Published in final edited form as:

Cell Calcium. 2008 August ; 44(2): 190–201. doi:10.1016/j.ceca.2007.11.003.

Lysosomes co-localize with ryanodine receptor subtype 3 to form a trigger zone for calcium signalling by NAADP in rat pulmonary arterial smooth muscle

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Summary

In arterial myocytes the Ca^{2+} mobilizing messenger NAADP evokes spatially restricted Ca^{2+} bursts from a lysosome-related store that are subsequently amplified into global Ca^{2+} waves by Ca^{2+} -induced Ca^{2+} -release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs). Lysosomes facilitate this process by forming clusters that co-localize with a subpopulation of RyRs on the SR. We determine here whether RyR subtypes 1, 2 or 3 selectively co-localize with lysosomal clusters in pulmonary arterial myocytes using affinity purified specific antibodies. The density of: (1) αlgP120 labelling, a lysosome-specific protein, in the perinuclear region of the cell (within 1.5 μm of the nucleus) was ~4-fold greater than in the sub-plasmalemmal (within 1.5 μm of the plasma membrane) and ~2-fold greater than in the extra-perinuclear (remainder) regions; (2) RyR3 labelling within the perinuclear region was ~4- and ~14-fold greater than that in the extra-perinuclear and sub-plasmalemmal regions, and ~2-fold greater than that for either RyR1 or RyR2; (3) despite there being no difference in the overall densities of fluorescent labelling of lysosomes and RyR subtypes between cells, co-localization with αlgp120 labelling within the perinuclear region was ~2-fold greater for RyR3 than for RyR2 or RyR1; (4) co-localization between αlgp120 and each RyR subtype declined markedly outside the perinuclear region. Furthermore, selective block of RyR3 and RyR1 with dantrolene (30 μM) abolished global Ca^{2+} waves but not Ca^{2+} bursts in response to intracellular dialysis of NAADP (10nM). We conclude that a subpopulation of lysosomes cluster in the perinuclear region of the cell and form junctions with SR containing a high density of RyR3 to comprise a trigger zone for Ca^{2+} signalling by NAADP.

Keywords

Lysosomes; Ryanodine receptors; NAADP

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, In the online version, at doi:10.1016/j.ceca.2007.11.003.

Introduction

The spatiotemporal pattern of Ca^{2+} signalling varies in an agonist-specific manner, and thus “calcium fingerprints” determine, in part, the cellular response initiated by a given agonist [1]. The precise mechanisms that underpin such stimulus-specific Ca^{2+} signalling patterns remain to be determined. However, it is now recognized that this may be a consequence of organellar selection by multiple Ca^{2+} mobilizing messengers [2,3]. Consistent with this proposal, intracellular Ca^{2+} stores may be mobilized differentially by inositol 1,4,5-trisphosphate (IP_3) and the pyridine nucleotides, cyclic adenosine diphosphate-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP); [4,5]. Of particular interest in this respect is NAADP. A growing body of evidence suggests that it mobilizes Ca^{2+} via a relatively complex, two-pool mechanism in a variety of cell types including arterial smooth muscle [6-8]. Briefly, it has been suggested that NAADP induces intracellular Ca^{2+} release from a lysosome-related Ca^{2+} store in a manner that may be amplified by Ca^{2+} -induced Ca^{2+} -release (CICR) from the sarco/endoplasmic reticulum (SR/ER). Furthermore, the precise nature of coupling between lysosome-related Ca^{2+} stores and the SR/ER may vary between cell types, in that CICR may be initiated via ryanodine receptors (RyRs) and/or IP_3 receptors (IP_3Rs) [2,3,7,9].

This two-pool mechanism of Ca^{2+} mobilization by NAADP is conserved within both coronary and pulmonary arterial smooth muscle cells (PASMCs): [3,10]. However, these studies suggest that NAADP initiates spatially restricted Ca^{2+} bursts from lysosome-related stores that then trigger a global Ca^{2+} wave by CICR via RyRs, but not via IP_3Rs . Thus, cells may co-ordinate and restrict the relationship between lysosomal Ca^{2+} stores and the SR/ER, such that NAADP may generate Ca^{2+} signals in a manner that is suited to its regulatory function in a given cell type. This proposal gained support from the fact that, in contrast to their diffuse granular pattern in some cell types, lysosome-related organelles formed highly organised, dense clusters within PASMCs [3]. Most striking, however, was the observation in this study that these lysosomal clusters co-localized with a subpopulation of RyRs on the SR to form a junctional complex or trigger zone for Ca^{2+} signalling by NAADP. The significance of this finding may lie in the fact that all 3 subtypes of RyR (RyR1, RyR2 and RyR3) are expressed in vascular smooth muscle [11-13].

We therefore sought to determine whether or not lysosomes co-localized selectively with RyR1, RyR2, or RyR3 in PASMCs. Our findings suggest that a junctional complex is formed between lysosomes and RyR3 to comprise a trigger zone for Ca^{2+} signalling by NAADP.

Methods

All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Cell isolation

Adult male Wistar rats (150–300 g) were sacrificed by cervical dislocation. The heart and lungs were removed *en bloc* and placed in physiological salt solution (PSS) of the following composition (mM): 130 NaCl, 5.2 KCl, 1 MgCl_2 , 1.7 CaCl_2 , 10 glucose, 10 Hepes, pH 7.4. Single arterial smooth muscle cells were isolated from second-order branches of the pulmonary artery. Briefly, arteries were dissected out and placed in low Ca^{2+} solution of the following composition (mM): 124 NaCl, 5 KCl, 1 MgCl_2 , 0.5 NaH_2PO_4 , 0.5 KH_2PO_4 , 15 NaHCO_3 , 0.16 CaCl_2 , 0.5 EDTA, 10 glucose, 10 taurine and 10 Hepes, pH 7.4. After 10 min the arteries were placed in the same solution containing 0.5mg/ml papain and 1 mg/ml

bovine serum albumin and kept at 4°C overnight. The following day 0.2mM 1,4-dithio-DL-threitol was added to the solution, to activate the protease, and the preparation was incubated for 1 h at room temperature (22°C). The tissue was then washed 3× in fresh low Ca²⁺ solution without enzymes, and single smooth muscle cells were isolated by gentle trituration with a fire-polished Pasteur pipette. Cells were stored in suspension at 4°C until required.

Immunocytochemistry

200 µl of cell suspension was placed onto poly-D-lysine-coated coverslips, fixed using ice cold methanol (−20°C, 15min), permeabilised by 3 × 5min washes with 0.6% Triton X-100 in phosphate buffered saline (PBS, pH 7.4), washed 3 × 5min in blocking solution (1% BSA, 4% goat serum and 0.3% Triton X-100 in PBS) and incubated overnight at 4°C with affinity-purified rabbit anti-RyR1 (raised against the peptide residues 4476–4486 [14]), anti-RyR2 (raised against the peptide residues 1344–1365 [15]) and anti-RyR3 (raised against the peptide residues 4236–4336 [16]) antibodies (1:500) and mouse antibodies (GM10 [17] raised against the lysosomal membrane protein α lgp120 [18]; 1:250, a gift from Professor Paul Luzio, University of Cambridge). Coverslips were washed 4 × 5min with blocking solution and incubated (1 h, 22°C, dark) with goat anti-rabbit Texas Red-conjugated secondary antibodies (1:200; excitation 555 nm, emission 617 nm) and goat anti-mouse FITC-conjugated secondary antibodies (1:200; excitation 490nm, emission 518nm), washed 6 × 5min with PBS and attached to slides by antifade mountant (2.4g mowiol 4–88, 6g of glycerol, 2ml of 0.2M Tris-HCl pH 8.5, 2.5% dabco) with DAPI (1 µg/ml; excitation 358nm, emission 461 nm). For control slides primary antibody was omitted. Images were acquired using a DeltaVision microscope system (Applied Precision, USA), on an Olympus 1X70 microscope using a 60×, 1.40 n.a., oil immersion objective and Photometric CH300 CCD camera. Single or multiple Z-sections (focal depth 0.28 µm, Z-step 0.2 µm) were taken through a cell. Images were deconvolved off-line via SoftWorx software (Applied Precision, USA). Following deconvolution, Z-sections and 3D reconstructions of each Z-stack obtained were analysed using Volocity software (Improvision, UK).

Analysis of fluorescent labelling

3D renderings of PASMCs were subdivided into three separate regions (Fig. 1), namely the perinuclear (the area of the cell located within 1.5 µm of the DAPI labelled nucleus; $478.5 \pm 48.7 \mu\text{m}^3$, $n = 35$), the sub-plasmalemmal (the area of the cell located within 1.5 µm of the plasma membrane; $587.7 \pm 59.1 \mu\text{m}^3$, $n = 35$) and the extra-perinuclear regions (the remaining volume of the cytoplasm; $358.0 \pm 34.1 \mu\text{m}^3$, $n = 35$), the volumes of which were determined using Volocity software (Improvision, UK). The DeltaVision imaging system is able to accurately resolve elements of labelling smaller than 0.2 µm in size in each of the X-, Y- and Z-planes. However, the precisely controlled environment under which these ‘optimal’ measurements are obtained cannot be recreated under our experimental conditions. Therefore, we set a more conservative value on the limit of resolution and only included those volumes of labelling measuring 0.5 µm in the X-, Y- and Z-planes (volume $0.125 \mu\text{m}^3$) with any element of labelling smaller than these limits excluded from consideration. Following determination of the volumes occupied by the three cellular regions, we determined the extent of fluorescent labelling within these regions. In order to allow for direct comparison of fluorescent labelling between different regions/cells, the volume occupied by fluorescent labelling for a given protein was divided by the volume of the region in which it was located to give a measure of the density of fluorescent labelling in that region. Thus, data are presented as the mean $\pm$ S.E.M. of the volume of labelling per μm^3 of a given region (μm^3 per μm^3). Likewise, the density of co-localization was determined.

Ca²⁺ imaging

PASMCs were incubated for 30 min with 5 μ M Fura-2-AM in Ca²⁺-free PSS in an experimental chamber on a Leica DMIRBE inverted microscope and then superfused with Fura-2 free PSS for at least 30 min prior to experimentation. Intracellular Ca²⁺ concentration was reported by Fura-2 fluorescence ratio (F340/F380 excitation; emission 510nm). Emitted fluorescence was recorded at 22 °C with a sampling frequency of 0.2 Hz, using a Hamamatsu 4880 CCD camera via a Zeiss Fluor 40 $\times$, 1.3 n.a. oil immersion lens and Leica DMIRBE microscope. Background subtraction was performed on-line. Analysis was via Openlab imaging software (Improvision, UK).

Intracellular dialysis of NAADP

NAADP was applied intracellularly in the whole-cell configuration of the patch-clamp technique, and in current clamp mode (*I*-0) as described previously [8]. The pipette solution contained (mM): 140 KCl, 10 Hepes, 1 MgCl₂, and 5 μ M Fura-2, pH 7.4. The seal resistance, as measured using an Axopatch 200B amplifier (Axon instruments, Foster City, CA), was ≥ 3 G Ω throughout each experiment. Series resistance and pipette resistance was 10M Ω and 3M Ω , respectively. All experiments were carried out at room temperature (~22 °C). Dantrolene (30 μ M) was applied directly into the experimental chamber.

Data presentation and statistical analysis

Data are presented as mean $\pm$ S.E.M. and statistical significance assessed by repeated measures analysis of variance (ANOVA). Differences between individual means were determined by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA. A value of *P* 0.05 was deemed to be statistically significant.

Drugs and chemicals

All compounds were from Sigma–Aldrich except Fura-2 (Molecular probes) and fluorescently tagged secondary antibodies (Jackson Immunoresearch). GM10 antibodies were a kind gift from Dr. Luzio (University of Cambridge, UK).

Results

Lysosomal labelling is predominantly located in the perinuclear region of pulmonary arterial smooth muscle cells

The spatial distribution of lysosomes within isolated PASMCs was examined using an antibody raised against the integral lysosomal membrane protein α lgp120, GM10; [17], which has been shown previously to be expressed predominantly on the surface of lysosomes, with little or no expression on endosomes, the golgi cisternae or the plasma membrane [18]. Deconvolved Z-sections of isolated PASMCs revealed clustered labelling of α lgp120, indicative of the distribution of lysosomes [3], throughout the cytoplasm of cells (Fig. 2Ab). However, examination of 3D reconstructions of deconvolved Z-sections indicated that the largest clusters of α lgp120 labelling were located in close proximity to the nucleus of the cell (Fig. 2Ac). To enable analysis of the spatial distribution of α lgp120-labelling, PASMCs were subdivided into three distinct regions, namely the perinuclear (within 1.5 μ m of, and excluding any labelling within, the DAPI labelled nucleus), the sub-plasmalemmal (within 1.5 μ m of the plasma membrane) and the extra-perinuclear region (the remainder of the cytoplasmic volume of the cell). The density of α lgp120-labelling within each region was then determined as described previously (see Section 2). Fig. 2Ad shows the spatial distribution of those volumes of α lgp120 labelling 0.125 nm³ in a 3D reconstruction of a deconvolved Z-stack of images obtained through a typical cell, with each

individual volume coloured to indicate the region of the cell in which it is located (perinuclear labelling in orange; extra-perinuclear labelling in pink and sub-plasmalemmal labelling in light blue; see also supplementary movie S.1.). Fig. 2B shows that the density of α lgp120 labelling was seen to decline markedly from the perinuclear region ($0.04 \pm 0.003 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $n=35$) to the extra-perinuclear region ($0.02 \pm 0.003 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$) and then to a minimum density in the sub-plasmalemmal region ($0.01 \pm 0.002 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$). Across all PASMCs studied, $58 \pm 3\%$ of the total volume of lysosomal labelling was observed within the perinuclear region. These data suggest, therefore, that not only was lysosomal labelling predominant in the perinuclear region of those PASMCs examined, but also that the density of labelling declines progressively as one moves from the nucleus to the plasma membrane of these cells.

As mentioned above, we have previously demonstrated that lysosomes form spatially restricted clusters within freshly isolated PASMCs [3]. We therefore sought to determine the mean volume of individual elements of lysosomal labelling within each defined region of the cell. Such measures may be used as an index of lysosomal clustering due to the fact that individual elements of α lgp120 labelling which are closer together than can be resolved under optimal conditions ($\sim 200 \text{ nm}$) will confer an increase in the observed volume of labelling. The mean volume of α lgp120 labelling within the perinuclear region of PASMCs was $0.76 \pm 0.06 \mu\text{m}^3$ ($n=35$; Fig. 2C), while the mean volumes of α lgp120 labelling were seen to decrease outside the perinuclear region, measuring $0.62 \pm 0.09 \mu\text{m}^3$ in the extra-perinuclear and $0.31 \pm 0.03 \mu\text{m}^3$ in the sub-plasmalemmal regions of PASMCs. Although there was a trend for a decrease in the mean volume of elements of labelling between the perinuclear and extra-perinuclear regions of cells, this decrease was not statistically significant ($P > 0.05$). However, the mean volume of individual elements of labelling within the sub-plasmalemmal region of cells was significantly smaller than those observed in either the perinuclear or extra-perinuclear regions ($P < 0.05$). Therefore, these findings suggest that a subpopulation of lysosomes form large clusters that are primarily located within the perinuclear region of PASMCs.

RyR1, RyR2 and RyR3 are differentially distributed within pulmonary arterial smooth muscle cells

Marked differences in the distribution of labelling for RyR1, RyR2 and RyR3 were observed within acutely isolated PASMCs.

Most strikingly, labelling for RyR3 was found to cluster primarily within the perinuclear region in all cells examined. Fig. 3A shows a typical example of a deconvolved Z-section (Panel b) and a 3D reconstruction of deconvolved Z-sections from a cell labelled for RyR3 (Panel c). However, the perinuclear clustering is most evident in Fig. 3Ad, which shows the spatial distribution of RyR3 labelling in a 3D reconstruction of the same cell with each individual volume of labelling, $0.125 \mu\text{m}^3$, coloured to indicate the region of the cell in which it is located (perinuclear labelling in orange; extra-perinuclear labelling in pink and sub-plasmalemmal labelling in light blue; see also supplementary movies S.2–S.4.). In fact the density of RyR3 labelling within the perinuclear region was $0.072 \pm 0.008 \mu\text{m}^3 \text{ per } \mu\text{m}^3$ ($n=811$; Fig. 4A), almost fourfold higher than that in the extra-perinuclear region ($0.019 \pm 0.005 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$) and approximately 14-fold higher than measured in the sub-plasmalemmal region ($0.005 \pm 0.001 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$). Furthermore, an examination of the mean volumes of distinct elements of RyR3 labelling within PASMCs identified significantly larger clusters of RyR3 labelling within the perinuclear region ($2.4 \pm 0.33 \mu\text{m}^3$, Fig. 4B) than was observed in either the extra-perinuclear ($0.72 \pm 0.15 \mu\text{m}^3$, $P < 0.05$) or the sub-plasmalemmal regions ($0.47 \pm 0.11 \mu\text{m}^3$, $P < 0.05$). Therefore, RyR3

appears to be preferentially targeted to the perinuclear region in PSMCs, as was found to be the case with respect to lysosomes.

In marked contrast to the perinuclear clustering of RyR3, labelling for RyR2 was found to be most concentrated in the extra-perinuclear region of those cells examined (Fig. 3Ba–c). Once more this is most evident in the 3D reconstruction which shows each individual volume of RyR2 labelling $0.125\mu\text{m}^3$ coloured to indicate the region of the cell in which it is located (perinuclear labelling in orange; extra-perinuclear labelling in pink and sub-plasmalemmal labelling in light blue; Fig. 3Bd). Analysis of the density of RyR2 labelling supported this view, with $0.065 \pm 0.009 \mu\text{m}^3$ of RyR2 labelling per μm^3 of the extra-perinuclear region ($n=14$; Fig. 4A), almost twofold greater than that observed in the perinuclear region ($0.039 \pm 0.008 \mu\text{m}^3$ per μm^3 ; $P<0.05$) and sixfold higher than was measured in the sub-plasmalemmal region ($0.011 \pm 0.002 \mu\text{m}^3$ per μm^3 ; $P<0.05$). Examination of the mean volumes of distinct elements of RyR2 labelling suggested that larger clusters of RyR2 labelling were observed in the extra-perinuclear region ($1.6 \pm 0.3 \mu\text{m}^3$, Fig. 4B) than in the perinuclear region ($0.97 \pm 0.15 \mu\text{m}^3$, $P=0.07$), although this difference was not statistically significant. There was, however, a significant decline in the mean volume of elements of RyR2 labelling in the sub-plasmalemmal region of PSMCs ($0.46 \pm 0.06 \mu\text{m}^3$, $P<0.05$) when compared to either the perinuclear or extra-perinuclear regions. Thus, unlike lysosomes and RyR3, RyR2 may be preferentially targeted to the extra-perinuclear region of PSMCs, although a significant proportion of this RyR subtype is also located in the perinuclear region.

The distribution of RyR1 within PSMCs was quite different from that observed for either RyR2 or RyR3. Fig. 3C shows a deconvolved Z-section (Panel b) and a 3D reconstruction (Panel c) from a typical example of a cell labelled for RyR1. Clearly, there is little pattern to the distribution of RyR1 labelling, and this is most evident in Fig. 3Cd which shows a 3D reconstruction of the same cell with each individual volume of RyR1 labelling $0.125 \mu\text{m}^3$, coloured to indicate the region of the cell in which it is located (perinuclear labelling in orange; extra-perinuclear labelling in pink and sub-plasmalemmal labelling in light blue). Consistent with this view no significant difference was found between the density of RyR1 labelling within each region, where it measured $0.041 \pm 0.007 \mu\text{m}^3$ per μm^3 of the perinuclear region ($n=10$; Fig. 4A), compared to $0.025 \pm 0.005 \mu\text{m}^3$ per μm^3 of the extra-perinuclear region ($P>0.05$) and $0.031 \pm 0.005 \mu\text{m}^3$ per μm^3 of the sub-plasmalemmal region ($P>0.05$). There was also little difference between the mean volumes of distinct elements of RyR1 labelling between the perinuclear ($1.04 \pm 0.27 \mu\text{m}^3$, Fig. 4B) the extra-perinuclear ($0.71 \pm 0.21 \mu\text{m}^3$, $P>0.05$) and the sub-plasmalemmal regions ($0.53 \pm 0.08 \mu\text{m}^3$, $P>0.05$). Therefore, RyR1 labelling appears to be distributed relatively evenly throughout PSMCs.

Furthermore, direct comparison of the density of labelling for RyR1, 2 and 3 within each of the specified regions of the PSMCs (Fig. 4A) revealed that: (1) RyR3 is the predominant subtype within the perinuclear region of these cells, with a twofold greater density of labelling than observed for the other subtypes; (2) RyR2 is the predominant subtype in the extra-perinuclear region, with a threefold greater density of labelling than observed for the other subtypes; (3) RyR1 predominates in the sub-plasmalemmal region, with a threefold and sixfold greater density of labelling than observed for RyR2 and RyR3, respectively.

That RyR3 alone forms dense clusters in the perinuclear region was evident from the fact that its mean volume of labelling was approximately threefold greater than that for RyR1 ($P<0.05$) and RyR2 ($P<0.05$; Fig. 4B). We also found evidence of clustering of RyR2 alone within the extra-perinuclear region of the cell, the mean volume of RyR2 labelling being approximately twofold greater than that measured for either RyR1 ($P<0.05$) or RyR3 ($P<0.05$) within the extra-perinuclear region.

Lysosomes cluster preferentially with RyR3 in the perinuclear region of isolated pulmonary arterial smooth muscle cells

Consistent with the predominantly perinuclear distribution of labelling for both RyR3 and α lgp120, a greater extent of co-localization was observed between α lgp120 and RyR3 when compared to either RyR1 or RyR2, with the observed co-localization predominately located in the perinuclear region rather than in the extra-perinuclear or sub-plasmalemmal regions of PASMCs.

Fig. 5A shows a typical example of a PASMC labelled for α lgp120 and RyR3, with 3D reconstruction of a series of deconvolved Z sections taken at the same depth through the cell showing the labelling for α lgp120 in green and RyR3 in red merged to show areas of co-localization between lysosomes and RyR3 in yellow (Panel Ab–d). Similarly, panel e shows a deconvolved Z section taken through the cell showing the spatial distribution of lysosomal labelling and RyR3 labelling within the cell. However, that RyR3 preferentially co-localizes with lysosomes in the perinuclear region of the cell is most evident from Fig. 5Af which shows each individual volume of co-localization $0.125\mu\text{m}^3$ coloured to indicate the region of the cell in which it is located (perinuclear labelling in orange; extra-perinuclear labelling in pink; see also supplementary movies S.5–S.7.). Similarly, typical examples of the distribution of volumes of co-localization between α lgp120 and RyR2 and RyR1 are shown in Fig. 5B and C, respectively. The density of co-localization between α lgp120 and RyR3 labelling in the perinuclear region was $0.012\pm 0.002\mu\text{m}^3\text{ per }\mu\text{m}^3$ ($n=11$; Fig. 6A), twofold greater than that observed with respect to either RyR1 ($0.0057\pm 0.0015\mu\text{m}^3\text{ per }\mu\text{m}^3$; $n=10$; $P<0.05$) or RyR2 ($0.0064\pm 0.0015\mu\text{m}^3\text{ per }\mu\text{m}^3$; $n=14$; $P<0.05$). Furthermore, the mean volume of co-localization between RyR3 and α lgp120 labelling within the perinuclear region measured $0.81\pm 0.15\mu\text{m}^3$ (Fig. 6B), twofold to threefold greater than that observed for either RyR1 ($0.3\pm 0.03\mu\text{m}^3$; $P<0.05$) or RyR2 ($0.38\pm 0.04\mu\text{m}^3$; $P<0.05$). We also found that within the perinuclear region there were 5.00 ± 1.3 elements of co-localization between α lgp120 and RyR3 labelling per cell with a volume greater than $0.5\mu\text{m}^3$, but only 1.43 ± 0.3 elements of co-localization between α lgp120 and RyR1 labelling, and 1.6 ± 0.2 elements of co-localization between α lgp120 and RyR2 labelling with a volume greater than $0.5\mu\text{m}^3$. When taken together with the fact that RyR3 labelling co-localized with $41\pm 6\%$ of the total volume of α lgp120 labelling within the perinuclear region, compared to $14\pm 3\%$ for RyR1 and $13\pm 2\%$ for RyR2, these data indicate that lysosomal clusters preferentially associate with RyR3 in the perinuclear region of PASMCs.

That lysosome-RyR3 clusters are targeted to the perinuclear region of PASMCs is emphasised in Fig. 6A and B. These bar charts show a fourfold decline in the density of co-localization between RyR3 and α lgp120 to $0.003\pm 0.001\mu\text{m}^3\text{ per }\mu\text{m}^3$ and a concomitant twofold decline in the mean volume of co-localization to $0.35\pm 0.08\mu\text{m}^3$ in the extra-perinuclear region when compared to the perinuclear region of PASMCs ($P<0.05$), at which point neither measure was found to be significantly different from that obtained for either RyR1 or RyR2. Perhaps most significantly, there was little or no co-localization between α lgp120 labelling and that for any of the three RyR subtypes within the sub-plasmalemmal region of PASMCs.

The marked differences observed with respect to the level of co-localization between RyR subtypes and lysosomes within PASMCs cannot be explained by differences in the overall density of labelling for each RyR subtype nor any variation in α lgp120 labelling between cells. This is clear from the fact that across all cells studied there was no significant difference in the total density of RyR1 ($0.0331\pm 0.0035\mu\text{m}^3\text{ per }\mu\text{m}^3$; $n=10$; Fig. 7A), RyR2 ($0.0328\pm 0.0038\mu\text{m}^3\text{ per }\mu\text{m}^3$; $n=14$; $P>0.05$) or RyR3 labelling ($0.031\pm 0.0028\mu\text{m}^3\text{ per }\mu\text{m}^3$; $n=11$; $P>0.05$), nor was there any significant difference between the density of α lgp120 labelling between cells co-labelled with either RyR1 ($0.019\pm 0.0036\mu\text{m}^3\text{ per }\mu\text{m}^3$; Fig.

7A), RyR2 ($0.02 \pm 0.0016 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P > 0.05$) or RyR3 ($0.022 \pm 0.002 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P > 0.05$). Despite these facts, when co-localization was examined across the entire volume of PSMCs (i.e. without regionalisation) a significantly greater degree of co-localization was still observed between αlgp120 labelling and RyR3 labelling ($0.006 \pm 0.0006 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; Fig. 7B) when compared to that for αlgp120 labelling and RyR2 labelling ($0.003434 \pm 0.0007 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$) or αlgp120 labelling and RyR1 labelling ($0.0031 \pm 0.0007 \mu\text{m}^3 \text{ per } \mu\text{m}^3$; $P < 0.05$). Furthermore, Fig. 7C shows that the Pearson product-moment correlation value suggests a significantly greater correlation between the spatial distribution of αlgp120 labelling and RyR3 labelling (0.26 ± 0.01) than observed for either αlgp120 labelling and RyR2 labelling (0.18 ± 0.01 , $P < 0.05$) or αlgp120 labelling and RyR1 labelling (0.16 ± 0.02 , $P < 0.05$). Taken together, these data provide further evidence in support of the view that lysosomes preferentially co-localize with RyR3 in PSMCs.

Dantrolene inhibits NAADP-induced global Ca^{2+} waves but not Ca^{2+} bursts in isolated pulmonary arterial smooth muscle cells

The measures of co-localization between αlgp120 and each RyR subtype provide a rank order of $\text{RyR3} \gg \text{RyR2} > \text{RyR1}$, which suggests that the proposed trigger zone for Ca^{2+} signalling by NAADP is likely formed between lysosomes and RyR3. To examine this hypothesis further we studied the effect on Ca^{2+} signalling by NAADP of selectively blocking RyR1 and RyR3 with dantrolene, which has been shown bind to RyR3 and RyR1 but not RyR2 and to block Ca^{2+} release via RyR3 and RyR1 but not via RyR2 [19]. Intracellular dialysis of NAADP (10 nM) from a patch pipette, in the whole-cell configuration and under current clamp conditions ($I=0$), induced spatially restricted Ca^{2+} burst events (as indicated by the arrow in Fig. 8A Image 2). These Ca^{2+} bursts preceded and then triggered global Ca^{2+} waves in isolated PSMCs as indicated by the Fura-2 fluorescence ratio (F340/F380) which increased from 0.65 ± 0.04 to 1.85 ± 0.14 ($n = 20$; Fig. 8A). Following preincubation (15 min) of PSMCs with dantrolene (30 μM) intracellular dialysis of NAADP (10 nM) failed to induce a global Ca^{2+} wave. However, consistent with our previous observations on the effects of ryanodine and thapsigargin [8], Ca^{2+} bursts were still evident (as indicated by the arrow in Fig. 8B Image 3). These Ca^{2+} bursts events resulted in an increase in the Fura-2 fluorescence ratio from 0.54 ± 0.04 to only 0.85 ± 0.04 ($n = 6$; Fig. 8B). In marked contrast, preincubation of PSMCs with dantrolene (30 μM) was without effect on the increase in the Fura-2 fluorescence ratio induced by lonomycin (1 μM), which measured 0.3 ± 0.04 ($n = 11$) in the absence of dantrolene and 0.36 ± 0.05 in its presence ($n = 12$).

Discussion

Our previous studies on pulmonary arterial smooth muscle have shown that NAADP induces spatially restricted Ca^{2+} bursts from lysosome-related Ca^{2+} stores that are subsequently amplified into global Ca^{2+} waves by CICR from the SR via RyRs [3]. Of further significance in this respect was our observation that lysosomal clusters form junctions with a subpopulation of RyRs on the SR to comprise a trigger zone for Ca^{2+} signalling by NAADP. We therefore sought to determine whether or not lysosomes selectively couple to one of the three RyR subtypes expressed in arterial smooth muscle, namely RyR1, RyR2, or RyR3. Our findings suggest that lysosomal clusters are primarily located in the perinuclear region of pulmonary arterial smooth muscle cells and that they preferentially co-localize with a region of the SR containing a high density of RyR3.

The distribution of labelling for a given protein by density was determined for each of three regions of each cell relative to the nucleus (defined by DAPI labelling), namely the perinuclear (within 1.5 μm of the nucleus), the sub-plasmalemmal (within 1.5 μm of the

plasma membrane) and the extra-perinuclear region (the remaining volume of the cytoplasm). The density of labelling for the lysosome marker (α lgp120) was ~2-fold greater in the perinuclear than observed within the extra-perinuclear region and ~4-fold greater than was observed in the sub-plasmalemmal region of cells, with dense clusters of labelling evident in the perinuclear region compared to a more diffuse distribution of labelling outwith the perinuclear region. In common with the distribution of lysosomes, the density of RyR3 labelling was concentrated within the perinuclear region of the cell where it was ~4- and ~14-fold greater than that in the extra-perinuclear and sub-plasmalemmal regions, respectively. Furthermore, the density of RyR3 labelling within the perinuclear region was ~2-fold higher than that for RyR2 and RyR1, respectively.

The marked decline in RyR3 labelling between the perinuclear to the extra-perinuclear regions was in sharp contrast to the increase in the density of RyR2 labelling. Indeed, the density of RyR2 labelling was ~2- and ~6-fold greater in the extra-perinuclear region of cells than that in the perinuclear and sub-plasmalemmal regions, respectively. Furthermore, RyR2 was the predominant RyR subtype detected by density of labelling in the extra-perinuclear region of cells, being ~3-fold greater than that for RyR3 and ~3-fold greater than that for RyR1. Significantly, and in common with our findings with respect to RyR3, the density of RyR2 labelling declined markedly within the sub-plasmalemmal region of cells when compared with either the perinuclear or extra-perinuclear regions.

In contrast to the above, we found no significant difference in the density of RyR1 labelling between the perinuclear, extra-perinuclear and sub-plasmalemmal regions. Therefore, RyR1 labelling was the dominant form of RyR sub-type within the sub-plasmalemmal region, with a ~3- and ~6-fold greater density of labelling than was observed for RyR2 and RyR3, respectively.

The aforementioned data suggest that RyR3 is the predominant RyR subtype in the perinuclear region of PSMCs, whilst RyR2 predominates within the extra-perinuclear region and RyR1 in the sub-plasmalemmal region.

Further insight was provided by analysis of the density of co-localization between lysosomes and each RyR subtype. Within the perinuclear region of the cell RyR3 was found to co-localize with ~41% of the total volume of lysosome labelling, with the density of co-localization being ~4- and ~60-fold greater than that observed in the extra-perinuclear or sub-plasmalemmal regions, respectively. In marked contrast, labelling for RyR2 and RyR1 co-localized with only 13% and 14%, respectively, of the total volume of lysosome labelling within the perinuclear region with their respective density of co-localization being approximately twofold lower than that for RyR3. Furthermore, the mean volume of co-localization between RyR3 and lysosomes was ~2-fold greater than that for either RyR1 or RyR2. We may conclude, therefore, that lysosome clusters predominantly co-localize with RyR3 within the perinuclear region of the cell and that the rank order of co-localization between lysosomes and the three RyR subtypes is $\text{RyR3} \gg \text{RyR2} > \text{RyR1}$.

Given the above, we were able to determine whether or not RyR3 was a pre-requisite for amplification of NAADP-induced Ca^{2+} bursts into global Ca^{2+} waves using dantrolene, which blocks Ca^{2+} signalling via RyR3 and RyR1 but not via RyR2 [19]. Consistent with a pivotal role for RyR3 in the amplification of NAADP-induced Ca^{2+} bursts, pre-incubation of cells with dantrolene abolished global Ca^{2+} waves but not the spatially restricted Ca^{2+} bursts evoked by intracellular dialysis of NAADP. This finding suggests, therefore, that CICR via RyR3 on the SR represents the Initial step in the development of a propagating Ca^{2+} wave from the point of initiation of Ca^{2+} bursts from lysosomes by NAADP.

The aforementioned findings raise the question as to why RyR3 might be targeted to the lysosome–SR junctions. A determining factor in this respect could be the relative sensitivity of each RyR subtype to CICR, the maximum gain in response to Ca^{2+} and the relative sensitivity of each receptor subtype to inactivation by Ca^{2+} . The threshold for activation of RyR1, RyR2 and RyR3 is similar, with channel activation at cytoplasmic Ca^{2+} concentrations $>100\text{nM}$ [20,21]. However, estimates of the EC_{50} (in the absence of Mg^{2+} and ATP) are different, with half maximal activation at $\sim 250\text{ nM}$ for RyR2 and $\sim 400\text{ nM}$ for RyR3 [20,21]. The higher EC_{50} exhibited by RyR3 could be significant, because this would provide for a higher margin of safety with respect to the all-or-none amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores by CICR via RyRs at the lysosome–SR junction, i.e. the probability of false events being initiated would be lower for RyR3 than for RyR2. Another factor that may be of significance is that while the mean open time vs. cytoplasmic Ca^{2+} concentration for RyR2 and RyR3 are comparable and increase approximately 10-fold over their activation range, the mean open time for RyR1 is much lower and increases only twofold over its activation range [20,21]. Furthermore, comparison of the Po vs. cytoplasmic Ca^{2+} concentration curves shows that RyR3 (0–1) exhibits a higher gain in Po than does RyR2 (0–0.9), while RyR1 (0–0.2) exhibits relatively little gain in Po with increasing cytoplasmic Ca^{2+} concentration [20,21]. Thus, once the threshold for activation is breached RyR3 would offer greater amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores than would RyR2, while amplification via RyR1 would be marginal. There is also marked variation in the relative sensitivity of each RyR subtype to inactivation by Ca^{2+} . RyR3 exhibits the lowest sensitivity to inactivation by Ca^{2+} with an IC_{50} of 3 mM while that for RyR2 is 2 mM ; in each case channel activity may still be observed at concentrations $>10\text{ mM}$ [20,21]. In marked contrast, RyR1 inactivation occurs within the μM range and full inactivation is achieved by 1 mM Ca^{2+} [21]; this may, in part, explain the low gain in Po for RyR1 in response to activation by Ca^{2+} . Its sensitivity to inactivation by Ca^{2+} would therefore render RyR1 unsuitable for a role in the amplification of Ca^{2+} bursts at lysosome–SR junctions because the local Ca^{2+} concentration may exceed the threshold for RyR1 inactivation. Thus, the functional properties of RyR3 make it best suited to a role in the amplification of Ca^{2+} bursts at lysosome–SR junctions.

However, the density of RyR3 labelling declines markedly (fourfold to the extra-perinuclear region and ~ 14 -fold to the sub-plasmalemmal region) outside of the perinuclear region of the cell, which was defined as being within $1.5\text{ }\mu\text{m}$ of the nucleus. It seems unlikely, therefore, that RyR3 functions to carry a propagating Ca^{2+} wave far beyond the point of initiation of CICR within the trigger zone for Ca^{2+} signalling by NAADP. Given this finding it may be of significance that the density of labelling for RyR2 increased markedly in the extra-perinuclear region when compared to the perinuclear region of cells, and exhibited a ~ 3 -fold greater density of labelling within this region than was observed for either RyR3 or RyR1. This suggests that RyR2, but not RyR1, may function to receive Ca^{2+} from RyR3 at the interface of the lysosome–SR junction and thereby allow for further propagation of the Ca^{2+} signal via CICR. Once more we can gain insight into the relative roles of RyR1 and RyR2 from their respective properties of regulation by Ca^{2+} . The lower EC_{50} for CICR via RyR2 than for RyR1 would help insure that once initiated a propagating Ca^{2+} wave would be less prone to failure. Furthermore, relative to RyR1, the greater intrinsic gain exhibited by RyR2 with respect to the increase in mean open time and Po with increasing cytoplasmic Ca^{2+} concentration would render it most suitable of these two RyR subtypes for a role in underpinning the wider propagation of a global Ca^{2+} wave.

The even distribution of RyR1 across the three specified regions of PSMCs suggests that it may contribute in some way to the regulation of Ca^{2+} signalling within each region. However, our data suggest that it is in the sub-plasmalemmal region of cells where RyR1 is predominant, exhibiting a threefold to fivefold greater density of labelling than was

observed for either RyR3 or RyR2. RyR1 may therefore be of primary importance with respect to Ca^{2+} signalling between the SR and Ca^{2+} -sensitive ion channels in the plasma membrane.

In conclusion, we propose that lysosomal clusters are primarily located in the perinuclear region of PSMCs, where they form junctions with a subsection of the SR that contains a high density of RyR3. These lysosome–SR junctions comprise the trigger zone for Ca^{2+} signalling by NAADP, within which CICR via RyR3 may be a prerequisite for initiation of a propagating Ca^{2+} wave in response to NAADP-dependent Ca^{2+} bursts from lysosome-related stores. Thereafter, CICR via RyR2 likely functions to carry the propagating Ca^{2+} wave beyond the perinuclear region of the cell.

Acknowledgments

We would like to thank Professor Paul Luzio for the kind gift of antibodies raised against αlgpl 20. This work was supported by a Wellcome Trust Project Grant (CNW, reference number 070772), British Heart Foundation Project Grants (NPK, reference number PG/05/128/19884; JHC, reference number PG/03/065) and a British Heart Foundation Studentship (PJC, reference number FS/05/050).

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Figure 1.

Schematic representation of the regionalisation of an isolated pulmonary arterial smooth muscle. Isolated cells were divided into three separate regions, namely the perinuclear, extra-perinuclear and sub-plasmalemmal regions. The perinuclear region (shown in orange) was determined as being the volume of the cytoplasm up to 1.5 μm from the DAPI labelled nucleus (shown in dark blue). The sub-plasmalemmal region was determined as being the volume of the cytoplasm within 1.5 μm of the plasma membrane (shown in light blue), while the extra-perinuclear region (shown in pink) encompassed the remaining volume of the cytoplasm located outwith either the perinuclear and sub-plasmalemmal regions. (For Interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Figure 2.

Lysosome clusters are predominantly located within the perinuclear region of pulmonary arterial smooth muscle cells. (A)a Transmitted light image of an isolated PASC. (A)b Deconvolved Z-section (focal depth $0.28 \mu\text{m}$) taken through the cell showing the distribution of α lgp120 labelling, indicative of the distribution of lysosomes, in relation to the nucleus of the cell (dark blue) and plasma membrane (dotted line). (A)c shows a 3D reconstruction of a series of Z-sections (Z step $0.2 \mu\text{m}$) obtained through the cell, labelled as in (A)b. (A)d shows a 3D representation of the distribution of individual volumes of α lgp120 labelling, $0.125 \mu\text{m}^3$, measured in the cell coloured to indicate the region of the cell in which lysosomes are located in relation to the nucleus; perinuclear volumes (orange), extra-perinuclear volumes (pink) and sub-plasmalemmal volumes (light blue). (B) and (C) Bar charts showing the densities (μm^3 of labelling per μm^3 of region; mean $\pm$ S.E.M.) of α lgp120 labelling and the mean volume of individual elements of α lgp120 labelling (μm^3 ; mean $\pm$ S.E.M.) within the three designated regions of isolated PASCs, respectively. * Indicates statistical difference ($P < 0.05$) when compared to α lgp120 labelling in the perinuclear region. ϕ indicates statistical difference ($P < 0.05$) when compared to α lgp120 labelling in the extra-perinuclear region ($n = 35$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Figure 3.

RyR3, 2 and 1 are differentially distributed within isolated pulmonary arterial smooth muscle cells. Images show the location of labelling for (A) RyR3, (B) RyR2 and (C) RyR1. In each case panels show the following: (a) transmitted light image of a PASM; (b) deconvolved Z-section taken through the cell with labelling for the given RyR subtype (red) in relation to the nucleus (dark blue) and the plasma membrane (dotted line); (c) 3D reconstruction of a series of Z-sections (Z step $0.2\ \mu\text{m}$) obtained through the cell fluorescently labelled as in (b); (d) 3D representation showing the distribution by colour of individual volumes of labelling, $0.125\ \mu\text{m}^3$, for the given RyR subtype in the perinuclear (orange), extra-perinuclear (pink) and sub-plasmalemmal (light blue) regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Figure 4.

Different RyR subtypes predominate within different regions of pulmonary arterial smooth muscle cells. (A) Bar chart showing the densities of labelling (μm^3 of labelling per μm^3 of region: mean $\pm$ S.E.M.) for each RyR subtype within the three designated regions of isolated PASCs. (B) Bar chart showing the mean volume (μm^3 : mean $\pm$ S.E.M.) of individual elements of RyR subtype labelling within the three designated regions of PASCs. * indicates statistical difference ($P < 0.05$) when compared to the density of RyR3 labelling in the perinuclear region, ϕ indicates statistical difference ($P < 0.05$) when compared to the density of RyR2 labelling in the extra-perinuclear region, θ indicates statistical difference ($P < 0.05$) when compared to RyR1 labelling in the sub-plasmalemmal region ($n = 10$ for RyR1; $n = 14$ for RyR2; $n = 11$ for RyR3).

Figure 5.

Lysosomes primarily co-localize with RyR3 in the perinuclear region of the pulmonary arterial smooth muscle cells. Images show the degree of co-localization between α lgp120 labelling, indicative of the distribution of lysosomes, and labelling for (A) RyR3 (B) RyR2 and (C) RyR1. In each case panels show the following: (a) transmitted light image of an isolated PASC; (b) 3D reconstruction of a series of deconvolved Z-stack images (Z step $0.2 \mu\text{m}$) showing the distribution of α lgp120 labelling (green) in relation to the nucleus (dark blue) and the plasma membrane (dotted line); (c) 3D representation of deconvolved Z-stack of images (Z step $0.2 \mu\text{m}$) showing the distribution of labelling for the given RyR subtype (red) in relation to the nucleus and the plasma membrane, as labelled in (b); (d) 3D representation of deconvolved Z-stack of images (Z step $0.2 \mu\text{m}$) showing the distribution of labelling for α lgp120, the given RyR subtype and the co-localization between the two in relation to the nucleus and the plasma membrane, with areas of co-localization shown in yellow; (e) merged, deconvolved Z-sections showing the distribution of α lgp120 labelling and the given RyR subtype labelling in relation to the nucleus and the plasma membrane, with areas of co-localization shown in yellow; (f) 3D representation showing by colour the spatial distribution of individual volumes of co-localization between α lgp120 and the given RyR subtype. $0.125 \mu\text{m}^3$. in the perinuclear (orange), extra-perinuclear (pink) and sub-plasmalemmal (light blue) regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Figure 6.

Lysosomes form significantly larger areas of co-localization with RyR3 than either RyR2 or RyR1. (A) Bar chart showing the densities (μm^3 of co-localization per μm^3 of volume; mean $\pm$ S.E.M.) of co-localization between αlgp120 and each RyR subtype within the three designated regions of isolated PSMCs. (B) Bar chart showing the mean volumes (μm^3 ; mean $\pm$ S.E.M.) of individual elements of co-localization between αlgp120 and each RyR subtype labelling, $0.125 \mu\text{m}^3$, in the three designated regions of PSMCs. * indicates statistical difference ($P < 0.05$) when compared to co-localization between αlgp120 and RyR3 labelling in the perinuclear region, θ indicates statistical difference ($P < 0.05$) when compared to co-localization between αlgp120 RyR1 labelling in the sub-plasmalemmal region ($n=10$ for $\alpha\text{lgp120/RyR1}$; $n=14$ for $\alpha\text{lgp120/RyR2}$; $n=11$ for $\alpha\text{lgp120/RyR3}$).

Figure 7.

There is a greater degree of correlation with respect to co-localization between RyR3 and lysosomes when compared to that for either RyR1 or RyR2. (A) Bar chart showing the densities (μm^3 of fluorescent labelling per μm^3 of cell volume; mean $\pm$ S.E.M.) for αlgp120 and RyR subtype labelling in isolated PSMCs without regionalisation of the cytoplasmic area. (B) Bar chart showing the mean densities (μm^3 of co-localization per μm^3 of cell volume; mean $\pm$ S.E.M.) of co-localization between αlgp120 labelling and labelling of each of the three RyR subtypes in isolated PSMCs without regionalisation. (C) Record of individual Pearson r values (spread) and the mean $\pm$ S.E.M. determined for the correlation between the spatial distribution of αlgp120 labelling and labelling of each of the RyR subtypes in isolated PSMCs. * indicates statistical difference ($P < 0.05$) when compared to co-localization/correlation between αlgp120 and RyR3 labelling in the perinuclear region ($n = 10$ for $\alpha\text{lgp120}/\text{RyR1}$; $n = 14$ for $\alpha\text{lgp120}/\text{RyR2}$; $n = 11$ for $\alpha\text{lgp120}/\text{RyR3}$).

Figure 8.

Dantrolene inhibits global Ca^{2+} signals in pulmonary arterial smooth muscle cells in response to NAADP. (A) Top panel, series of pseudocolour images of the Fura-2 fluorescence ratio recorded in an isolated PASMC during intracellular dialysis of 10 nM NAADP. The arrow in image 2 indicates a Ca^{2+} burst event preceding the global Ca^{2+} wave. Bottom panel, record of the Fura-2 fluorescence ratio against time. Note, the loss of a 3 GΩ seal is indicated at the end of the record by three bars. (B) Top panel, series of pseudocolour images of the Fura-2 fluorescence ratio recorded in an isolated PASMC during intracellular dialysis of 10 nM NAADP following preincubation (15 min) with 30 μM dantrolene. The arrow in image 3 indicates the presence of a Ca^{2+} burst event. Bottom panel, record of the Fura-2 fluorescence ratio against time. (For Interpretation of the references to colour In this figure legend, the reader is referred to the web version of the article.)