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Research paper

Flow cytometric calcium flux assay: Evaluation of cytoplasmic calcium kinetics in whole blood leukocytes

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ABSTRACT

In leukocytes, as in many other cell types, cytoplasmic calcium ($[Ca^{2+}]_i$) changes play a key role in a series of pathways leading to activation. Here we describe a flow cytometric method allowing the simultaneous kinetic analysis of changes in $[Ca^{2+}]_i$ in the three types of leukocytes, i.e. monocytes, granulocytes and lymphocytes. Heparinised whole blood was diluted in phosphate buffered saline with Ca^{2+} and 1 mM sodium pyruvate and incubated with the Ca^{2+} indicator fluo3-acetoxymethyl ester. Leukocytes were identified by labelling with the phycoerythrin-conjugated antibody against CD45, the leukocyte common antigen. Resuspension of the cells in PBS with or without Ca^{2+} allowed us to detect the origin of Ca^{2+} changes. During flow cytometric analysis only CD45-positive cells were counted and monocytes, granulocytes and lymphocytes were evaluated separately. Baseline fluorescence of the fluo3–Ca^{2+}-complex was determined and changes in $[Ca^{2+}]_i$ after stimulation with the calcium ionophore A23187 or the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) were recorded over a time period of 150 s. Stimulation with A23187 resulted in a rise in $[Ca^{2+}]_i$ in all three leukocyte subpopulations. This rise was sustained in the presence of extracellular Ca^{2+} (Ca^{2+}_{ex}) but had a transient character in the absence of Ca^{2+}_{ex}. For fMLP, $[Ca^{2+}]_i$ changes occurred only in monocytes and granulocytes and were transient irrespective of the presence or absence of Ca^{2+}_{ex}.

In conclusion, the present method is a simple, fast and easy tool to analyse in vitro $[Ca^{2+}]_i$ changes over time in leukocytes under physiologically relevant conditions, without the need for their isolation or the lysis of erythrocytes. The whole blood approach allows a continuous interaction between the different leukocyte subpopulations and other blood components and a minimum of preparative manipulations avoids artefactual activation of the cells. A distinction can be made between Ca^{2+} release from the intracellular stores and the entry of Ca^{2+} from outside the cell. The approach allows to evaluate the effect of various agonists on $[Ca^{2+}]_i$ changes in leukocytes, with physiological, patho-physiological or therapeutic purposes.

1. Introduction

A primary event following stimulation of leukocytes is a change in cytoplasmic calcium concentration ($[Ca^{2+}]_i$) which results in numerous cellular activation processes like chemotaxis, oxidative burst, cytokine production and phagocytosis (Scharff and Foder, 1993). Measurement of changes in leukocyte $[Ca^{2+}]_i$ can contribute to the elucidation of patho-physiologically relevant cell activation mechanisms, leading to susceptibility to infection (Chonchol, 2006), inflammation

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic calcium; fMLP, N-formyl-methionyl-leucyl-phenylalanine; Ca^{2+}_{ex}, extracellular calcium; PBS, phosphate buffered saline; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); BAPTA-AM, 1,2-bis(2-aminophenoxyl)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester; EGTA, ethylene glycol-bis-(2-aminoethyl ether)-tetraacetic acid; fluo3-AM, fluo3-acetoxymethyl ester; PE, phycoerythrin; $Ca^{2+}{}_i$, intracellular calcium; RT, room temperature; FL, fluorescence; MFI, mean fluorescence intensity.

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(Tintinger et al., 2005), wasting and malnutrition (Stenvinkel et al., 1999) and cardiovascular damage (Ross, 1999).

Upon stimulation, $[Ca^{2+}]_i$ in leukocytes augments, which can originate from the release of Ca^{2+} from internal Ca^{2+}-stores and/or from an entry of Ca^{2+} from outside the cell into the cytosol (Krause et al., 1990; Liu et al., 2007; Oh-hora and Rao, 2008; Rasmussen and Barrett, 1984). Flow cytometry is a valuable tool to study the different types of leukocytes (Cognasse et al., 2003; Avendano et al., 2008) and $[Ca^{2+}]_i$ changes can be evaluated by making use of fluorescent Ca^{2+} indicators, such as fluo3, that can be loaded into intact cells (Bailey and Macardle, 2006; Merritt et al., 1990).

Fluo3, a Ca^{2+}-chelator probe derived from the chromophore fluorescein, combines a large enhancement of fluorescence upon Ca^{2+} binding with an absorption and emission spectrum similar to fluorescein (Minta et al., 1989). The excitation and emission wavelength of fluo3 make it suitable for use in flow cytometers equipped with a 488 nm argon laser and fluorescein filter settings and its high affinity for Ca^{2+} makes it sensitive to physiologically important $[Ca^{2+}]_i$ changes (Merritt et al., 1990; Minta et al., 1989).

In contrast to previously described methods to investigate $[Ca^{2+}]_i$ requiring isolation of leukocytes (Burchiel et al., 2000; Vandenberghe and Ceuppens, 1990) or lysis of erythrocytes (Lund-Johansen and Olweus, 1992), the purpose of the present method was to maintain leukocytes in a milieu where all blood components remain present. By studying whole blood, artefacts due to sample preparation were minimized. In addition, assessing monocytes, granulocytes and lymphocytes together makes it possible to evaluate the effects without neutralizing the possibilities for cross-talk among the different leukocyte types, which is physiologically more relevant.

In the presently described method, leukocytes are identified in whole blood based on their constitutive expression of the leukocyte common antigen CD45 and their light scatter properties. The use of buffers of different composition enabled the distinction between $[Ca^{2+}]_i$ changes caused by release from intracellular stores and/or influx of extracellular Ca^{2+} (Ca^{2+}_{ex}). In addition the kinetics of the $[Ca^{2+}]_i$ changes could be analysed by measuring over time.

This method provides a direct, sensitive and simple tool to assess ex vivo the immediate and time-dependent changes in $[Ca^{2+}]_i$ in leukocytes under physiologically relevant conditions. It is applicable in combination with different types of compounds, as demonstrated here for the chemotactic peptide formyl N-methionyl-leucyl-phenylalanine (fMLP) and the Ca^{2+} ionophore A23187, and it can contribute in the elucidation of patho-physiological pathways, finally leading to therapeutic intervention.

2. Materials and methods

2.1. Materials

Dulbecco's phosphate buffered saline (PBS) with and without Ca^{2+} and Mg^{2+} (PBS+Ca^{2+} and PBS–Ca^{2+} respectively), sodium pyruvate and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were purchased from Gibco (Paisley, UK). The compounds 1,2-bis(2-aminophenoxyl)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) and its solvent dimethyl sulfoxide, ethylene glycol-bis-(2-aminoethyl ether)-tetraacetic acid tetrasodium salt (Na_4-EGTA) and the calcium ionophore A23187 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fluo3-AM was delivered by Molecular Probes (Eugene, OR, USA) and Becton Dickinson (San Jose, CA, USA) provided the human monoclonal phycoerythrin (PE)-labelled antibody against CD45 and fMLP.

2.2. Blood collection

After informed consent whole blood from healthy volunteers was collected in Vacutainer™ tubes containing sodium heparin (Becton Dickinson) and was processed within the hour. Exclusion criteria were smoking, pregnancy, current infection or medication intake. The study was approved by the local Ethics Committee.

2.3. Experimental set-up

A flow cytometric protocol to determine the kinetics of $[Ca^{2+}]_i$ mobilization in leukocytes was developed. The application of three experimental conditions allowed us to gain insight in the origin of $[Ca^{2+}]_i$ fluxes: in condition (a) extracellular calcium (Ca^{2+}_{ex}) is present and intracellular calcium (Ca^{2+}_i) is available; in condition (b) only Ca^{2+}_i is available and in condition (c) Ca^{2+}_{ex} is absent and Ca^{2+}_i changes are buffered with BAPTA.

2.3.1. Cell preparation and loading with fluo3-AM

Heparinised whole blood was diluted 1:10 in PBS + Ca^{2+} containing 1 mM sodium pyruvate. Fluo3-AM was added at a final concentration of 5 μM and the samples were incubated for 30 min in the dark at room temperature (RT). Fluo3, the fluorescent Ca^{2+} chelator probe, is coupled to an acetoxymethyl ester (AM) which masks the Ca^{2+}-binding region and makes the fluo3-molecule lipid soluble allowing it to cross the cell membrane. Following de-esterification by the cytoplasmic esterases, the fluo3 is trapped inside the cell. Upon binding the cytoplasmic Ca^{2+}-ions, the fluo3–Ca^{2+}-complex emits a substantially increased fluorescence following excitation, with an intensity that is proportional to $[Ca^{2+}]_i$. This fluorescent Ca^{2+} signal can be detected by flow cytometry (Hallett et al., 1999).

2.3.2. Labelling of the leukocytes

The identification of the leukocyte population in the whole blood samples was performed by direct immunostaining for the leukocyte common antigen CD45. Therefore the fluo3-loaded samples were incubated with the PE-conjugated human monoclonal anti-CD45 antibody for 15 min in the dark at RT.

2.3.3. Experimental conditions applied to investigate cellular Ca^{2+} handling

After a washing step in PBS–Ca^{2+}, the cells were resuspended in the appropriate buffer in accordance to the different conditions and kept at 37 °C until analysis.

For condition (a) a PBS + Ca^{2+} buffer was used containing 1 mM sodium pyruvate and 25 mM HEPES. This condition allows changes of $[Ca^{2+}]_i$ originating from the release of Ca^{2+} from the intracellular stores and from the entry of Ca^{2+}_{ex} into the cytosol and thus corresponds to the *in vivo* condition.

Ca^{2+}-entry is prevented by the use of a PBS–Ca^{2+} buffer containing 1 mM sodium pyruvate, 25 mM HEPES and 5 mM