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Contemporary Issues

Caveats and limitations of plate reader-based high-throughput kinetic measurements of intracellular calcium levels

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ABSTRACT

Calcium plays a crucial role in virtually all cellular processes, including neurotransmission. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is therefore an important readout in neurotoxicological and neuropharmacological studies. Consequently, there is an increasing demand for high-throughput measurements of $[Ca^{2+}]_i$, e.g. using multi-well microplate readers, in hazard characterization, human risk assessment and drug development. However, changes in $[Ca^{2+}]_i$ are highly dynamic, thereby creating challenges for high-throughput measurements. Nonetheless, several protocols are now available for real-time kinetic measurement of $[Ca^{2+}]_i$ in plate reader systems, though the results of such plate reader-based measurements have been questioned. In view of the increasing use of plate reader systems for measurements of $[Ca^{2+}]_i$ a careful evaluation of current technologies is warranted. We therefore performed an extensive set of experiments, using two cell lines (PC12 and B35) and two fluorescent calcium-sensitive dyes (Fluo-4 and Fura-2), for comparison of a linear plate reader system with single cell fluorescence microscopy. Our data demonstrate that the use of plate reader systems for high-throughput real-time kinetic measurements of $[Ca^{2+}]_i$ is associated with many pitfalls and limitations, including erroneous sustained increases in fluorescence, limited sensitivity and lack of single cell resolution. Additionally, our data demonstrate that probenecid, which is often used to prevent dye leakage, effectively inhibits the depolarization-evoked increase in $[Ca^{2+}]_i$. Overall, the data indicate that the use of current plate reader-based strategies for high-throughput real-time kinetic measurements of $[Ca^{2+}]_i$ is associated with caveats and limitations that require further investigation.

Introduction

There is an increasing demand for high-throughput screening (HTS) in toxicity testing, mainly because of its cost-effectiveness and speed. Until recent years, HTS was largely limited to screening for cytotoxicity, using e.g., the MTT assay (Denizot and Lang, 1986). Nowadays, several high-throughput assays are available to assess cell viability, cytotoxicity and apoptosis (see e.g., Lövborg et al., 2005; Schoonen et al., 2009). Though useful as a first step to determine non-cytotoxic concentrations for further testing, several more subtle and organ- and cell type-specific endpoints that do not necessarily lead to acute cytotoxicity (e.g., disruption of intracellular calcium homeostasis, mitochondrial disregulation, or inhibition of transporters or channels) need to be assessed for proper hazard characterization.

This requirement makes the use of HTS challenging, particularly in neuroscience, neuropharmacology and neurotoxicology. Neuron-specific endpoints, including calcium homeostasis, electrical activity, neurotransmitter release as well as receptor and channel activation, are highly dynamic. Although static fluorescent or luminescent signals are, due to experimental design of plate reader, the favored endpoints for high-throughput (neurotoxicity) studies, efforts have been made to use HTS for real-time kinetic measurements of neuronal signals, including agonist or chemical-induced dynamic changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Ca^{2+} plays an essential role in a large number of cellular processes, including neurotransmission (for reviews see: Garcia et al., 2006; Westerink, 2006), gene expression (for review see: Carrasco and Hidalgo, 2006) and programmed (apoptosis) as well as necrotic (e.g., via mitochondrial disruption or release of degradative enzymes) cell death (for review see: Orrenius et al., 2011). Neuronal cells therefore exert strong control over the dynamics of their Ca^{2+} signals, i.e., tightly regulate the balance between Ca^{2+} influx, Ca^{2+} extrusion, Ca^{2+} sequestration and Ca^{2+} buffering by cytosolic Ca^{2+} binding proteins (for reviews see: Berridge et al., 2003; Garcia et al., 2006; Kostyuk and Verkhratsky, 1994; Miller, 1991; Westerink, 2006). As a result, Ca^{2+} signals are highly dynamic with fast, transient increases and oscillations that occur in seconds to minutes (Eilers et al., 1995). Due to the dynamics of Ca^{2+} signals, real-time kinetic measurements of Ca^{2+} are required. Also, Ca^{2+} fluorescent dyes and equipment to measure the Ca^{2+}-derived transient fluorescent signals need to have sufficient sensitivity and temporal resolution. While single cell fluorescent microscopy meets these demands it is also labor intensive and time consuming. Despite the

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fact that the sensitivity of plate readers is rather low compared to fluorescent microscopy, measurements of $[Ca^{2+}]_i$ kinetics with plate readers would thus be favorable. Consequently, many laboratories now use plate readers and a large amount of HTS data on changes in $[Ca^{2+}]_i$ has become available. However, the accuracy and suitability of plate readers to determine dynamic changes in $[Ca^{2+}]_i$ has recently been questioned as results obtained with plate readers differ between studies and in many cases do not match electrophysiological recordings and fluorescent microscopy data (Westerink and Hondebrink, 2010).

As there is a clear need for reliable HTS strategies in (neuro) toxicity testing and drug development, the aim of this study is to carefully compare current high-throughput real-time kinetic Ca^{2+} measurement strategies with (single cell) fluorescent microscopy. We therefore used extensively characterized dopaminergic PC12 cells (Westerink and Ewing, 2008) as well as non-dopaminergic B35 neuroblastoma cells (Otey et al., 2003) for measurements of $[Ca^{2+}]_i$ using two common fluorescent dyes (Fura-2 and Fluo-4) to compare plate reader and single cell microscopy experiments.

Materials and methods

Chemicals. NaCl, KCl and HEPES were obtained from Merck (Whitehouse Station, NJ, USA); $MgCl_2$, $CaCl_2$, glucose, sucrose, and NaOH were obtained from BDH Laboratory Supplies (Poole, UK). Fura-2 (pentapotassium salt), Fura-2 AM, and Fluo-4 AM were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). All other chemicals were obtained from Sigma-Aldrich (St. Louis MO, USA) unless otherwise noted. Saline solutions were prepared with de-ionized water (Milli-Q®; resistivity > 10 MΩ cm). Stock solutions were prepared just prior to the experiments.

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands) as described previously (Heusinkveld et al., 2010).

Rat neuroblastoma (B35) cells (Otey et al., 2003) were cultured in DMEM (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (ICN Biomedicals, Zoetermeer, The Netherlands) and 1% additional amino acids (stock solution containing 40 mM of L-Cys, L-Ala, L-Asp, L-Pro, L-Glu and L-Asx).

For fluorescent microscopy Ca^{2+} imaging experiments, undifferentiated PC12 (1.4×10^6 cells/dish; ±75% confluency) or B35 (1.4×10^6 cells/dish; ±75% confluency) cells were subcultured in poly-L-lysine (50 μg/ml) coated glass-bottom dishes (MatTek, Ashland, MA) as described previously (Heusinkveld et al., 2010). For fluorescent plate reader Ca^{2+} imaging experiments, undifferentiated PC12 ($1.5\ 10^5$ cells/well; ±100% confluency) or B35 ($1.2\ 10^5$ cells/well; ±100% confluency) cells were subcultured in poly-L-lysine (50 μg/ml) coated, black, clear-bottom, 96-well plates (Greiner Bio-one, Solingen, Germany). Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

Fluorescent Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 or the Ca^{2+}-sensitive fluorescent single wavelength dye Fluo-4. Briefly, cells were loaded with 5 μM Fura-2 AM or 5 μM Fluo-4 AM (Molecular Probes; Invitrogen, Breda, The Netherlands) in saline (containing in mM: 1.8 $CaCl_2$, 24 glucose, 10 HEPES, 5.5 KCl, 0.8 $MgCl_2$, 125 NaCl, and 36.5 sucrose at pH 7.3, adjusted with NaOH) for 20 min at room temperature (RT), followed by 15 min (Fura-2 AM) or 30 min (Fluo-4 AM) de-esterification in external saline at RT.

In specific experiments, probenecid was used to prevent/reduce dye-leakage. Briefly, probenecid was dissolved in external saline and pH was adjusted to 7.3. Unless otherwise noted cells were loaded for 15 min with probenecid (2.5 mM) during de-esterification (protocol adapted from Di Virgilio et al., 1990). Cells were continuously exposed to probenecid during measurements.

For microplate reader experiments, cells were placed in an Infinite M200 microplate reader equipped with a Xenon Flash light source (10W; Tecan Trading AG, Männedorf, Switzerland) controlled by iControl software (version 7.1). For fluorescence microscopy, cells were placed on the stage of an Axiovert 35 M inverted microscope (40× oil-immersion objective, NA 1.0; Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (Xenon Short Arc lamp, 150W) and an Image SensiCam digital camera (TILL Photonics GmBH, Gräfelfing, Germany). Camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing.

Fluorescence was evoked by 340- and 380-nm excitation wavelengths (F340 and F380, Fura-2) or 488-nm excitation wavelength (F488, Fluo-4) and collected at 510 nm (Fura-2) or 520 nm (Fluo-4). Data was collected every 3 s in the plate reader (exposure: 15 flashes at 40 Hz) and every 6 s in fluorescence microscopy (exposure: 2 ms). Significant dye bleaching did neither occur in plate reader nor in microscopy. To assess dynamic ranges for both plate reader and microscopy, maximum and minimum fluorescence values (at 340 and 380 nm excitation wavelength) were determined in separate experiments in which Fura-2 loaded PC12 cells were incubated with ionomycin (5 μM) and ethylenediamine-tetraacetic acid (EDTA; 17 mM).

Data on changes in F340/F380 (Fura-2) from fluorescence microscopy were analyzed using a custom-made MS-Excel macro which calculates F340/F380 ratio values from the raw F340 and F380 data reflecting changes in $[Ca^{2+}]_i$ and includes background correction. Data on changes in F488 (Fluo-4) from fluorescence microscopy as well as all data from the plate reader were analyzed manually with correction for background where applicable.

Data analysis and statistics. All data are presented as mean F340/F380 (± SD; normalized to baseline; Fura-2 AM) or as F488 (± SD; normalized to baseline; Fluo-4 AM) from the number of cells (n; fluorescence microscopy) or wells (n; plate reader) in 3–5 independent experiments unless otherwise noted. Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, California, USA). Continuous data were compared using the Student's t-test, paired or unpaired where applicable. A p value <0.05 is considered statistically significant; n.s. indicates the absence of a significant effect.

Results

Initial experiments were performed to check reproducibility of the plate reader and to identify those conditions that yield the best signal and signal-to-noise ratio for measurements of $[Ca^{2+}]_i$. As most plate readers can operate in top-read and in bottom-read mode, experiments were performed with the plate reader either in top or in bottom fluorescence reading mode. The results presented in Fig. 1A demonstrate a sharp but limited increase in F340/F380 in Fura-2-loaded PC12 cells upon injection of 100 μl high-K^+ containing saline into 200 μl starting volume per well (final concentration: 50 mM K^+), both in top- and in bottom-read mode. Although data obtained in bottom-read mode reveal a larger change in F340/F380 compared to top-read measurements, suggestive of a higher sensitivity, raw experimental data (not shown) revealed that the fluorescence yield (F340 and F380) in bottom-read experiments is often only 10–20% of the yield in top-read experiments in the same well plate. Similarly, a sharp but limited increase in F340/380 is observed upon injection of 100 μl ATP-containing saline (final concentration 100 μM ATP; Fig. S1) in both top- and bottom-read mode, with the bottom-read mode yielding a lower total fluorescence yield (raw data; not shown). In summary, the change in F340/F380 is highest in bottom-read experiments but the