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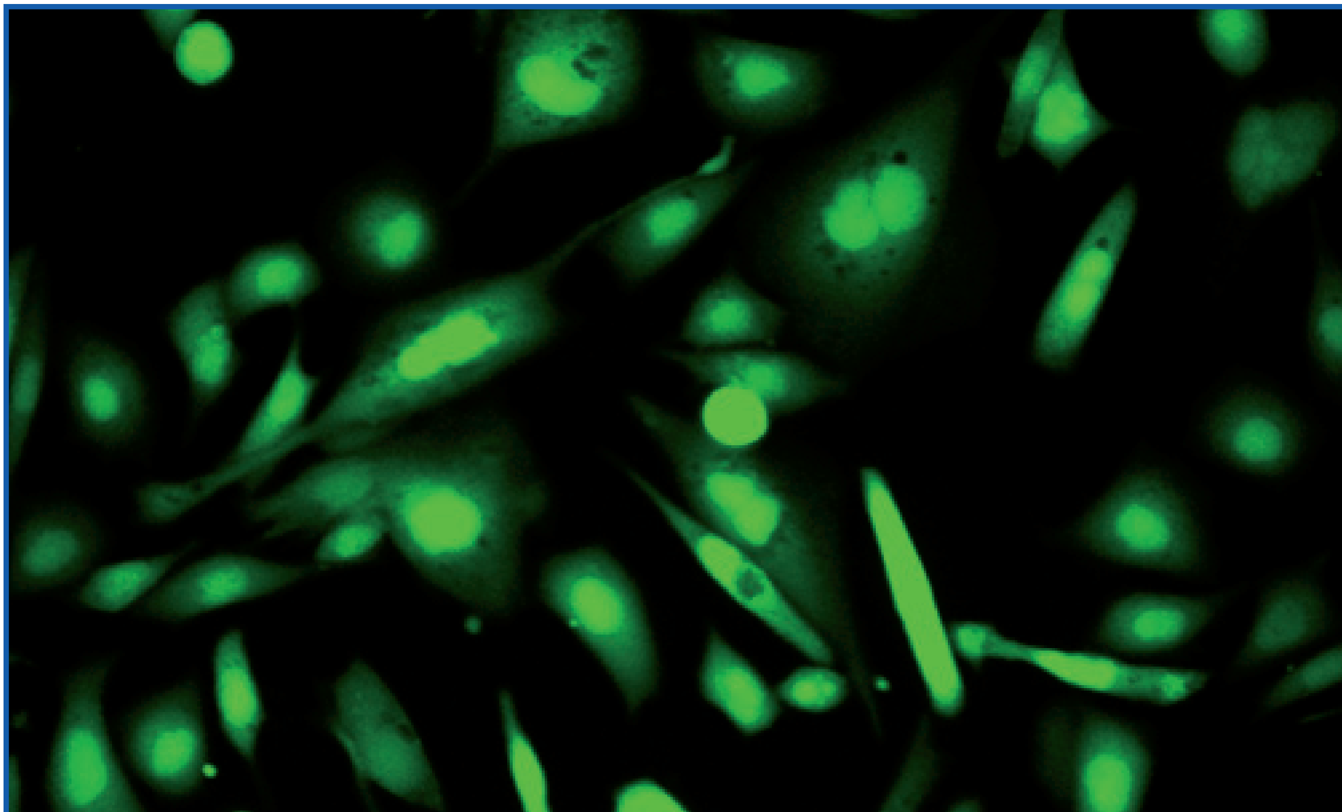
Measurement Of Concentration-Dependent Increases In $[Ca^{2+}]_c$ ATP-Stimulated Chinese Hamster Ovary (CHO-K1) Cells



Introduction

Analysis of temporal changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is amongst the most important methods in drug discovery including G-protein research (Kiselyov *et al*., 2003), receptor activation and apoptosis studies. Unlike other second messengers, changes in $[Ca^{2+}]_c$ are often short-lived, show high signal/noise and are often referred to as the "molecular switch" of a cell in organisms as diverse as nematodes (Mathews *et al*., 2003, humans and plants (Scrase-Field & Knight, 2003). The ability to measure the temporal aspects of $[Ca^{2+}]$ has greatly enhanced the knowledge of signal transduction mechanisms.

In drug research, the kinetic measurement of Ca^{2+} responses is widely performed with plate reader type systems. These systems allow the reading of multiple wells at the same time and excel in throughput. However, they do not visualize single cells due to their low resolution and hence assume that the cellular population is uniform and shows homogeneous responses. This is not necessarily the case as shown in many studies from cell cultures (e.g. Suzuki *et al*., 1991) to isolated tissue (e.g. Ruehlmann *et al*., 2000). Only microscopy allows the investigation of individual cells but this technique traditionally had limitations on throughput.

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BD

BD Bioscience has developed the BD Pathway Bioimager, a sophisticated automated cellular imaging platform for advanced academic research and assay development. The BD Pathway Bioimager uses lamp excitation and can take advantage of the entire visible, near-UV and near-infrared spectrum for excitation of multiple probes. The system's proprietary confocal disk can be switched into and out of the light path by the user in order to accommodate virtually all imaging needs.

Unlike other systems, the BD Pathway Bioimager moves the objective underneath the plate thus ensuring optimal sample stability. Loosely attached cells can be imaged as well as those that ordinarily would respond to mechanical stress with an increase in $[Ca^{2+}]_c$ even in absence of a pharmacological stimulus (e.g. Arora *et al.*, 1994).

Aim

The aim of this experiment was to investigate the effects of ATP on the $[Ca^{2+}]_c$ signaling properties of Chinese Hamster Ovary (CHO) Cells and to illustrate the versatility of the BD Pathway Bioimager system.

Methods

Cell Culture

Chinese Hamster Ovary cells (CHO-K1, American Type Culture Collection, CCL-61) were grown and maintained in Minimum Essential Medium, Eagle's, Dulbecco's Modification (DMEM, Biofluids, P104G) containing 10% Fetal Bovine Serum (Gibco, 26140-079), 1% Penicillin / Streptomycin (Biofluids, 303), 1% Non-Essential Amino Acids solution (Biofluids, P332). For experimentation, 7000 cells / well were seeded into 96 well plates (Costar, 3614) and grown overnight at 37ºC, 5% CO_2/95% Air.

Dye Loading

Cells were loaded in growth medium with 2 µM Fluo4/AM molecular probes, F14201 and probenecid (1.25mM in 5mM HEPES) for 30 minutes at 37ºC. Cells were washed with HBSS/probenecid (Hanks Buffered Saline Solution, pH 7.2) to remove excess dye and left to sit for another 10 minutes at room temperature to complete de-esterification of the dye.

These experiments were performed without the use of a nuclear dye.

Imaging

Plates were moved into the environmental chamber of the BD Pathway 855 Bioimager. The drug treatment plate was placed into its holder where it is accessible to the 3-dimensional automated dispenser. The dispenser uses disposable tips obviating the need for a wash station and allows addition of drug directly above the imaging station without having to move the sample plate. Drugs were added as a bolus in non-contact mode. Although the system is capable of active mixing, it was found unnecessary in this experiment (data not shown). For these experiments, a 20X U-Apo 340 objective (Olympus, NA 0.75) was used. Cells were imaged at 37ºC and under 5% CO_2/95% Air.

Pharmacological Treatment

Cells were exposed to ATP after the sixth datapoint was taken. The drug was added as bolus and the response was over the course of the experiment.

Data Handling

Preliminary experiments had shown that CHO cells respond well to ATP in a concentration dependent manner (data not shown). In Fluo-4 loaded cells, a

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subpopulation often shows a spontaneous rise in $[Ca^{2+}]_c$ even before the agonist is added. This response may be triggered by the generation of oxygen-free radicals or be truly spontaneous activity, however, for most pharmacological applications it presents a non-specific activity that can be excluded from the analysis. The presented data is derived from cells that do not show this early rise. The method to exclude these cells is explained elsewhere in a separate application note.

The classified data is exported directly into a text (.txt) file and opened using the proprietary add-in for Microsoft™ Excel™. Here, various calculations can be performed including concentration response curves and further thresholding operations. For publication purposes, analyzed data was exported into Graphpad (Prism).

Results

Cells exposed to ATP show a pronounced, steep increase in $[Ca^{2+}]_c$, followed by a short decline and a plateau ("peak-plateau response"). The amplitude of the plateau is indicative of a balance between Ca^{2+} channel / store-release mechanisms and Ca^{2+} store sequestering and pumping.

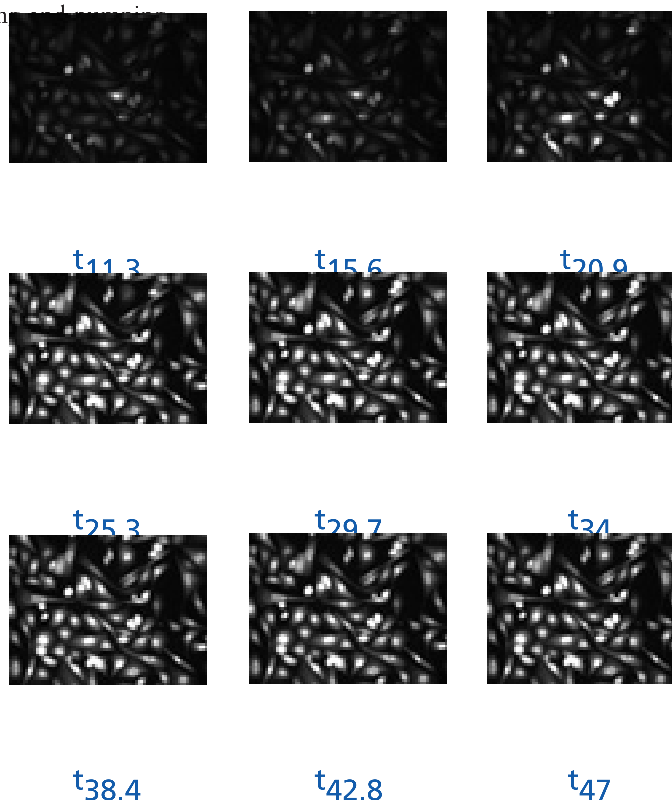

Figure 1 $[Ca^{2+}]_c$ in CHO-K1 cells in response to 100µM ATP. Drug addition was performed after $t_{20.9}$

Prior experiments had shown that the cells respond within 5-10 seconds after drug addition. The acquisition frequency was chosen according to balance the need for high temporal resolution with the obvious issue of photobleaching and image-storage requirements in mind. The response pattern is somewhat typical for cultured cells. Primary cells and excitable cells such as neurons, skeletal muscle and cardiac myocytes can respond much faster and for those cells, a higher sampling frequency can be selected.

The images shown in Figure 1 show a uniform increase of virtually all cell $[Ca^{2+}]_c$ over time. Since data points were acquired between stored image, thus increasing the sampling frequency, the variability of the cells is more evident in the data traces (see

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Figure 2)

Data Classification

AttoVision allows distinct "Treatment Zones" (TZ) to be analyzed for each phase of the response. The first TZ was defined from the start of the experiment to the time of the drug addition and serves as the baseline. The second TZ identifies the peak

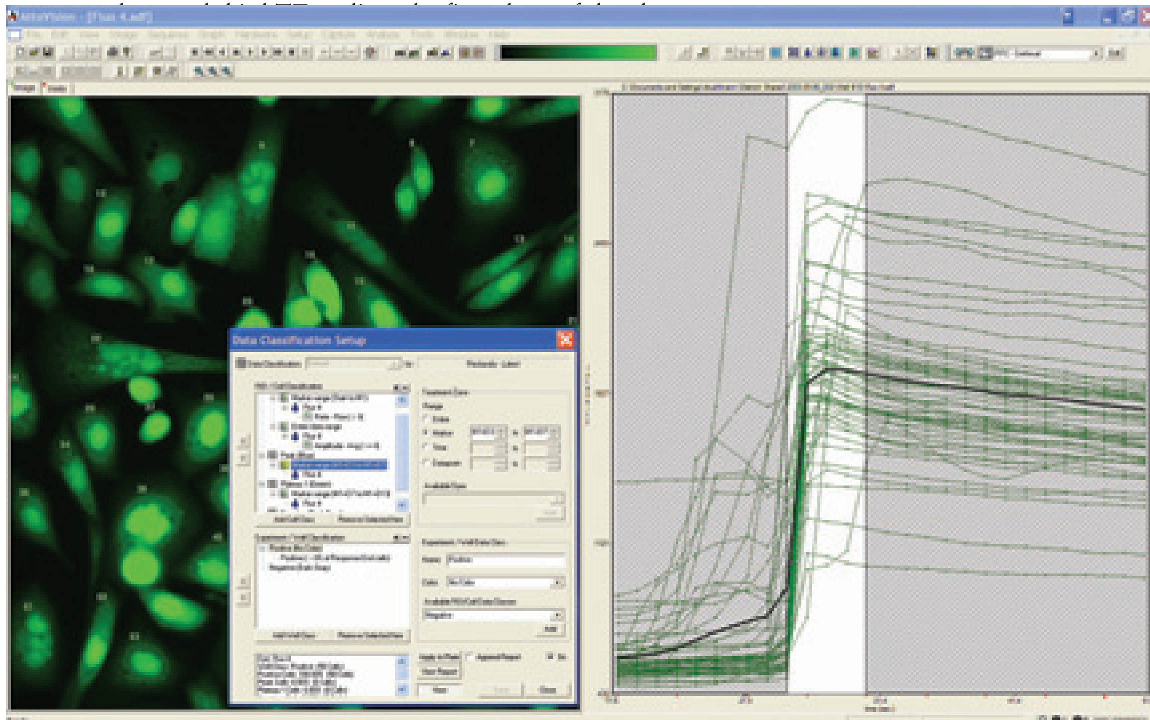

Figure 2 Data classification menu and setup of Treatment Zones

Treatments zones can be allocated in different ways, depending on the experiment. Figure 2 shows the treatment-zone set-up menu in relation to the time course of the cellular data (right). In this example, a robust response of the cells can be seen after ATP was added at t_{20}s. Addition of a drug creates a "Marker" which serves as a reference point for the rest of the experiment. It is also possible to arrange the TZs by time after the start of the experiment or by the number of the data point acquired.

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Concentration Response Curves

Normalized Peak Fluorescence Intensity

Normalized Plateau Fluorescence Intensity

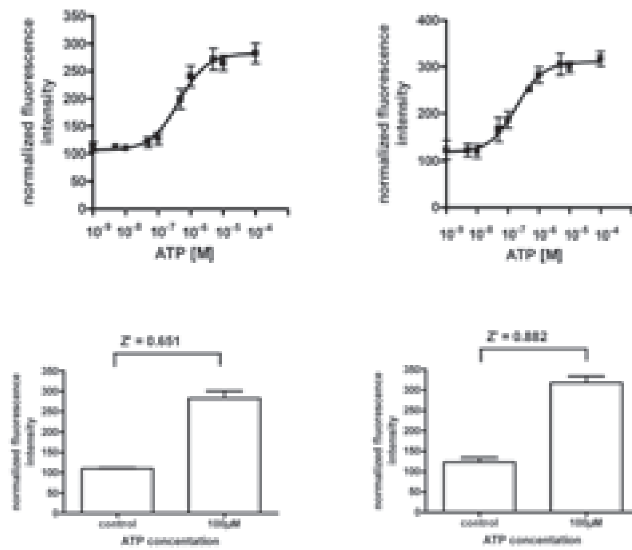

Figure 3 Concentration response curve to ATP in CHO-K1 cell $[Ca^{2+}]_c$

To normalize the data, the average fluorescence intensity of each cell in the plateau or peak TZ is divided by its own fluorescence intensity at basal level (TZ1). The normalized cell-by-cell data is averaged and plotted on a log scale. The left panel of Figure 3 shows the normalized peak response of the cells exposed to either control or rising concentration of ATP. The right shows the same calculation for the plateau of the $[Ca^{2+}]_c$ response.

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Discussion

CHO-K1 cells show robust, concentration-dependent increases in $[Ca^{2+}]_c$ when stimulated with ATP.

A Z' of 0.882 with n=4 wells is considered very suitable for a live-cell assay. The robustness of the analysis is in part due to the software's ability to identify specific subpopulations of cells and classify them either as outliers or include them in a separate analysis.

The responses are fast and very reproducible. The AttoVision software environment allows imaging with real-time analysis and to perform advanced calculations on distinct periods within the time-course. Together with the Microsoft Excel ™ Add-In BD Image Data Explorer, BD Bioscience has established a dedicated and specialized software suite for live-cell kinetic imaging.

Cells in culture respond typically in "Peak-Plateau" form in their $[Ca^{2+}]_c$ rise. This is not necessarily the case in all cell types, especially primary cells or cells can show distinct pattern. For example, rabbit vena cava vascular smooth muscle cells in situ respond to phenylephrine stimulation by "firing" a succession of waves, the frequency and the speed of which is concentration-dependent (Ruehlmann *et al*. 2000). In addition, these cells show incremental recruitment but not incremental rise in $[Ca^{2+}]_c$ amplitude. When cells respond in this way, traditional, plate-reader type systems fail to differentiate the response types and thus are of limited use. The BD Pathway Bioimagers high-resolution approach paired with dedicated kinetic software can not only quantify the amplitude of the response but also score the number of cells responding per well and identify discrete subpopulations.

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