INFLUENCE OF ER LEAK ON RESTING CYTOPLASMIC CA2+ AND RECEPTOR-MEDIATED CA2+ 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.

KEYWORDS: Endoplasmic reticulum; calcium leak; translocon; macrophage; purinergic

1 INTRODUCTION

The endoplasmic reticulum (ER) plays important roles in many cellular processes, including protein folding and cellular Ca^{2+} (Ca^{2+}) homeostasis (Lam & Galione, 2013). 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 (Berridge, 1993). Released Ca^{2+} can stimulate further release via activation of ER ryanodine receptors, a process termed Ca^{2+}-induced Ca^{2+} release (CICR) (Collier et al., 2000). 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 orai family of plasma membrane Ca^{2+} channels (Sogkas et al., 2015). 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 (Myers & Swanson, 2002; Desai & Leitinger, 2015).

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 (Camello et al., 2002). Though candidates such as presenilins (Supnet & Bezprozvanny, 2011) and the ER translocon (Van Coppenolle et al., 2004; Amer et al., 2009; Hammadi et al., 2013) 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 (Seimon et al., 2006; Lim et al., 2008). ER-stressed mediated apoptosis in lesional macrophage is a central event during plaque necrosis in advanced atherosclerosis (Tabas et al., 2009). In this study we sought to identify channels that mediate ER 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 2mM L-glutamine, 10% foetal bovine serum and 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37^{o}C with 5% CO_2. To generate THP-1 differentiated macrophages, cells were stimulated with 320 nM of phorbol 12-myristate 13-acetate for 48h, 37^{o}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 x *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 2h at 37^{o}C, washed with dPBS (Lonza, UK) and cultured in RPMI-160 with 2mM 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^{o}C for 6d.

2.4 *Intracellular Ca^{2+} measurements*

Cells were loaded for 1h at 37^{o}C with 2µM Fura-2 AM and measurements were made at 37^{o}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 2mM 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.

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) (**Figure 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≤0.001; *N*=4) and reduced maxima (F ratio 1.341 ± 0.036 *vs* 2.67 ± 0.054 control μM; P≤0.001; *N*=4) (**Figure 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 (**Figure 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≤0.01; *N*=3) (**Figure 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 irreversibe 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≤0.001; *N*=4), though ionomycin was able to elevate cytoplasmic Ca^{2+} at concentrations 1 – 10μM (**Figure 1E**). However, the response to ionomycin in the absence of extracellular Ca^{2+} was abolished by Tg (**Figure 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≤0.001; *N*=4) (**Figure 1G**). In the absence of extracellular Ca^{2+}, ionomycin pre-treatment abolished Tg-evoked Ca^{2+} response (**Figure 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≤0.001; *N*=4) **(Figure 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 ER Ca^{2+} channels underpin the response. In initial experiments we investigated the role of classical ER Ca^{2+} release channels, namely the inositol 1,4,5-triphosphate (IP_3) receptor and Ca^{2+}-activated ryanodine receptors. 2-aminoethoxydiphenyl borate (2-APB) blocks SOCE via inhibition of both store-operated Ca^{2+} channels and the IP_3 receptor at concentrations >10μM reported in other cell types. Concurrent with this mechanism we observed inhibition of ionomycin-evoked Ca^{2+} responses both in the presence and absence of extracellular Ca^{2+} (**Figure 2A-B**). In agreement with a requirement for the IP_3 receptor in mediating ionomycin responses in the absence of extracellular Ca^{2+}, we observed inhibition with xestospongin C (XeC), a membrane permeable selective IP_3 receptor antagonist (**Figure 2C-D**). The inhibitory action of 2-APB and XeC in the absence of extracellular Ca^{2+} suggest that open IP_3 receptors contribute to the elevation in cytoplasmic Ca^{2+} evoked by ionomycin. Ionomycin-evoked responses were also inhibited by dantrolene (**Figure 2E-F**), revealing an involvement of ryanodine receptors in elevating $[Ca^{2+}]_{cyto}$. Neither 2-APB, XeC or dantrolene influence resting Ca^{2+} in macrophage. Therefore, although a contribution of IP_3 and ryanodine receptor activity to ER Ca^{2+} can be observed under experimental conditions with ionomycin present, our data does not support a physiological contribution of these channels to resting $[Ca^{2+}]_{ER}$.

3.4 Effect of translocon inhibition on resting cytoplasmic Ca^{2+} and receptor-mediated Ca^{2+} signalling

It has been reported in other cell types that the ER translocon, when open, can act as a Ca^{2+} channel and can mediate ER passive Ca^{2+} leak. In macrophage we employed anisomycin, an antibiotic inhibitor of peptidyl-transferase activity, which leaves the translocon closed, to test the hypothesis that the ER translocon contributes to ER Ca^{2+} leak and influences resting $[Ca^{2+}]_{cyto}$. In the absence of extracellular Ca^{2+}, anisomycin (200μM) pre-treatment significantly inhibited ionomycin-evoked Ca^{2+} responses in THP-1 macrophage, suggesting translocon activity can facilitate ER Ca^{2+} leak (**Figure 3A-C**). However, anisomycin also significantly reduced resting cytoplasmic Ca^{2+} in THP-1 macrophage (F ratio 0.963 ± 0.01 *vs* 1.07 ± 0.01 control; P≤0.05; *N*=4), in contrast to the effect of IP_3 or ryanodine receptor blockade. These data suggest that translocon activity is a determinant of ER Ca^{2+} homeostasis and resting cytoplasmic Ca^{2+} concentration under physiological conditions. In primary cells, the inhibitory action of anisomycin on the ionomycin response was observed as a reduction in net Ca^{2+} movement (**Figure 3E**), as judged by area under the curve of the ionomycin response in the absence of extracellular Ca^{2+} (**Figure 3E**). In contrast to model macrophage, anisomycin had no apparent effect on the resting $[Ca^{2+}]_{cyto}$. To further test the importance of translocon activity in ER Ca^{2+} homeostasis, we examined the effect of anisomycin on receptor-mediated Ca^{2+} signalling. Our previous data shows that P2Y-mediated Ca^{2+} responses elicted by ADP in THP-1 cells are totally dependent on release of ER Ca^{2+} (Sivaramakrishnan et al., 2012), and inhibited when the ER is depleted with thapsigargin or ionomycin (data not shown). Translocon inhibition caused a significant increase in ADP potency and response maxima in both model macrophage (**Figure 4A-B**) and primary macrophage (**Figure 4C-E**). These data suggest that limiting ER Ca^{2+} leak via

the translocon increases the amount of ER Ca^{2+} mobilisation by receptor activation in macrophage. Taken together, the effects of anisomycin 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.

3.5 Summary

In aggregate, under conditions of ionomycin-stimulated ER Ca^{2+} leak in human macrophage, we observe contributions of the IP_3 receptor, ryanodine receptor and open ER translocon. Neither IP_3 receptor nor ryanodine receptor activity contribute to $[Ca^{2+}]_{cyto}$. However, the activity of the translocon influences both $[Ca^{2+}]_{cyto}$ in resting macrophage under physiological conditions, and the magnitude of receptor-mediated Ca^{2+} signals in model macrophage, in this case P2Y receptor activation. In primary human macrophage, a contribution of translocon activity can be observed under conditions of ionomycin-stimulated Ca^{2+} leak, however there is no apparent contribution of translocon-mediated ER Ca^{2+} leak $[Ca^{2+}]_{cyto}$. Despite this, inhibition of the translocon augments receptor-mediated Ca^{2+} signalling in primary cells suggesting its involvement in Ca^{2+} homeostasis during cell signalling.

4 DISCUSSION

The study demonstrates ER Ca^{2+} leak via the ER translocon as an important regulator of ER Ca^{2+} homeostasis and receptor-mediated Ca^{2+} signalling in human macrophage. Blockade of translocon-mediated Ca^{2+} leak from the ER using anisomycin augments the Ca^{2+} signal produced by external cues, in this study ADP, and therefore translocon activity is likely to modulate the ability of macrophage to respond to their environment. To the best of our knowledge, this is the first demonstration of the role ER translocon plays in controlling receptor-mediated Ca^{2+} signalling in leukocytes. The contribution of translocon-mediated leak appears more apparent in THP-1 model macrophage, where inhibition with anisomycin reveals a contribution to both resting $[Ca^{2+}]_{cyto}$ and receptor-mediated Ca^{2+} signalling, though a contribution to resting $[Ca^{2+}]_{cyto}$ is not observed in primary cells. Despite this, the pharmacology suggests translocon leak of ER Ca^{2+} does impact on receptor-mediated Ca^{2+} signalling in human primary macrophage. We can also infer that the translocon is efficiently closed in primary macrophage at rest and that this is not the case for THP-1 model macrophage, where a leak is apparent at rest. In other cell types such as vascular smooth muscle (Amer et al., 2009) and cancer cells (LNCaP cells; Van Coppenolle et al., 2004), the contribution of translocon-mediated ER Ca^{2+} leak has been investigated using puromycin. In such studies, puromycin elicits an expectant elevation of $[Ca^{2+}]_{cyto}$, however there is no discernible contribution of translocon-mediated leak to receptor-mediated Ca^{2+} signalling (Amer M et al., 2009). Macrophage are highly dynamic cells and their function is tightly linked to ER Ca^{2+} homeostasis. For example, ER stress caused by a reduction in $[Ca^{2+}]_{ER}$ is a trigger for macrophage apoptosis (Pinton et al., 2008; Sano & Reed, 2013), and ER stress modulates macrophage plasticity (Oh et al., 2012).

Ca^{2+} ionophores, like ionomycin, are generally assumed to elevate intracellular Ca^{2+} by facilitating Ca^{2+} transport across the plasma membrane. In the presence of extracellular Ca^{2+}, ionomycin elicits a Ca^{2+} response that is sustained in nature in both THP-1 model and human primary macrophage. Removal of extracellular Ca^{2+} diminishes the magnitude of response, but a robust transient response persists. Our data therefore indicates that ionomycin is capable of elevating intracellular Ca^{2+} in the absence of extracellular Ca^{2+}, revealing an additional mode of action independent of Ca^{2+} influx. Ionomycin is more potent at elevated $[Ca^{2+}]_{cyto}$ in the presence of extracellular Ca^{2+}, reflecting a dominant contribution due to Ca^{2+} influx via ionomycin itself and a likely contribution of SOCE (Morgan & Jacob , 1994; Dedkova et al., 2000; Muller et al., 2013). Dedkova et al. (2000) identified a component of Ca^{2+} ionophore-mediated Ca^{2+} responses which was dependent on phospholipase C activity, and consequent IP_3 production. Although we have not directly explored this pathway in macrophage, this may fit with our observation that open IP_3 receptors participate to the apparent ER Ca^{2+} leak when ionomycin is applied in the absence of extracellular Ca^{2+}. Open IP_3 receptors have been identified as potential conduits of ER Ca^{2+} leak in other cell types (Szlufcik et al., 2006), where proteolytic cleavage or biochemical modification, such as hyperphosphorylation or nitrosylation, lead to opening of 'uncoupled' IP_3 receptors. In this current study, IP_3 receptor activity was probed using antagonists XeC and 2-APB. While XeC is a reported selective inhibitor of IP_3-induced ER Ca^{2+} release by antagonism of IP_3 receptor (Gafni et al., 1997; Miyamoto et al., 2000), 2-APB is thought to block SOCE through two possible routes. One mode is via antagonism of the IP_3 receptor and thus preventing IP_3-mediated Ca^{2+} release from the ER. A second route is via direct inhibition of store-operated Ca^{2+} channels at the plasma membrane. Here, we show that IP_3 receptor activity contributes to ionomycin-evoked ER Ca^{2+} depletion but

does not affect resting $[Ca^{2+}]_{cyto}$. This study does not support a role for the IP_3 receptor Ca^{2+} leakiness in resting cells, as neither antagonists influence resting $[Ca^{2+}]_{cyto}$ in THP-1 model macrophage. These findings are similar to those observed for A7r5 rat vascular smooth muscle cell line where XeC or 2-APB have no influence on ER Ca^{2+} leak (De Smet et al., 1999; Missiaen et al., 2001).

In this study, we observed inhibitory actions of 2-APB on ionomycin-evoked Ca^{2+} responses both in the presence and absence of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, the contribution of SOCE in elevating intracellular Ca^{2+} is negated, and therefore the inhibitory action of 2-APB cannot be via inhibition of plasma membrane Ca^{2+} channels. We therefore reason that 2-APB is acting to antagonise the IP_3 in THP-1 model macrophage and thus limiting ionomycin-mediated ER Ca^{2+} leak in the absence of extracellular Ca^{2+}. However it is interesting to observe the ablation of responses to ionomycin in the absence of extracellular Ca^{2+} following 2-APB treatment, suggesting a total dependency on a 2-APB-sensitive Ca^{2+} conduit. The concentration of 2-APB used in this study was 100μM, in other cell types this concentration has been shown to block SERCA (Bilmen et al., 2002) and therefore the effect of 2-APB in this study could be attributed to ER Ca^{2+} store depletion. However, this is unlikely to be the case as 2-APB has no effect on resting $[Ca^{2+}]_{cyto}$, unlike thaspigargin. Our study also reveals a role of ryanodine receptors in mediating the responses to ionomycin both in the presence and absence of extracellular Ca^{2+}, where they likely contribute as part of Ca^{2+}-induced-Ca^{2+} release in response to Ca^{2+} influx (Goussakov et al., 2010) and ER release. Our study therefore identifies a major contribution of ER Ca^{2+} channels in mediating Ca^{2+} responses to ionomycin. When used as a control for Ca^{2+} dye loaded cells, ionomycin must therefore be used with caution, as the ER Ca^{2+} state, not just plasma membrane influx, will influence responsivity to ionomycin.

5 CONCLUSIONS

In summary, we identify an important role for the ER translocon in facilitating ER Ca^{2+} leak, and regulating the amount of Ca^{2+} mobilisable from the ER during receptor-mediated Ca^{2+} signalling in human macrophage.

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REFERENCES

Amer MS, Li J, O'Regan DJ, Steele DS, Porter KE, Sivaprasadarao A, Beech DJ (2009) Translocon closure to Ca2+ leak in proliferating vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 296(4):H910-916.

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361(6410):315-325.

Bilmen JG, Wootton LL, Godfrey RE, Smart OS, Michelangeli F (2002) Inhibition of SERCA Ca2+ pumps by 2-aminoethoxydiphenyl borate (2-APB). 2-APB reduces both Ca2+ binding and phosphoryl transfer from ATP, by interfering with the pathway leading to the Ca2+-binding sites. Eur J Biochem 269(15):3678-3687

Camello C, Lomax R, Petersen OH, Tepikin AV (2002) Calcium leak from intracellular stores--the enigma of calcium signalling. Cell Calcium 32(5-6):355-361

Collier ML, Ji G, Wang Y, Kotlikoff MI (2000) Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release. J Gen Physiol 115 (5):653-662

De Smet P, Parys JB, Callewaert G, Weidema AF, Hill E, De Smedt H, Erneux C, Sorrentino V, Missiaen L (1999) Xestospongin C is an equally potent inhibitor of the inositol 1,4,5-trisphosphate receptor and the endoplasmic-reticulum Ca(2+) pumps. Cell Calcium 26(1-2):9-13

Dedkova EN, Sigova AA, Zinchenko VP (2000) Mechanism of action of calcium ionophores on intact cells: ionophore-resistant cells. Membr Cell Biol 13(3):357-368

Desai BN, Leitinger N (2014) Purinergic and calcium signaling in macrophage function and plasticity. Front Immunol 5:580.

Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN (1997) Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 19(3):723-733

Goussakov I, Miller MB, Stutzmann GE (2010) NMDA-mediated Ca(2+) influx drives aberrant ryanodine receptor activation in dendrites of young Alzheimer's disease mice. J Neurosci 30(36):12128-12137.

Hammadi M, Oulidi A, Gackiere F, Katsogiannou M, Slomianny C, Roudbaraki M, Dewailly E, Delcourt P, Lepage G, Lotteau S, Ducreux S, Prevarskaya N, Van Coppenolle F (2013) Modulation of ER stress and apoptosis by endoplasmic reticulum calcium leak via translocon during unfolded protein response: involvement of GRP78. FASEB J 27(4):1600-1609.

Lam AK, Galione A (2013) The endoplasmic reticulum and junctional membrane communication during calcium signaling. Biochim Biophys Acta 1833(11):2542-2559.

Lim WS, Timmins JM, Seimon TA, Sadler A, Kolodgie FD, Virmani R, Tabas I (2008) Signal transducer and activator of transcription-1 is critical for apoptosis in macrophages

subjected to endoplasmic reticulum stress in vitro and in advanced atherosclerotic lesions in vivo. Circulation 117(7):940-951.

Missiaen L, Callewaert G, De Smedt H, Parys JB (2001) 2-Aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca2+ pump and the non-specific Ca2+ leak from the non-mitochondrial Ca2+ stores in permeabilized A7r5 cells. Cell Calcium 29(2):111-116.

Miyamoto S, Izumi M, Hori M, Kobayashi M, Ozaki H, Karaki H (2000) Xestospongin C, a selective and membrane-permeable inhibitor of IP(3) receptor, attenuates the positive inotropic effect of alpha-adrenergic stimulation in guinea-pig papillary muscle. Br J Pharmacol 130(3):650-654.

Morgan AJ, Jacob R (1994) Ionomycin enhances Ca2+ influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. Biochem J 300(Pt 3):665-672

Muller MS, Obel LF, Waagepetersen HS, Schousboe A, Bak LK (2013) Complex actions of ionomycin in cultured cerebellar astrocytes affecting both calcium-induced calcium release and store-operated calcium entry. Neurochem Res 38(6):1260-1265.

Myers JT, Swanson JA (2002) Calcium spikes in activated macrophages during Fcgamma receptor-mediated phagocytosis. J Leukoc Biol 72(4):677-684

Oh J, Riek AE, Weng S, Petty M, Kim D, Colonna M, Cella M, Bernal-Mizrachi C (2012) Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. J Biol Chem 287(15):11629-11641.

Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R (2008) Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. Oncogene 27(50):6407-6418.

Sano R, Reed JC (2013) ER stress-induced cell death mechanisms. Biochim Biophys Acta 1833(12):3460-3470.

Seimon TA, Obstfeld A, Moore KJ, Golenbock DT, Tabas I (2006) Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. Proc Natl Acad Sci U S A 103(52):19794-19799.

Sivaramakrishnan V, Bidula S, Campwala H, Katikaneni D, Fountain SJ (2012) Constitutive lysosome exocytosis releases ATP and engages P2Y receptors in human monocytes. J Cell Sci 125(Pt 19):4567-4575.

Sogkas G, Stegner D, Syed SN, Vogtle T, Rau E, Gewecke B, Schmidt RE, Nieswandt B, Gessner JE (2015) Cooperative and alternate functions for STIM1 and STIM2 in macrophage activation and in the context of inflammation. Immun Inflamm Dis 3(3):154-170.

Supnet C, Bezprozvanny I (2011) Presenilins function in ER calcium leak and Alzheimer's disease pathogenesis. Cell Calcium 50(3):303-309.

Szlufcik K, Missiaen L, Parys JB, Callewaert G, De Smedt H (2006) Uncoupled IP3 receptor can function as a Ca2+-leak channel: cell biological and pathological consequences. Biol Cell 98 (1):1-14.

Tabas I, Seimon T, Timmins J, Li G, Lim W (2009) Macrophage apoptosis in advanced atherosclerosis. Ann N Y Acad Sci 1173 Suppl 1:E40-45.

Van Coppenolle F, Vanden Abeele F, Slomianny C, Flourakis M, Hesketh J, Dewailly E, Prevarskaya N (2004) Ribosome-translocon complex mediates calcium leakage from endoplasmic reticulum stores. J Cell Sci 117(Pt 18):4135-4142.

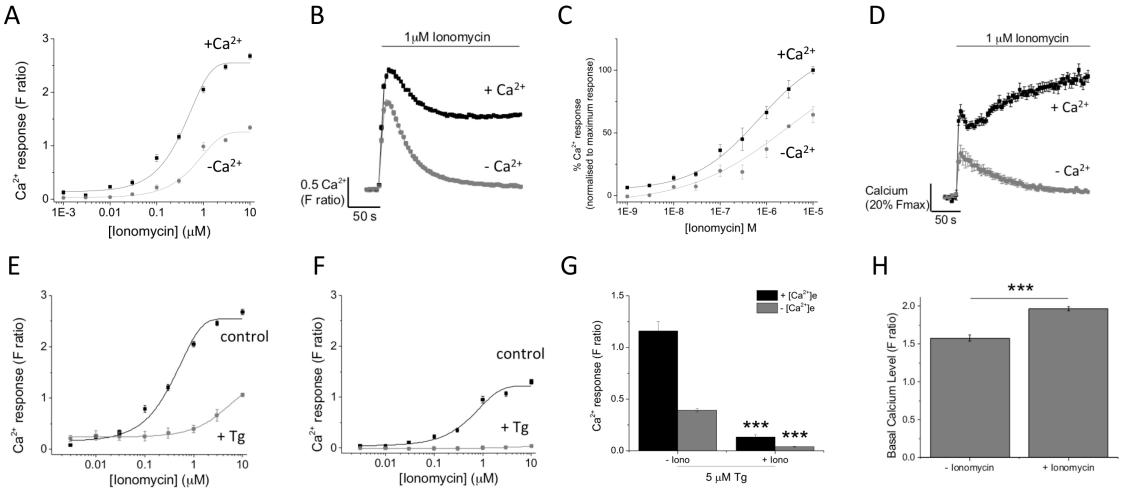
Figure 1 Ionomycin can elevate intracellular Ca^{2+} independent of Ca^{2+} influx but dependent on the ER Ca^{2+} store. Ionomycin-evoked intracellular Ca^{2+} responses in THP-1 model macrophage (A; *N*=4) and human primary macrophage (C; *N*=3) in the presence (*+Ca*$^{2+}$) and absence (*-Ca*$^{2+}$) of 1.5 mM extracellular Ca^{2+}. (B, D) Averaged (*N*=3-4) time traces showing intracellular Ca^{2+} responses evoked by 1 μM ionomycin in the presence and absence of extracellular Ca^{2+}. THP-1 macrophage responses are represented as absolute Fura-2 excitation/emission ratio (*F ratio*), and responses in primary macrophage are expressed normalised to maximal ionomycin-evoked response (*F max*) to control for donor variability. Dose-response curves for ionomycin-evoked Ca^{2+} responses for THP-1 macrophage in the presence *(+Tg*) and absence (control) of 5μM thapsigargin (*N*=4), in the presence (E) and absence (F) of 1.5mM extracellular Ca^{2+}. (G) Effect of preincubation with 1μM ionomycin (*iono*) on the magnitude of thapsigargin (*Tg*) evoked Ca^{2+} response in the presence (black bar) and absence (grey bar) of 1.5 mM extracellular Ca^{2+} (*N*=4). (H) Effect of 1μM ionomycin preincubation on resting cytoplasmic Ca^{2+} concentration (*N*=4) represented as absolute Fura-2 excititation/emission ratio (*F ratio*). ***P<0.001.

Figure 2 Involvement of ER Ca^{2+} channels in mediated response to ionomycin in the presence and absence of extracellular Ca^{2+} in THP-1 macrophage. Effect of 2-APB (100 μM) on ionomycin concentration-response in the presence (A) and absence (B) of 1.5 mM extracellular Ca^{2+} (*N*=3). Effect of xestopsongin C (5 μM) on ionomycin concentration-response in the presence (C) and absence (D) of 1.5 mM extracellular Ca^{2+} (*N*=3). Effect of dantrolene (20 μM) on ionomycin concentration-response in the presence (E) and absence (F) of 1.5 mM extracellular Ca^{2+} (*N*=3).

Figure 3 Effect of translocon inhibition on ionomycin-evoked Ca^{2+} leak. (A) Dose-response curves for ionomycin-evoked Ca^{2+} responses for THP-1 macrophage in the presence *(+anisomycin*) and absence (control) of 200 μM anisomycin (*N*=4), experiments are performed in the absence of extracellular Ca^{2+}. (B-C) Averaged (*N*=4) time traces from THP-1 macrophage showing intracellular Ca^{2+} responses evoked by low (0.3 μM) and high (1 μM) concentrations of ionomycin in the presence and absence of 200 μM anisomycin. Experiments performed in the absence of extracellular Ca^{2+}. (D-E) Effect of translocon inhibition in human primary macrophage. (D) Averaged (*N*=4) time traces from primary macrophage showing effect of 200 μM anisomycin on ionomycin-evoked intracellular Ca^{2+} response in the absence of extracellular Ca^{2+}. (E) Effect of 200 μM anisomycin on net Ca^{2+} movement (area under the curve) evoked by 1 μM ionomycin in the absence of extracellular Ca^{2+} (*N*=4). *P<0.05

Figure 4 ER Translocon inhibition attenuates receptor-mediated Ca^{2+} signaling. (A) Effect of translocon inhibition with anisomycin (200 μM) on ADP concentration-response curve in THP-1 macrophage (*N*=4). (B) Averaged (*N*=4) Ca^{2+} responses to 100 μM ADP in the presence (*anisomycin*) and absence (*control*) of anisomycin (200 μM). (C) Averaged *(N*=4) Ca^{2+} responses to 100 μM ADP in human primary macrophage performed in the presence (*anisomycin*) and absence (*control*) of anisomycin (200 μM). Averaged data showing augmentation of peak response (D) and net Ca^{2+} movement (E) elicited by ADP in human primary macrophage (*N*=4). *P<0.05 ***P<0.001

Figure 1


A
+Ca2+
-Ca2+
Ca2+ response (F ratio)
[Ionomycin] (μM)
B
1μM Ionomycin
+ Ca2+
- Ca2+
0.5 Ca2+ (F ratio)
50 s
C
+Ca2+
-Ca2+
% Ca2+ response (normalised to maximum response)
[Ionomycin] M
D
1 μM Ionomycin
+ Ca2+
- Ca2+
Calcium (20% Fmax)
50 s
E
control
+ Tg
Ca2+ response (F ratio)
[Ionomycin] (μM)
F
control
+ Tg
Ca2+ response (F ratio)
[Ionomycin] (μM)
G
+ [Ca2+]e
- [Ca2+]e
Ca2+ response (F ratio)
***
***
- Iono
+ Iono
5 μM Tg
H
***
Basal Calcium Level (F ratio)
- Ionomycin
+ Ionomycin

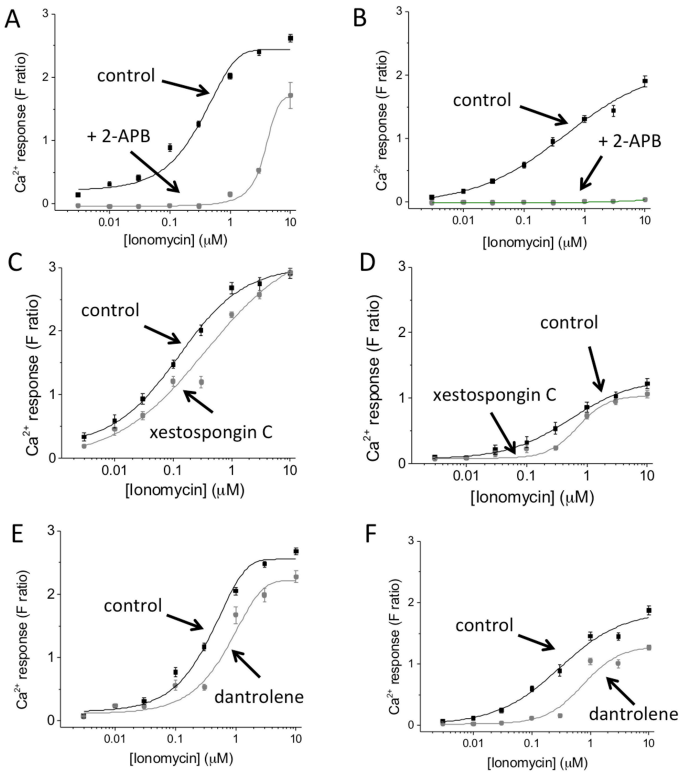

A
control
+ 2-APB
Ca2+ response (F ratio)
[Ionomycin] (μM)
B
control
+ 2-APB
C
control
xestospongin C
D
control
xestospongin C
E
control
dantrolene
F
control
dantrolene

Figure 2

Figure 3

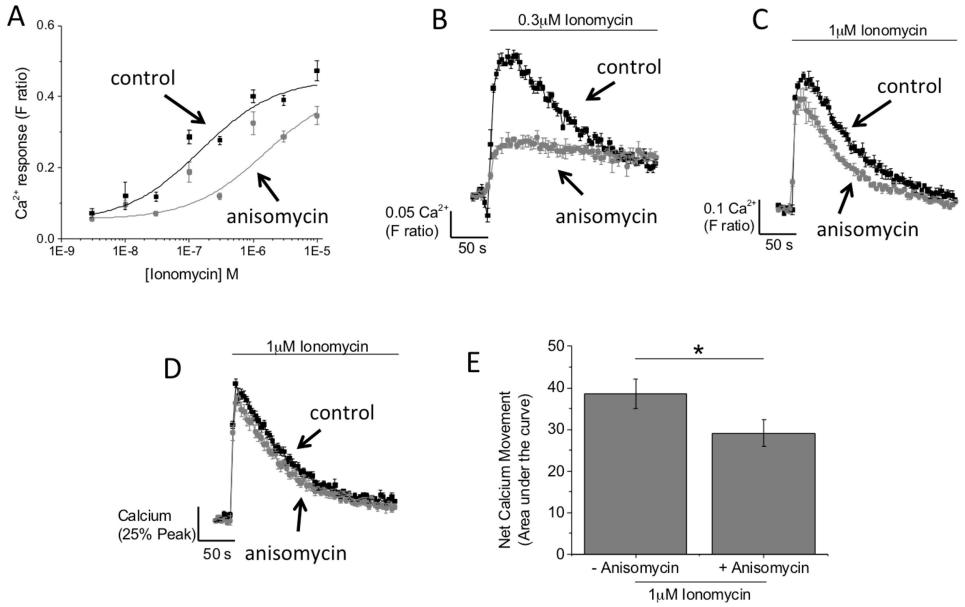
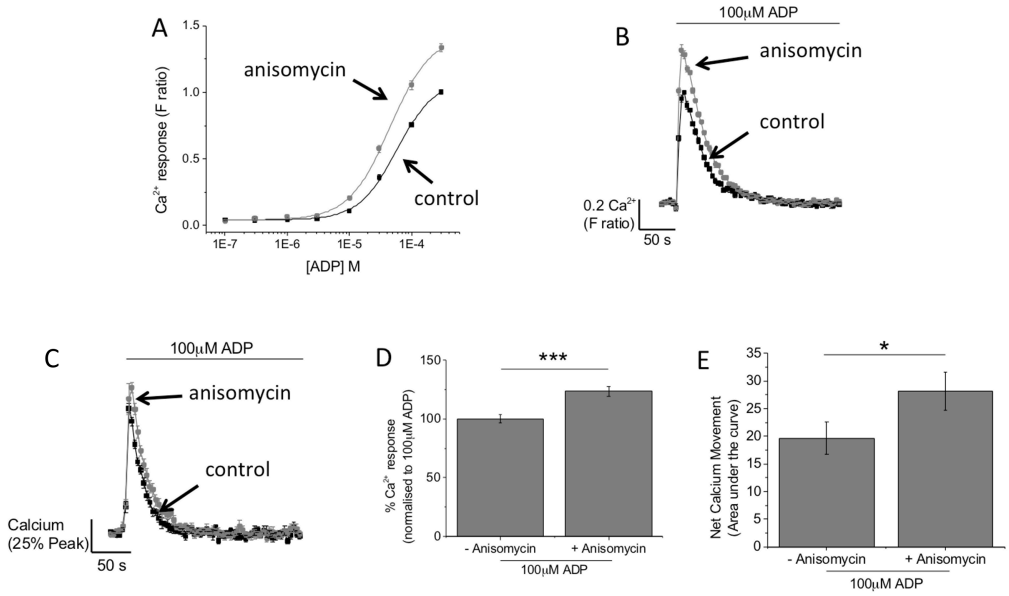

A
control
anisomycin
Ca2+ response (F ratio)
[Ionomycin] M
B
0.3μM Ionomycin
control
anisomycin
0.05 Ca2+
(F ratio)
50 s
C
1μM Ionomycin
control
anisomycin
0.1 Ca2+
(F ratio)
50 s
D
1μM Ionomycin
control
anisomycin
Calcium
(25% Peak)
50 s
E
*
Net Calcium Movement
(Area under the curve)
- Anisomycin
+ Anisomycin
1μM Ionomycin

Figure 4


A
anisomycin
control
Ca2+ response (F ratio)
[ADP] M
B
100μM ADP
anisomycin
control
0.2 Ca2+
(F ratio)
50 s
C
100μM ADP
anisomycin
control
Calcium
(25% Peak)
50 s
D
***
% Ca2+ response
(normalised to 100μM ADP)
- Anisomycin
+ Anisomycin
100μM ADP
E
*
Net Calcium Movement
(Area under the curve)
- Anisomycin
+ Anisomycin
100μM ADP