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# Influence of ER leak on resting cytoplasmic $Ca^{2+}$ and receptor-mediated $Ca^{2+}$ signalling in human macrophage

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ABSTRACT

Mechanisms controlling endoplasmic reticulum (ER) $Ca^{2+}$ homeostasis are important regulators of resting cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyto}$) and receptor-mediated $Ca^{2+}$ signalling. Here we investigate channels responsible for ER $Ca^{2+}$ leak in THP-1 macrophage and human primary macrophage. In the absence of extracellular $Ca^{2+}$ we employ ionomycin action at the plasma membrane to stimulate ER $Ca^{2+}$ leak. Under these conditions ionomycin elevates $[Ca^{2+}]_{cyto}$ revealing a $Ca^{2+}$ leak response which is abolished by thapsigargin. $IP_3$ receptors (Xestospongin C, 2-APB), ryanodine receptors (dantrolene), and translocon (anisomycin) inhibition facilitated ER $Ca^{2+}$ leak in model macrophage, with translocon inhibition also reducing resting $[Ca^{2+}]_{cyto}$. In primary macrophage, translocon inhibition blocks $Ca^{2+}$ leak but does not influence resting $[Ca^{2+}]_{cyto}$. We identify a role for translocon-mediated ER $Ca^{2+}$ leak in receptor-mediated $Ca^{2+}$ signalling in both model and primary human macrophage, whereby the $Ca^{2+}$ response to ADP (P2Y receptor agonist) is augmented following anisomycin treatment. In conclusion, we demonstrate a role of ER $Ca^{2+}$ leak via the translocon in controlling resting cytoplasmic $Ca^{2+}$ in model macrophage and receptor-mediated $Ca^{2+}$ signalling in model macrophage and primary macrophage.


## 1. Introduction

The endoplasmic reticulum (ER) plays important roles in many cellular processes, including protein folding and cellular calcium ($Ca^{2+}$) homeostasis [1]. During receptor-mediated intracellular $Ca^{2+}$ signal generation, $Ca^{2+}$ stored by the ER can be mobilised causing rapid elevation in the concentration of free cytoplasmic $Ca^{2+}$ ($[Ca^{2+}]_{cyto}$). Receptor-mediated processes coupled to phospholipase C (PLC) mobilise ER $Ca^{2+}$ through the production of inositol 1,4,5-triphosphate ($IP_3$), which open $IP_3$ receptors on the ER membrane through which $Ca^{2+}$ rapidly permeates into the cytoplasm [2]. Released $Ca^{2+}$ can stimulate further release via activation of ER ryanodine receptors, a process termed $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) [3]. During receptor-mediated $Ca^{2+}$signalling, mobilisation of the ER store lowers the concentration of free $Ca^{2+}$ within the ER lumen ($[Ca^{2+}]_{ER}$). The decrease in $[Ca^{2+}]_{ER}$ is sensed by the ER resident stromal interaction molecule (STIM), which in turn stimulates cellular $Ca^{2+}$ entry via the activation of the ora family of plasma membrane $Ca^{2+}$ channels [4]. This process is termed store-operated $Ca^{2+}$ entry (SOCE). Receptor-mediated intracellular $Ca^{2+}$ signals are therefore often a composition of ER $Ca^{2+}$ release and $Ca^{2+}$ entry via SOCE. In macrophage, receptor-mediated $Ca^{2+}$ signals are generated in response to environmental cues which are important for cellular migration [5,6].

Under resting conditions $[Ca^{2+}]_{ER}$ reflects a balance between the activity of SERCA and passive efflux of $Ca^{2+}$ into the cytoplasm via $Ca^{2+}$ leak channels. Hence the magnitude of ER $Ca^{2+}$ leak can influence resting $[Ca^{2+}]_{cyto}$ and the amount of ER $Ca^{2+}$ mobilisable in response to receptor-mediated signalling. The molecular identity of ER $Ca^{2+}$ leak channels is poorly defined for mammalian cells [7]. Though candidates such as presenilins [8] and the ER translocon [9–11] have been proposed as leak channels in some cell types, there is currently no published description of candidate channels in leukocytes. Processes of ER $Ca^{2+}$ leak are therefore likely to play important homeostatic roles in controlling both $[Ca^{2+}]_{cyto}$ and $[Ca^{2+}]_{ER}$ in macrophage. Understanding mechanisms of ER $Ca^{2+}$ leak in macrophage is also of importance, as a decrease in $[Ca^{2+}]_{ER}$ is a key initiator of apoptosis in ER-stressed macrophage, and ER $Ca^{2+}$ release is necessary for apoptotic signalling in macrophage [12,13]. ER-stressed mediated apoptosis in lesional macrophage is a central event during plaque necrosis in advanced atherosclerosis [14]. In this study we sought to identify channels that mediate ER

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$Ca^{2+}$ leak in human macrophage and determine the influence of ER $Ca^{2+}$ leak channel activity on receptor-mediated $Ca^{2+}$ signalling.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ionomycin, Thapsigargin (Tg) and 2-APB were obtained from Santa-Cruz Biotechnologies. ADP, Anisomycin and Dantrolene were obtained from Sigma-Aldrich (UK). Xestospongin C was obtained from Abcam (UK).

### 2.2. Cells

THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC). Human THP-1 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 10% foetal bovine serum and 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37 °C with 5% $CO_2$. To generate THP-1 differentiated macrophages, cells were stimulated with 320 nM of phorbol 12-myristate 13-acetate for 48 h, 37 °C, 5% $CO_2$.

### 2.3. Isolation of PBMCs and generation of monocyte-derived macrophages

Peripheral venous blood was collected from healthy human volunteers through the National Health Service (NHS) Blood and Transplant. Blood was layered on top of Histopaque-1077 (Sigma-Aldrich, UK) for centrifugation at 1000 × *g* for 25 min. Buffy coat layer containing the PBMCs was removed, washed and counted using trypan blue exclusion. PBMCs were allowed to adhere onto T75 flasks for 2 h at 37 °C, washed with dPBS (Lonza, UK) and cultured in RPMI-160 with 2 mM L-glutamine, 5% heat-inactivated autologous serum and 50 IU/ml penicillin and 50 μg/ml streptomycin, in the presence of 10 ng/ml recombinant human GM-CSF (Peprotech, UK) at 37 °C for 6 d.

### 2.4. Intracellular $Ca^{2+}$ measurements

Cells were loaded for 1 h at 37 °C with 2 μM Fura-2 AM and measurements were made at 37 °C on a 96-well plate reader (FlexStation III, Molecular Devices). Change in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) is indicated as ratio of fura-2 emission intensities for 340- and 380-nm excitation (F ratio). SBS buffer contained (mM): 130 NaCl, 5 KCL, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 8 D-glucose, 10 HEPES pH 7.4. $Ca^{2+}$ free SBS was prepared by excluding $CaCl_2$ and supplemented with 2 mM EGTA. Loading of cells with Fura-2 was performed in SBS buffer supplemented with 0.01% (w/v) pluronic acid. Pre-treatment of all compounds, except for anisomycin (1hr), were done at 30 min.

### 2.5. Statistical analysis

Data were analysed using OriginPro 9.0 software (Origin Lab, USA). Concentration-response curves were fitted assuming a Hill coefficient of 1. Hypothesis testing for experiments with paired datasets were performed by means of paired Student's *t*-test using Origin Pro 9.0. Data are expressed as mean ± SEM of at least three independent experiments. *N* represents number of technical repeats for THP-1 model macrophage and number of donors for primary macrophage.

## 3. Results

### 3.1. Ionomycin elevates cytoplasmic $Ca^{2+}$ independent of $Ca^{2+}$ influx

In THP-1 model macrophage, application of ionomycin in the presence of extracellular $Ca^{2+}$ elevated $[Ca^{2+}]_{cyto}$ in a concentration-dependent manner ($EC_{50}$ 0.365 ± 0.01 μM; $N = 4$) (Fig. 1A). In the absence of extracellular $Ca^{2+}$, ionomycin retained its ability to elevate cytoplasmic $Ca^{2+}$ but with a reduced potency ($EC_{50}$ 0.61 ± 0.11 μM *vs* control with extracellular $Ca^{2+}$; $P \leq 0.001$; $N = 4$) and reduced maxima (F ratio 1.341 ± 0.036 *vs* 2.67 ± 0.054 control μM; $P \leq 0.001$; $N = 4$) (Fig. 1A). The kinetics of $Ca^{2+}$ response generated by maximal concentrations of ionomycin differed in the presence or absence of extracellular $Ca^{2+}$, with responses in the absence of extracellular $Ca^{2+}$ lacking a sustained phase (Fig. 1B). Similar results were observed in primary macrophage. In primary cells, ionomycin evoked a $Ca^{2+}$ response in the presence ($EC_{50}$ 0.332 ± 0.19 μM; $N = 3$) and absence of extracellular $Ca^{2+}$ ($EC_{50}$ 3.47 ± 2.01 μM *vs* control with extracellular $Ca^{2+}$; $P \leq 0.01$; $N = 3$) (Fig. 1C–D). Data from primary and model macrophage are consistent with the notion that ionomycin acts as a $Ca^{2+}$-selective pore at the plasma membrane and that the sustained phase is dependent upon $Ca^{2+}$ influx. However, the data also demonstrate that ionomycin can elevate cytoplasmic $Ca^{2+}$ in the absence of extracellular $Ca^{2+}$ and therefore in the absence of $Ca^{2+}$ influx.

### 3.2. Ionomycin stimulates release of ER $Ca^{2+}$ store independent of $Ca^{2+}$ influx and dependent on passive $Ca^{2+}$ leak

We initially investigated the requirement of the ER $Ca^{2+}$ store in mediating ionomycin-evoked responses by depleting the store with the irreversible SERCA inhibitor thapsigargin (Tg). In these experiments, Tg reduced the ability of ionomycin to elevate cytoplasmic $Ca^{2+}$ in the presence of extracellular $Ca^{2+}$ ($EC_{50}$ 9.28 ± 6.91 μM *vs* control; $P \leq 0.001$; $N = 4$), though ionomycin was able to elevate cytoplasmic $Ca^{2+}$ at concentrations 1–10 μM (Fig. 1E). However, the response to ionomycin in the absence of extracellular $Ca^{2+}$ was abolished by Tg (Fig. 1F), suggesting that ionomycin can elevate cytoplasmic $Ca^{2+}$ independent of $Ca^{2+}$ influx and dependent upon the ER $Ca^{2+}$ store. To further test the contribution of the ER $Ca^{2+}$ store for ionomycin-evoked response, we examined the effect of ionomycin pre-treatment of the magnitude of $Ca^{2+}$ mobilisation following SERCA inhibition with thapsigargin. In the presence of extracellular $Ca^{2+}$, pre-incubation with ionomycin significantly attenuated the magnitude of Tg-evoked elevation in cytoplasmic $Ca^{2+}$ (88.5± 1.93%; $P \leq 0.001$; $N = 4$) (Fig. 1G). In the absence of extracellular $Ca^{2+}$, ionomycin pre-treatment abolished Tg-evoked $Ca^{2+}$ response (Fig. 1G). As the magnitude of Tg-evoked response in the absence of extracellular $Ca^{2+}$ is directly proportional to the ER $Ca^{2+}$ content, these data suggest ionomycin in the absence of extracellular $Ca^{2+}$ elevates cytoplasmic $Ca^{2+}$ by stimulating ER store mobilisation. Finally, we observed a significant elevation in basal cytoplasmic $Ca^{2+}$ in cells pre-treated with ionomycin in the absence of extracellular $Ca^{2+}$ (F ratio 1.57 ± 0.039 *vs* 1.96 ± 0.026 control; $P \leq 0.001$; $N = 4$) (Fig. 1H).

### 3.3. ER $Ca^{2+}$ channels mediate ionomycin-evoked cytoplasmic $Ca^{2+}$ elevation in the absence of extracellular $Ca^{2+}$

Data thus far indicate a primary role for ER $Ca^{2+}$ store release in mediating the effect of ionomycin independent of $Ca^{2+}$ influx. On the assumption that ionomycin is acting as a plasma membrane conduit for $Ca^{2+}$ and stimulating store leak, one would expect that