Analytical Biochemistry 527 (2017) 13–19

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

The use of flow cytometry to examine calcium signalling by TRPV1 in mixed cell populations

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ARTICLE INFO

Article history:
Received 23 August 2016
Received in revised form
24 March 2017
Accepted 30 March 2017
Available online 31 March 2017

Keywords:
Flow cytometry
TRPV1
Calcium signaling
Indo-1 AM
Immune phenotyping

ABSTRACT

Flow cytometric analysis of calcium mobilisation has been in use for many years in the study of specific receptor engagement or isolated cell:cell communication. However, calcium mobilisation/signaling is key to many cell functions including apoptosis, mobility and immune responses. Here we combine multiplex surface staining of whole spleen with Indo-1 AM to visualise calcium mobilisation and examine calcium signaling in a mixed immune cell culture over time. We demonstrate responses to a TRPV1 agonist in distinct cell subtypes without the need for cell separation. Multi parameter staining alongside Indo-1 AM to demonstrate calcium mobilization allows the study of real time calcium signaling in a complex environment.

Introduction

The ability to investigate calcium influx in response to known calcium inducers is of increasing importance [1]. The use of Indo-1 acetoxymethylester (Indo-1 AM) emission in flow cytometry has been known for many years [2] in the study of cell specific calcium channels on isolated single cell populations [3]. Most frequently, flow cytometric analysis is combined with microscopy or cuvette based techniques to detect calcium signaling in distinct populations [4]. However, the ability to compare cells of different types simultaneously would be beneficial where particular calcium channels are not uniquely expressed on specific cell types, or where the initiation of calcium signaling needs to be studied in mixed populations undergoing stimulation, for instance in whole blood or lymphoid populations [5]. During our study of immune/nerve cell interaction, we wanted to determine Transient Receptor Potential Vanilloid-1 (TRPV1) expression and function in a splenic population. Classically known as a vital neural receptor and calcium channel, TRPV1 plays a critical role in the release of calcitonin gene related peptide (CGRP) [6]. TRPV1 shapes the thermal regulating system in the body and is found on peripheral nerves and immune cells [7], although comparative expression in lymphoid tissue has never been shown. Given the mechanosensitive and thermal responsive nature of TRPV1, we needed to study calcium signaling with as little cell manipulation as possible. Furthermore we were interested in signaling within distinct immune cell populations in a co-culture environment. We chose to investigate calcium influx using Indo-1 AM (Fluorescence Ex/Em 335/405 and 475 nm) which provided the opportunity to maximise additional surface staining on remaining channels using an LSRII. To maximize surface receptor detection, we used a dilution staining gradient approach as described previously [8]. This staining method was initially described for monitoring immune cell populations of mouse blood and has since been developed for 10 channel flow cytometric analysis [9]. Here we combine five fluorescence parameter staining across four fluorochromes with Indo-1 AM to demonstrate the potential for calcium signaling as part of a multiplex analysis with minimum intervention.

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http://dx.doi.org/10.1016/j.ab.2017.03.025
0003-2697/

Materials and methods

Mice

Male C57BL/6Ola mice (6–8weeks old, Harlan Olac) were housed in the University of Manchester Biological Services Unit (BSU) in ventilated cages under specific pathogen free conditions at 21 °C with a 12 h light/dark cycle and free access to food and water. TRPV1 deficient mice (B6.129X1-*Trpv1tm1Jul*/J) were sourced from The Jackson laboratory and bred at the University of Manchester. Procedures were carried out under the Home Office Scientific Procedures Act 1986 (revised January 2013). For euthanasia, Mice were culled via CO_2 inhalation.

Ethics, consent and permission

All experiments were approved by the University of Manchester's ethics committee (http://www.manchester.ac.uk/research/environment/governance/ethics/), and the UK Home Office in accordance with Home Office Scientific Procedures Act 1986 (revised January 2013), adhering to the basic principles of replacement, reduction and refinement (3Rs). Dr Assas holds a personal license from the UK Home Office.

Splenocyte islolation

Isolated spleens were disaggregated through 100 μm sieves (BD Phamingen, Oxford, UK). Cells were centrifuged (400 g, 5 min, room temp), and re-suspended in complete media (RPMI-1640, supplemented with 50 μg/ml FCS, 100 μg/ml penicillin/streptomycin and 1 mM L-glutamine 0.42 mM calcium (Sigma Aldrich, UK)) at 5×10^7 cells/ml.

Intracellular Indo-1 AM splenocyte staining

This protocol is modified from Flow cytometry core facility, Camelia Botner Laboratories (https://www.ucl.ac.uk/ich/services/lab-services/FCCF/protocols/calcium_flux). 1×10^7 splenocytes were stained with Indo-1 AM at 4 μM for 30 min at room temperature (diluted in either complete media or calcium free media as detailed in the text). Cells were centrifuged (400 g, 5 min), washed and placed in a 96 well plate (5×10^5 cells per well). Cells were centrifuged (400 g, 2 min) and resuspended (1 mM calcium, 1 mM magnesium and 0.5% FBS (Sigma Aldrich, UK)) then rested (15 min) in preparation for surface antibody staining.

Cell surface staining

This protocol was modified from Frischmann *et al* (2006). Selected splenocytes were first incubated with anti-CD16/32 (2.5 μg/ml BDbiosciences, San Jose, USA) to block non-specific binding, washed and then labelled with the following antibody cocktail (20min, ice): anti-CD4-PE (0.1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD8-PE (1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD19-Alexa fluor 700 (0.25 μg/ml, eBioscience, San Diego, CA, USA), anti-F480-APC (0.1 μg/ml, eBioscience, San Diego, CA, USA), anti-CD11c-APC-Cy7 (0.5 μg/ml, eBioscience, San Diego, CA, USA). Cells were washed and re-suspended in complete media (400 μl of RPMI-1640, supplemented with 50 μg/ml FCS, 100 μg/ml penicillin/streptomycin and 1 mM L-glutamine 0.42 mM calcium (Sigma Aldrich, UK) or calcium free media for acquisition.

Instrument details (LSRII), configuration and settings

Instrument Model BD LSRII Flow Cytometer, Serial Number H08800001 (BD Biosciences, Oxford Science Park, Oxford). The instrument flow cell (fixed-alignment quartz cuvette), fluidics, light source, excitation optics or optical detectors/paths were not altered. There is a four-laser configuration as follows: 488-nm Coherent® SapphireTM air-cooled argon-ion laser, 50 mW; 640-nmCoherent® Cube air-cooled laser, 40 mW; 405-nm Coherent® Radius air-cooled laser, 50 mW; 355 nm Coherent Genesis 355-20 Air cooled laser, 20 mW. Detector arrays consist of BD octagons (561 and 488 nm laser lines) and BD Trigons (405 and 640 nm laser lines). The entire PMT arrangements are illustrated in the supplementary Table S1.

LSRII instrument setup

This protocol was modified from Flow cytometry core facility, Camelia Botner Laboratories (https://www.ucl.ac.uk/ich/services/lab-services/FCCF/protocols/calcium_flux) and the BD LSRII user's guide (http://www.gla.ac.uk/media/media_231654_en.pdf). The filters were configured as follows: 530/30 PMT A (Indo-1 blue) and 405/20 PMT B (Indo-1 violet) on the UV trigon and close violet laser shutter. This prevented the violet laser from interfering with the Indo-1 violet emission. Indo emission and excitation were monitored on a linear scale, and viewed on a time versus ratio (Indo-1 violet (numerator), Indo-1 blue (denominator)) plot. Parameters were set using Indo-1 single stain and surface only stained samples. Compensation was achieved using single stained beads (eBioscience, UK).

Sample acquisition

All samples were rested for 15 min at 30 °C prior to acquisition. Each sample was checked for clear Indo-1 blue staining (i.e. no calcium signaling) prior to acquisition and addition of agonists. Indo-1 blue staining was set up at approximately a 70° angle to allow movement to Indo-1 violet gate (Supplementary Fig. S1). Time ratio plots were monitored during acquisition. Events were acquired at 200 cells/second for all samples. Firstly, each sample was recorded for 10 s to establish a baseline (Fig.S1 B-D). With continuous recording, samples were treated with an agonist (capsaicin (100 μl in media, 100 μM, Sigma Aldrich, UK) or ionomycin (positive control, 100 μl in media, 2 μM, Sigma Aldrich, UK)) or vehicle (100 μl, bringing the total volume to 500 μl), vortexed and returned to the LSRII to record. Each sample was acquired for 360 s in total ($2–3x10^5$ events). The acquisition rod was cleaned between each sample, and distilled water was run for 5 min between each sample type (control, tests and ionomycin) to ensure no agonist was carried over.

Analysis

The data was compensated and analysed using FlowJo_V10.Ink (Treestar, Ashland USA). Labelled beads were used as compensation controls (eBioscience, San Diego, CA, USA) and analyzed as follows: splenocytes were gated on FSC-A/FSC-H to remove aggregates, and debris was viewed on FSC-A/SSC-A. T and B cells were gated on $CD4^+$ and $CD8^+$ (PE) and $CD19^+$ (Alexa flour 700) channels, with non-T non-B cells remaining in the unstained gate. CD11c + cells were analyzed on the APC-Cy7 channel, and F480 + cells in the non-T non-B cell gate on APC/APC-Cy7 channels. F480 + CD11c+ were also gated for interest (data not shown). Negative controls (media treated) were used to define the gates. The Indo-1 blue vs Indo-1 violet plot was then examined in each cell population. Data were plotted as number of cells over time migrating from Indo-blue (450 LP mirror) to Indo-violet (405/20 filter). Analysis was performed on FlowJo_V10.Ink (Treestar, Ashland USA).