylor, Tesfai, Herman, & Lemasters, 1995) and washed twice with BSA–HBS solution for 15 min at 37 °C. After this initial loading procedure, cells were incubated with 2.5 μM fura-2/AM for 60 min at room temperature in the dark and washed twice with BSA–HBS solution for 15 min at room temperature. Cells were excited at 550, 340, and 380 nm wavelengths, and the emission intensities at 580 and 510 nm were monitored for mitochondrial and cytosolic Ca^{2+} levels, respectively. Mitochondrial Ca^{2+} levels were expressed as normalized fluorescent ratio (F/F_0, where F_0 is the base-line fluorescence).

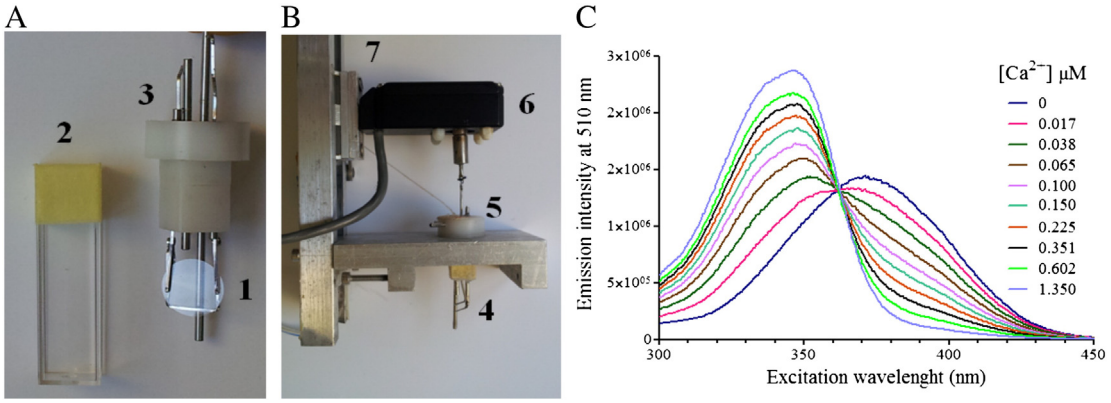

Fig. 1. Apparatus for measurement of Ca^{2+} levels in adherent cells (A) and for simultaneous measurement of isometric force and cytosolic Ca^{2+} levels in intact tissues (B). Cells grown on round glass coverslips (1) were loaded with indicator dyes and mounted into the spectrofluorometer cuvette (2) with the aid of the apparatus (3). Inverted aortic ring stretched by the hooks (4) was mounted into the cuvette via the apparatus (5) attached to an isometric force-displacement transducer (6) and micrometer (7) allowing fine adjustment in tone. Titration of fura-2 by Ca^{2+} (C). Fura-2 excitation spectrum was obtained in the presence of increasing Ca^{2+} concentrations (0–1.350 μM).