

Inhibition of T Cell Receptor Signaling-Mediated Calcium Flux in T cells

Author

Agilent Technologies, Inc.

Abstract

Increased intracellular calcium (Ca^{2+}) content is an essential and irreversible step towards the activation of T lymphocytes, and is involved in the regulation of cell survival, proliferation, and differentiation. This application note demonstrates the capability of the Agilent NovoCyte flow cytometer to quantify dynamic changes in intracellular Ca^{2+} by measuring calcium flux in Jurkat T cells. These data show that the NovoCyte flow cytometer can be used to measure dynamic alterations in intracellular Ca^{2+} concentration in real time at the single-cell level.

Introduction

Ca^{2+} is an important secondary messenger and a key component of many cell processes. It is an extremely versatile molecule used by all cells for various signaling pathways, occurring during the activation of G protein-coupled receptors, synaptic transmission of neurons, muscle contraction, and antigen-specific activation of T cells.

Unlike many other secondary messengers, Ca^{2+} is not synthesized or metabolized, but stored and released by channels/pumps that maintain Ca^{2+} concentrations in distinct cellular compartments. Alterations in intracellular Ca^{2+} concentrations are among the most rapid cellular responses, ranging from faster than a microsecond to several minutes depending on the specific stimuli.

Methods that are currently used to detect Ca^{2+} flux include confocal microscopy, plate-based assays, and flow cytometry. Flow cytometry has the unique capability to determine Ca^{2+} levels in several millions of individual cells over time as well as in an entire population. Intracellular Ca^{2+} measurements use fluorescent Ca^{2+} indicators that exhibit an increase in fluorescence upon binding to Ca^{2+} . Ca^{2+} indicators such as Quin-2, Fura-2, Indo-1, Fluo-3, and Fluo-4/AM can be used to quantify Ca^{2+} concentrations by flow cytometry. This application note describes the measurement of calcium flux in a cultured T cell line using the NovoCyte flow cytometer to assess the inhibition of T cell receptor (TCR)-mediated calcium flux by ibrutinib.

Calcium flux in Jurkat T cells on the NovoCyte flow cytometer

Increased intracellular Ca^{2+} content is an essential and irreversible step towards the activation of T lymphocytes and in regulating their cell survival, proliferation, and differentiation. Upon antigen recognition, activation of the TCR initiates numerous signaling cascades, resulting in cellular activation and proliferation. During TCR signaling, IL-2-inducible kinase (ITK) activates phospholipase C (PLC) and subsequently releases intracellular Ca^{2+} stores from the endoplasmic reticulum (ER), resulting in a rapid elevation of intracellular Ca^{2+} levels up to 500 nM.

This process subsequently triggers the opening of ion channels in the plasma membrane allowing an influx of extracellular Ca^{2+} , which in turn activates key molecules, such as calcineurin. Inhibition of calcium flux has been shown to affect T cell function; therefore, measuring the Ca^{2+} content in T cells presents important information about the status and kinetics of T cell activation.

The capability of the NovoCyte flow cytometer to quantify dynamic changes in intracellular Ca^{2+} was demonstrated by measuring calcium flux in Jurkat T cells. Calcium flux was induced by A23187 or ionomycin, both effective Ca^{2+} carriers that increase the ability of divalent ions to cross biological membranes. Resting Jurkat T cells were loaded with the Ca^{2+} indicator dye, Fluo-4/AM. Fluo-4/AM binds to free Ca^{2+} , and the intensity of the fluorescence signal is proportional to the concentration. Ca^{2+} concentration was measured by the change in Fluo-4/AM fluorescence over time.

A stable background of Fluo-4/AM is seen before the addition of calcium flux stimulants (Figures 1A to 1C). Upon addition of A23187 or ionomycin, the mean fluorescence signal increases immediately, indicating an elevation in intracellular Ca^{2+} concentration. Notably, the effect of A23187 is more robust than the addition of ionomycin (Figures 1B to 1D). These data demonstrate that dynamic alterations in intracellular Ca^{2+} concentration can be measured on the NovoCyte flow cytometer.

Inhibition of CD3-mediated calcium flux in Jurkat T cells by ibrutinib

T cell calcium flux is initiated by TCR signaling, which leads to a sequence of events culminating in the release of Ca^{2+} stores from the ER and an influx of extracellular Ca^{2+} into the cell. Ibrutinib is a small molecule that specifically blocks TCR signaling by inhibition of IL-2-inducible kinase (ITK). We used the NovoCyte flow cytometer to investigate the inhibitory effect of ibrutinib on TCR activation through calcium flux induced by CD3 crosslinking. The addition of αCD3 antibody results in a rapid increase of Ca^{2+} in Jurkat T cells, attaining an intracellular Ca^{2+} concentration 3.5 times higher than the baseline (Figure 2).

Peak Ca^{2+} concentrations are reached approximately 1.5 minutes after the addition of αCD3 antibody (Figure 2D). Next, the effect of the ibrutinib on calcium flux was assayed; Jurkat T cells were either pretreated with DMSO or ibrutinib. Treatment with DMSO alone had no effect on TCR-induced calcium flux in Jurkat T cells (Figure 2B). However, calcium flux was completely abolished with ibrutinib (Figure 2C). By measuring the kinetics of Ca^{2+} mobilization on the NovoCyte flow cytometer, we can observe defects or inhibition of TCR signaling, as was shown with ibrutinib treatment.

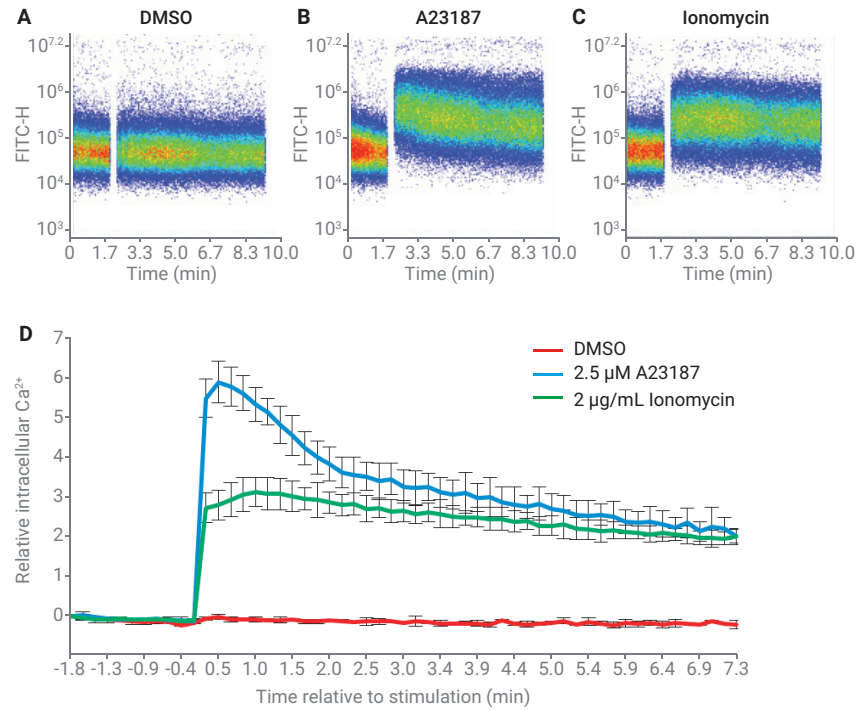


Figure 1. Calcium flux in Jurkat cells. Jurkat T cells were loaded with Fluo-4/AM, and acquired on the NovoCyte flow cytometer. The baseline for each sample was acquired, and the rest of the cells were analyzed after the addition of Ca^{2+} influx stimulants, and responses were measured over time. A) DMSO; B) 2.5 μM A23187; C) 2 $\mu\text{g}/\text{mL}$ ionomycin; D) time-dependent changes of intracellular Ca^{2+} concentration. Data were normalized to baseline by the equation: Relative Intracellular Ca^{2+} (RIC) = fold of baseline (initial stage) – 1. Time points depicted on the X-axis are the time relative to stimulation. Zero is the stimulation time, and the initial detection point was ~20 seconds relative to simulation. Data were normalized to the baseline ($n = 3$, means \pm SEM).

Conclusion

Accurate measurements of intracellular Ca^{2+} concentration allows for a more comprehensive understanding of Ca^{2+} -regulated cell functions and signaling pathways that affect a wide range of physiological and pathological processes. Calcium flux can be induced by Ca^{2+} stimulants and measured on the NovoCyt flow cytometer to allow real-time Ca^{2+} measurement at the single-cell level. The NovoCyt flow cytometer offers high-performance flow cytometry, making the application of calcium flux assays and related studies easy to perform.

References

- Berridge, M. J.; Lipp, P.; Bootman, M. D. The Versatility and Universality of Calcium Signalling. *Nat. Rev. Mol. Cell Biol.* **2000**, 1(1), 11.
- Feske, S.; Calcium Signaling in Lymphocyte Activation and Disease. *Nat. Rev. Immunol.* **2007**, 7, 690–702.
- Smith-Garvin, J. E.; Koretzky, G. A.; Jordan, M. S. T Cell Activation. *Annu. Rev. Immunol.* **2009**, 27, 591.
- Parekh, A. B. Functional Consequences of Activating Store-Operated CRAC Channels. *Cell Calcium* **2007**, 42, 111–121.
- Lewis, R. S.; Cahalan, M. D. Mitogen-Induced Oscillations of Cytosolic Ca^{2+} and Transmembrane Ca^{2+} Current in Human Leukemic T Cells. *Cell Regul.* **1989**, 1, 99–112.
- Guy, C. S.; Vignali, D. A. Organization of proximal signal initiation at the TCR:CD3 complex. *Immunol. Rev.* **2009**, 232, pp. 7–21.
- Dubovsky, J. A.; et al. Ibrutinib is an Irreversible Molecular Inhibitor of ITK Driving a Th1-Selective Pressure in T Lymphocytes. *Blood* **2013**, 122(15), 2539–2549.

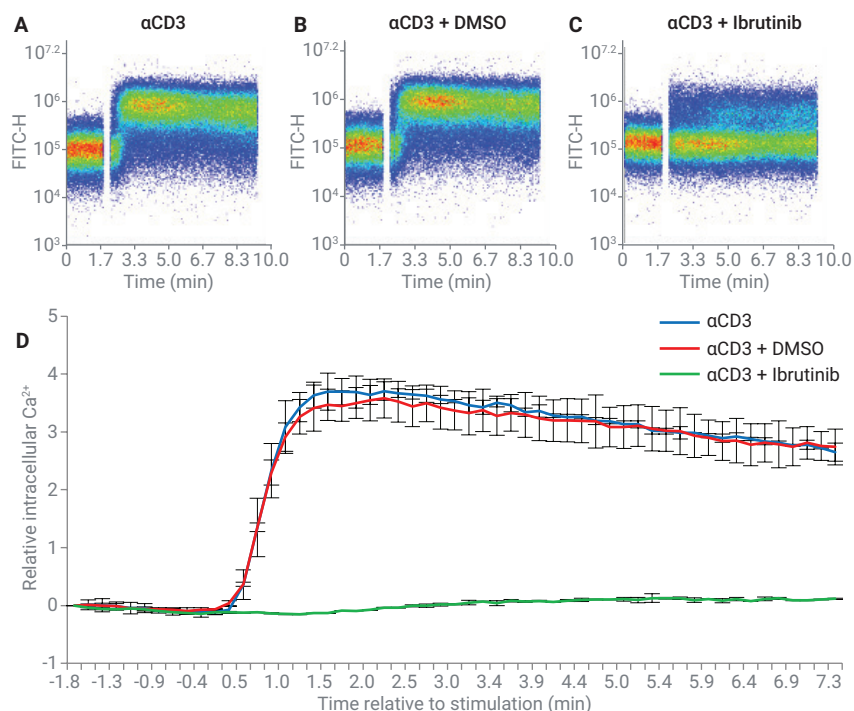


Figure 2. Ibrutinib inhibition of CD3-mediated calcium flux in Jurkat T cells. Jurkat T cells were loaded with Fluo-4/AM then treated with DMSO or 1 μM ibrutinib. The baseline was acquired, then the rest of the cells were stimulated with 5 $\mu\text{g/mL}$ anti-CD3 and further analyzed. A) Without treatment; B) treated with DMSO; C) treated with 1 μM Ibrutinib; D) time-dependent changes of intracellular Ca^{2+} concentration. Data were normalized to the baseline by the equation: $\text{RIC} = \text{fold of baseline (initial stage)} - 1$. Time points depicted on the X-axis are the time relative to stimulation. Zero is the stimulation time, and the initial detection point was ~20 seconds relative to stimulation. Data were normalized to the baseline ($n = 3$, means \pm SEM).

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.