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

Calcium (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 amongst 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 advantage of 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 utilize fluorescent Ca^{2+} indicators which exhibit an increase in fluorescence upon binding to Ca^{2+}. Ca^{2+} indicators such as Quin-2, Fura-2, Indo-1, Fluo-3, Fluo-4/AM can be used to quantify Ca^{2+} concentrations by flow cytometry. In this application note, calcium flux was measured 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 indicated 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 the subsequent release of intracellular Ca^{2+} stores from the endoplasmic reticulum (ER) resulting in a rapid elevation of intracellular Ca^{2+} levels up to 500nM. 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 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 (Figure 1A-C, left section of plot). Upon addition of A23187 or Ionomycin, 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 (Figure 1B-D). This data demonstrates that dynamic alterations in intracellular Ca^{2+} concentration can be measured on the NovoCyte flow cytometer.

Figure 1. Calcium flux in Jurkat cells. Jurkat T cells were loaded with Fluo-4/AM and acquired on the NovoCyte flow cytometer. Baseline for each sample was acquired and the rest of the cells were analyzed after the addition of Ca^{2+} influx stimulants and response measured over time. A. DMSO B. 2.5μM A23187; C. 2μg/mL Ionomycin ; D. Time-dependent changes of intracellular Ca^{2+} concentration. Data was normalized to baseline by the equation of Relative Intracellular Ca^{2+} (RIC) = fold of baseline (initial stage) - 1. Time points depicted on X axis are the time relative to stimulation. Zero is the stimulation time, and the initial detection point was ~ 20s relative to simulation. Data was normalized to baseline (n = 3, means +/- SEM).
Inhibition of CD3-mediated calcium flux in Jurkat T cells by Ibrutinib

T cell calcium flux is initiated by TCR signaling leading to a sequence of events culminating in the release of Ca\(^{2+}\) stores from the ER and 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. Addition of αCD3 antibody results in a rapid increase of Ca\(^{2+}\) in Jurkat T cells attaining an intracellular Ca\(^{2+}\) concentration 3.5 fold higher than the baseline (Figure 2). Peak Ca\(^{2+}\) concentrations are reached about 1.5 minutes after 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 monitor defects or inhibition of TCR signaling, as was shown with Ibrutinib treatment.

Figure 2. Ibrutinib inhibition of CD3-mediated calcium flux in Jurkat T cells. Jurkat T cells were loaded with Fluo-4/AM and then treated with DMSO or 1 μM Ibrutinib. Baseline was acquired than the rest of the cells were stimulated with 5 μg/mL anti-CD3 and further analyzed. A. Without treatment; B. Treated with DMSO; C. Treated with 1μM Ibrutinib; D. Time-dependent changes of intracellular Ca\(^{2+}\) concentration. Data was normalized to baseline by the equation of Relative Intracellular Calcium (RIC) = fold of baseline (initial stage) - 1. Time points depicted on X axis are the time relative to stimulation. Zero is the stimulation time, and the initial detection point was ~20s relative to simulation. Data was normalized to baseline (n = 3, means +/- 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 NovoCyte flow cytometer to allow real time Ca\(^{2+}\) measurement at the single cell level. The NovoCyte flow cytometer offers high performance flow cytometry making the application of calcium flux assays and related studies easy to perform.

References


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