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CELLULAR NEUROPHYSIOLOGY

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Neuronal endoplasmic reticulum acts as a single functional Ca^{2+} store shared by ryanodine and inositol-1,4,5-trisphosphate receptors as revealed by intra-ER $[Ca^{2+}]$ recordings in single rat sensory neurones

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Abstract We addressed the fundamentally important question of functional continuity of endoplasmic reticulum (ER) Ca^{2+} store in nerve cells. In cultured rat dorsal root ganglion neurones we measured dynamic changes in free Ca^{2+} concentration within the ER lumen ($[Ca^{2+}]_L$) in response to activation of inositol-1,4,5-trisphosphate receptors ($InsP_3Rs$) and ryanodine receptors (RyRs). We found that both receptors co-exist in these neurones and their activation results in Ca^{2+} release from the ER as judged by a decrease in $[Ca^{2+}]_L$. Depletion of Ca^{2+} stores following an inhibition of sarco(endoplasmic)reticulum Ca^{2+}-ATPase by thapsigargin or cyclopiazonic acid completely eliminated Ca^{2+} release via both $InsP_3Rs$ and RyRs. Similarly, when the store was depleted by continuous activation of $InsP_3Rs$, activation of RyRs (by caffeine or 0.5 μM ryanodine) failed to produce Ca^{2+} release, and vice versa, when the stores were depleted by activators of RyRs, the $InsP_3$-induced Ca^{2+} release disappeared. We conclude that in mammalian neurones $InsP_3Rs$ and RyRs share the common continuous Ca^{2+} pool associated with ER.

Keywords Calcium signalling · $InsP_3R$/RyR · Endoplasmic reticulum calcium stores · Sensory neurones

Introduction

The endoplasmic reticulum (ER), represented by a three-dimensional intracellular network of tubules and cisternae, serves as an integrating signalling organelle, which co-ordinates fast physiological Ca^{2+} signalling and long-lasting adaptive responses controlled by post-translational protein processing within the ER lumen [2, 4, 6, 9, 14]. Many ER-resident chaperones responsible for correct folding of proteins are regulated by the concentration of free Ca^{2+} within the ER lumen ($[Ca^{2+}]_L$), and therefore the latter bestows the link between fast physiological events and protein turnover [28, 51]. Fluctuations of $[Ca^{2+}]_L$ are determined by the balance between Ca^{2+} release and Ca^{2+} uptake.

Two types of ligand-gated Ca^{2+} channels, the inositol-1,4,5-trisphosphate receptors ($InsP_3Rs$) and ryanodine receptors (RyRs), provide the route for Ca^{2+} release and underlie the excitability of the ER membrane [3, 32, 36, 47]. Both types of Ca^{2+} release channels are abundantly expressed in nerve cells [50, 52], although their intracellular distribution shows considerable heterogeneity. That is, dendritic spines of Purkinje neurones are rich in $InsP_3Rs$ but are devoid of RyRs, although the latter are found in quantity in the dendritic shafts and in the cell body [27, 37]. In contrast, RyRs are predominant in dendrites of CA1 hippocampal neurones [38]; they are also preferentially expressed in axons and synaptic terminals of cerebellar basket neurones [25]. In agreement with such peculiar distribution, different types of Ca^{2+} release are activated upon physiological stimulation in distinct neuronal sub-compartments. Stimulation of synaptic inputs triggers $InsP_3$-mediated Ca^{2+} release in the spines of Purkinje cells [13, 46], whereas Ca^{2+}-induced Ca^{2+} release generated through RyRs plays an important role in postsynaptic Ca^{2+} signalling in hippocampus ([1, 12] but see [24]) and controls multivesicular neurotransmitter release in cerebellar synaptic terminals [25].

Therefore heterogeneous localization of Ca^{2+} release channels provides for a spatial control of Ca^{2+} signals, which is particularly important for highly polarized nerve cells. Yet, such a heterogeneity does not implicitly entail the existence of separate Ca^{2+} pools associated with different Ca^{2+} release mechanisms. This particular issue recently became a matter of controversy [5, 34].

Experiments on neuronal preparations have shown that depletion of RyR-sensitive Ca^{2+} store in Purkinje neurones completely abolished responses to photoreleased

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$InsP_3$ suggesting that both receptors share the same interconnected Ca^{2+} pool [21]. Similar overlap between $InsP_3$-sensitive and caffeine-sensitive Ca^{2+} pools was suggested for hippocampal [19], cerebellar granule [20, 39] and cultured myenteric [23] neurones. In contrast $[Ca^{2+}]_i$ imaging in adrenal chromaffin cells revealed two distinct Ca^{2+} pools sensitive to caffeine and $InsP_3$ respectively [8]. Finally the existence of separate Ca^{2+} pools in nerve cells was recently suggested by Blaustein and Golovina [5], who based their theory on a direct measurements of $[Ca^{2+}]_L$ in astrocytes and atrial myocytes [15, 16, 17]. In the present paper we addressed the question of internal continuity of the ER Ca^{2+} pools in mammalian sensory neurones using direct monitoring of $[Ca^{2+}]_L$. Our evidence suggests that in this preparation the $InsP_3$Rs and RyRs share the same functional Ca^{2+} pool.

Materials and methods

Real-time imaging of Ca2+ concentration in the store

Dorsal root ganglion neurones were enzymatically isolated from new-born (1–3 days old) Sprague-Dawley rats using a conventional treatment with 0.1% protease (type XIV) in HEPES-buffered MEM for 8 min at 37°C. Individual cells were separated mechanically and plated on poly-L-ornitine (1 mg/ml) and laminin (0.01 mg/ml) covered glass coverslips. Neurones were maintained in culture media (DMEM, supplemented with 10% horse serum, 50 U/ml penicillin/streptomycin mixture and 6 μg/ml insulin) at 37°C in an atmosphere of air supplemented with 5% CO_2 for 1–2 days prior to the experiment. In the present study we investigated only large (proprioceptive) neurones with somas larger than 35 μm in diameter.

For $[Ca^{2+}]_L$ recordings we have used Mag-Fura-2 (K_D~50 μM) suitable for detecting high intraluminal $[Ca^{2+}]$ levels [7, 31, 42]. The neurones were incubated with 5 μM Mag-Fura-2 for 30 min at 37°C, and washed at 37°C for 1 h prior to the experiment (loading at 37°C promotes dye compartmentalization within the ER lumen [49]).

The cytoplasmic portion of dye was removed either via intracellular dialysis through the patch pipette (as described in our previous paper [43]) or by permeabilization of the plasmalemma with saponin [41, 42]. In the latter case the cellular membrane was permeabilized by brief (7–10 s) application of saponin (0.001%) in "intracellular" solution. The permeabilization technique was used exclusively for probing the neurones for $InsP_3$-induced Ca^{2+} release as $InsP_3$ does not penetrate through intact plasmalemma.

Calibration of Mag-Fura-2 signals

The $[Ca^{2+}]_L$ values were calculated using the 340/380 nm ratio with the equation $[Ca^{2+}]_L = K^*(R-R_{min})/(R_{max}-R)$. R_{min}, R_{max} and K^* were determined using exposure of Mag-Fura-2/AM loaded intact neurones or saponin-permeabilized neurones to 20 μM ionomycin and four calibrating solutions with $[Ca^{2+}]$<10 nM (10 mM EGTA); 100 μM; 400 μM and 10 mM; solutions were prepared as described previously [43]. The calibration procedure on permeabilized cells consistently yielded higher (~40%) values for K*, most likely due to a higher Mag-Fura-2 K_D within the ER lumen. As we assumed this being more accurate, the values from the latter procedure were used throughout. Values of R_{min}, R_{max} and K^* were 0.3, 1.9 and 287 μM respectively.

Real-time video-imaging

Fluorescence images were captured using an Olympus IX70 inverted microscope (40× UV objective) equipped with a charge-coupled device (CCD) cooled intensified camera (Pentamax Gene IV, Roper Scientific, UK). The specimen was alternately illuminated at 340, 380 and 488 nm by a monochromator (Polychrom IV, TILL Photonics, Germany) at a cycle frequency 0.5–5 Hz. Control over the experiment, image storage and off-line analysis was performed by use of MetaFluor/MetaMorph software (Universal Imaging Corporation, USA) running on a Windows 98 workstation.

Electrophysiology and solution exchange

Whole-cell recordings were made by using EPC-9 amplifier run by the PC-based PULS software (both from HEKA, Germany). The pipette resistance was 3–5 MΩ. All solutions were applied using a fast local superfusion technique [53] which ensured complete exchange of the milieu surrounding the cell within 100 ms.

Solutions and reagents

The extracellular bathing solution contained (in mM): NaCl 135, KCl 3, $CaCl_2$ 2, glucose 20, HEPES/NaOH 20, pH 7.4. The Ca^{2+}-free solution contained 5 mM EGTA with no $CaCl_2$ added. The "intracellular" solution used in permeabilization experiments contained (in mM): KCl 140, Na_2ATP 3, $MgCl_2$ 2, $CaCl_2$ 0.4, BAPTA 5, HEPES/KOH 20, pH 7.2, free Ca^{2+} concentration ~70 nM). The intra-pipette solution used for intracellular dialysis contained (in mM): $CsCl_2$ 122, TEA-Cl 20; Na_2ATP 3, HEPES/CsOH 10, EGTA 0.1, pH 7.3. All reagents were purchased from Sigma (Dorset, UK), and fluorescent probes were obtained from Molecular Probes (Ore., USA).

Results

Sensory neurones co-express functional $InsP_3$Rs and RyRs

We monitored intraluminal Ca^{2+} dynamics in single sensory neurones using low-affinity Ca^{2+} probe Mag-Fura-2 compartmentalized within the ER lumen. The cytosolic portion of the dye was removed either by intracellular dialysis under whole-cell patch-clamp configuration [43], or by permeabilization of the plasmalemma by brief application of saponin [41, 42]. We used the latter technique to permit direct activation of $InsP_3$Rs by $InsP_3$, which otherwise cannot penetrate through the cell membrane.

The resting $[Ca^{2+}]_L$ determined with both techniques varied between 100 and 400 μM. Since $[Ca^{2+}]_L$ is an important determinant of the velocity and magnitude of Ca^{2+} release [43] we restricted our analysis to neurones with $[Ca^{2+}]_L$ higher than ~300 μM.

Brief extracellular applications of both $InsP_3$ (3–10 μM; 10 s) and caffeine (20 mM, 5 s) to permeabilized neurones triggered transient fall in $[Ca^{2+}]_L$, which recovered to the pre-stimulated level after washout (Fig. 1). We found these responses in all neurones subjected to such an application protocol (n=12). The $InsP_3$-induced $[Ca^{2+}]_L$ decrease was substantially slower as compared to that induced by caffeine: on average maximal velocity of

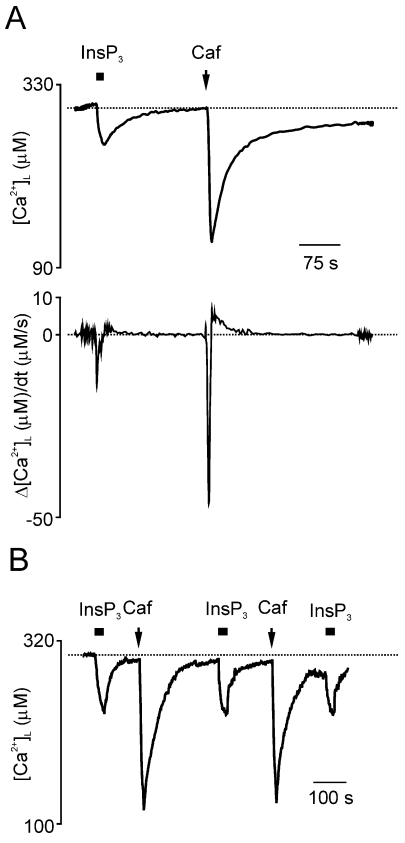

Fig. 1A, B $InsP_3Rs$ and RyRs coexist in DRG neurones. **A** The endoplasmic reticulum lumen $[Ca^{2+}]$ ($[Ca^{2+}]_L$) recording (*top trace*) and its first derivative (*bottom trace*) obtained from permeabilized DRG neurone pre-loaded with Mag-Fura-2/AM. The neurone was challenged by 10 μM $InsP_3$ and 20 mM caffeine as indicated on the graph. **B** The $[Ca^{2+}]_L$ recordings from another permeabilized DRG neurone alternately treated with 10 μM inositol-1,4,5-trisphosphate ($InsP_3$) and 20 mM caffeine as indicated on the graph

$[Ca^{2+}]_L$ decrease induced by $InsP_3$ was about three times lower as compared with $[Ca^{2+}]_L$ decrease induced by caffeine (12±6 μM/s versus 41±10 μM/s, respectively; n=12, see also Fig. 3). Quite obviously the depletion of ER store by $InsP_3$ required much more time as compared with caffeine (see for example Fig. 5). Such a difference may result from either lower density of $InsP_3Rs$ or from denial of Ca^{2+}-induced potentiation of IICR in heavily buffered "intracellular" solution in permeabilized experiments.

Both agents, caffeine and $InsP_3$, triggered $[Ca^{2+}]_L$ decrease in a dose-dependent fashion (Fig. 2). The responses to $InsP_3$ saturated at 10 μM, i.e. at a rather high concentration, which might reflect either hampered diffusion of $InsP_3$ towards ER membrane even in permeabilized preparation, or generally lower sensitivity of neuronal $InsP_3Rs$ to $InsP_3$ [22]. Responses to caffeine saturated at concentrations higher than 10 mM. Therefore, these experiments demonstrate that ER membrane in DRG neurones possesses both $InsP_3$-induced and Ca^{2+}-induced Ca^{2+}-release mechanisms (IICR and CICR, respectively).

Inhibition of SERCA pumps and direct activation of RyR deplete the Ca^{2+} store

The ability of the ER Ca^{2+} store to generate Ca^{2+} signals is regulated by intraluminal free Ca^{2+} concentration, so that store depletion prevents development of Ca^{2+} release. We have already demonstrated that the store replenishment following CICR (induced either by caffeine or by Ca^{2+} entry) is determined by thapsigargin (TG)-sensitive sarco(endoplasmic)reticulum Ca^{2+}-ATPase (SERCA) pumps [43]. Here we investigated the mechanisms of

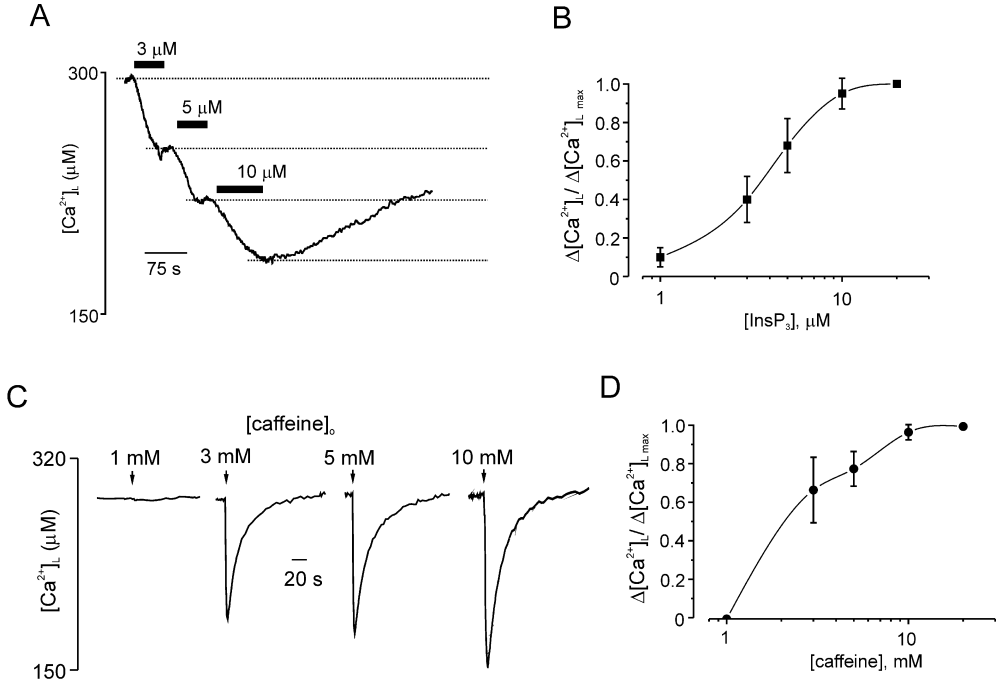

Fig. 2A–D Dose-dependence of $InsP_3$-induced and caffeine-induced Ca^{2+} release in DRG neurones. **A** The changes in $[Ca^{2+}]_L$ measured from permeabilized DRG neurone in response to applications of increasing concentrations of $InsP_3$. **B** The concentration dependence of $InsP_3$-induced decrease in $[Ca^{2+}]_L$. The data are mean±SD from 5 cells. **C** The changes in $[Ca^{2+}]_L$ measured from dialysed DRG neurone in response to applications of increasing concentrations of caffeine. **D** The concentration dependence of caffeine-induced decrease in $[Ca^{2+}]_L$. The data are mean±SD from 5 cells

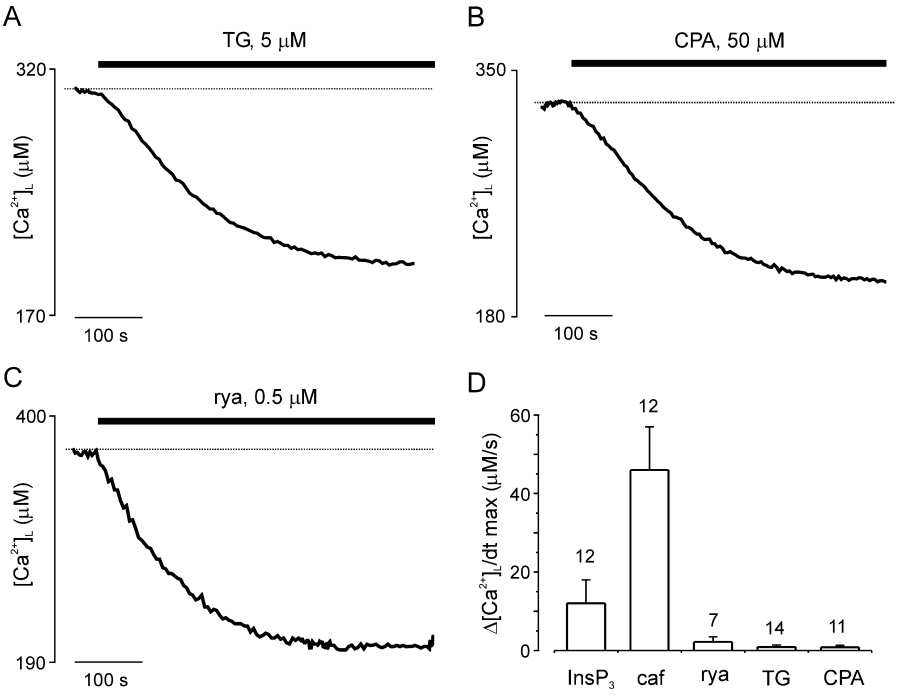

Fig. 3A–D Activation of RyRs and inhibition of SERCA pumps deplete the Ca^{2+} store. **A–C** Examples of $[Ca^{2+}]_L$ recordings from Mag-Fura-2 pre-loaded dialysed DRG neurones in response to extracellular application of thapsigargin (*TG*), cyclopiazonic acid (*CPA*) and ryanodine (*rya*). **D** Average values (mean±SD, *n* is indicated on the graph) of maximal rate of $[Ca^{2+}]_L$ decrease induced by 10 μM $InsP_3$, 20 mM caffeine, 0.5 μM ryanodine, 5 μM TG and 50 μM CPA

store depletion in greater detail. Conceptually the stores can be depleted either by inhibition of Ca^{2+} uptake, which leaves the resting Ca^{2+} leakage unopposed, or by stimulation of Ca^{2+} release channels. In DRG neurones the inhibition of SERCA pumps by both TG (5 μM; *n*=14) and cyclopiazonic acid (CPA, 50 μM; *n*=11) triggered a progressive decrease in $[Ca^{2+}]_L$ which stabilized after complete depletion of the store (Fig. 3A, B). Alternatively the stores can be depleted by activation of the Ca^{2+}-release route. For this purpose we incubated the neurones with a low (0.5 μM) concentration of ryanodine. At this concentration the latter is known to promote opening of RyRs either by direct interaction with gating mechanisms [40] or by a dramatic (~1,000-fold) increase in RyRs sensitivity to cytosolic Ca^{2+} [26]. As shown in Fig. 3C, application of 0.5 μM of ryanodine effectively decreased $[Ca^{2+}]_L$, thus indicating the depletion of the ER store. The maximal velocities of $[Ca^{2+}]_L$ decrease initiated by various pharmacological agents (caffeine, $InsP_3$, ryanodine, TG and CPA) are compared in Fig. 3D.

RyRs and $InsP_3$Rs share the common pool sensitive to TG and CPA

The experiments described above have evidently demonstrated that the ER in DRG neurones is endowed with functional $InsP_3$Rs, RyRs as well as with TG and CPA-sensitive SERCA pumps. These data enabled us to address the central question of this study, i.e. whether all these mechanisms operate within a single Ca^{2+} pool or the ER Ca^{2+} store is represented by several independent compartments. For this purpose we utilized the ability of the agents described above to deplete the ER store. After Ca^{2+} pool was depleted by one of these agents, we tested the ability of others to initiate a further decrease in $[Ca^{2+}]_L$. The appearance of such a decrease would indicate the coexistence of separate Ca^{2+} pools.

First we tested the ability of TG and CPA to deplete the caffeine-sensitive Ca^{2+} pool. As shown in Fig. 4A, dialysed neurones were initially challenged with caffeine to probe for the existence of a caffeine-sensitive pool. Subsequently the neurones were incubated with 5 μM TG which resulted in a decrease in $[Ca^{2+}]_L$. After complete depletion of the pool (as was judged by stabilization of $[Ca^{2+}]_L$ in the presence of TG) the cells were challenged with 20 mM caffeine and 50 μM CPA. In all nine neurones exposed to such a protocol neither caffeine nor CPA were able to affect $[Ca^{2+}]_L$ after the stores were depleted by TG. Similar results were obtained when Ca^{2+} pool was initially depleted by 50 μM CPA: both caffeine and TG applied in the presence of CPA failed to affect $[Ca^{2+}]_L$ (Fig. 4B, *n*=8). Likewise, depletion of stores with 0.5 μM ryanodine rendered caffeine, CPA and TG, applied in the presence of ryanodine, totally ineffective (*n*=7; Fig. 4C, D).

After completion of these experiments we switched to permeabilized neurones, thus gaining the possibility to test for $InsP_3$-induced Ca^{2+} release. The cells were initially incubated with 10 μM $InsP_3$, which resulted in a drop in $[Ca^{2+}]_L$. The $[Ca^{2+}]_L$ stabilized at a steady-state level after complete depletion of the $InsP_3$-sensitive pool. Application of caffeine performed at this moment failed to further affect $[Ca^{2+}]_L$ (Fig. 5A, *n*=7). Vice versa, when the stores were depleted in the presence of 20 mM of caffeine, application of 10 μM $InsP_3$ did not induce any changes in $[Ca^{2+}]_L$ (Fig. 5B, *n*=6). Despite the clarity of the traces resulting from the protocols described above,

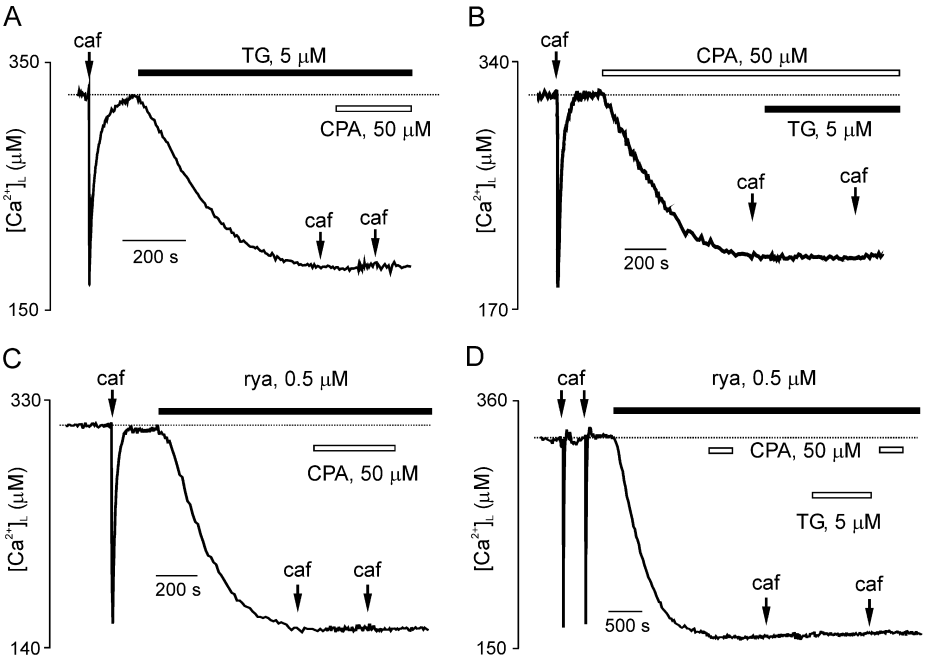

Fig. 4A–D Ryanodine- and caffeine-releasable Ca^{2+} pool is sensitive to sarco(endoplasmic)reticulum Ca^{2+}-ATPase (SERCA) inhibition by TG and CPA. All traces represent $[Ca^{2+}]_L$ recordings from Mag-Fura-2 pre-loaded dialysed DRG neurones. **A** $[Ca^{2+}]_L$ recording in response to applications of 20 mM caffeine, TG and CPA. Depletion of Ca^{2+} store by TG completely eliminates responses to caffeine and CPA. **B** Similarly to **A** depletion of Ca^{2+} store by CPA prevents $[Ca^{2+}]_L$ responses to 20 mM caffeine and to TG. **C**, **D** Activation of RyRs by 0.5 μM ryanodine depletes Ca^{2+} store and abolishes $[Ca^{2+}]_L$ responses to CPA, TG and 20 mM caffeine

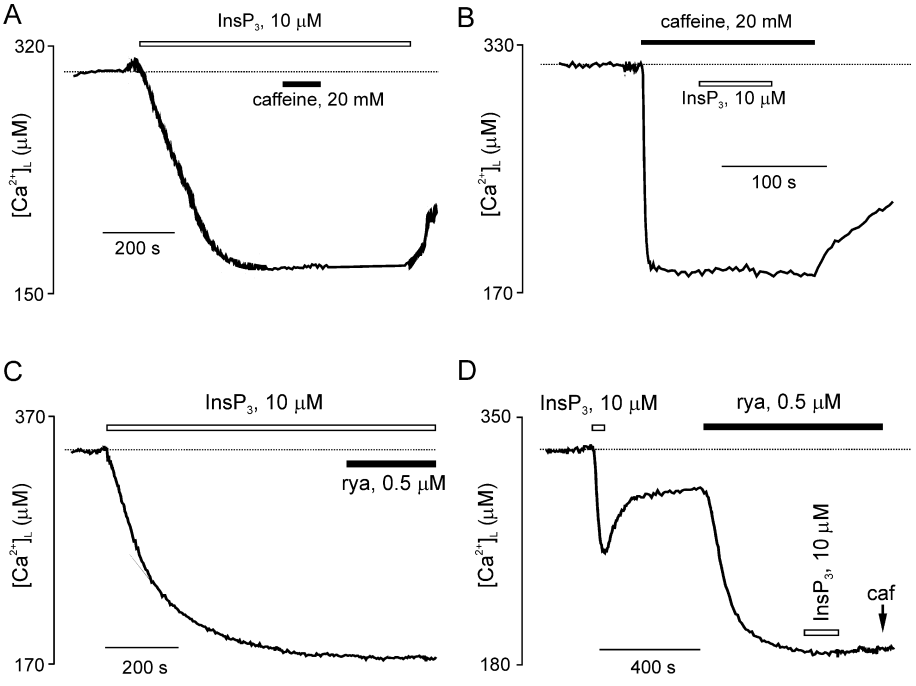

Fig. 5A–D $InsP_3$Rs and RyRs share a common Ca^{2+} pool in sensory neurones. All traces represent $[Ca^{2+}]_L$ recordings from Mag-Fura-2 pre-loaded permeabilized DRG neurones. **A** Depletion of Ca^{2+} store by incubation with 10 μM $InsP_3$ completely abolishes $[Ca^{2+}]_L$ response to 20 mM caffeine. **B** Similarly, depletion of the Ca^{2+} store in continuous presence of caffeine eliminates the $InsP_3$-induced Ca^{2+} release. **C** Depletion of Ca^{2+} store by incubation with 10 μM $InsP_3$ completely abolishes $[Ca^{2+}]_L$ response to ryanodine. **D** When the Ca^{2+} store was depleted by 0.5 μM ryanodine neither $InsP_3$ nor caffeine (20 mM) were able to produce further Ca^{2+} release

doubt remained, as caffeine is known as an effective inhibitor of $InsP_3$Rs [11, 33, 54]. Therefore, we looked for alternative means to specifically activate RyRs, and used 0.5 μM ryanodine for this purpose. Once more, after the Ca^{2+} pool was depleted by incubation with 10 μM of $InsP_3$, ryanodine was unable to induce any further fluctuations in $[Ca^{2+}]_L$ (Fig. 5C; n=5). Likewise, when the permeabilized neurone was treated with 0.5 μM ryanodine to achieve full exhaustion of the Ca^{2+} pool, neither $InsP_3$ nor caffeine were able to activate any additional Ca^{2+} release (Fig. 5D, n=5).

It has to be noted that SERCA inhibitors, caffeine and $InsP_3$ all decreased $[Ca^{2+}]_L$ to the same, relatively high residual level. As we have demonstrated before [43] application of ionomycin in Ca^{2+}-free extracellular solution following TG and/or CPA treatment reduced $[Ca^{2+}]_L$ to zero (R_{min}). This high residual $[Ca^{2+}]_L$ can reflect a limitation of the method, suggesting that part of the signal comes from an intracellular compartment not connected with the caffeine- or TG-sensitive portion of the ER. Nonetheless, as we already discussed in our previous paper [43] it may also represent an intrinsic property of the ER store, when severe depletion of the later may inhibit further release through Ca^{2+}release/k leakage channels.

Discussion

Here we report the first direct measurements of intraluminal Ca^{2+} dynamics in mammalian neurones during Ca^{2+} release produced by activation of $InsP_3Rs$ and RyRs. We found that these two types of Ca^{2+} release, the IICR and the CICR, not only coexist in sensory neurones but they share a common Ca^{2+} pool. Our suggestion contradicts a recent hypothesis postulating the existence of functionally separate ER Ca^{2+} pools in both excitable and non-excitable cells, including neurones [5]. The idea of separate Ca^{2+} pools was instigated by experiments employing direct imaging of intra-ER Ca^{2+} movements in astrocytes and atrial myocytes [15, 16, 17]. These studies concluded that excitable and non-excitable cells have at least two separate Ca^{2+} pools sensitive to $InsP_3$ and caffeine, respectively. This was based on the finding that inhibition of SERCA pumps by TG and CPA depletes the $InsP_3$-sensitive Ca^{2+} stores and abolishes responses to metabotropic agonists; however, this manipulation does not affect Ca^{2+} release triggered by caffeine. Although this finding explicitly implies the existence of a specific Ca^{2+}-uptake pathway replenishing the caffeine-sensitive pool, the authors of the cited studies failed to hypothesize on it.

The experiments described in this paper do not support the hypothesis of separate Ca^{2+} pools. On the contrary, in a series of direct approaches, we have demonstrated that depletion of Ca^{2+} stores by either opening of RyRs or $InsP_3Rs$ or by SERCA inhibition is always complete, and it precludes any further Ca^{2+} release irrespective of its mechanism. The most direct evidence for a common Ca^{2+} pool was obtained from the protocols shown in Fig. 5. These experiments show that depletion of the Ca^{2+} store due to an activation of RyRs by caffeine or ryanodine completely abolishes $InsP_3$-induced Ca^{2+} release, and vice versa, when the stores are depleted following $InsP_3Rs$ activation, the RyR-mediated Ca^{2+} release is fully blocked.

Our suggestion of the continuity of the neuronal ER Ca^{2+} store is in line with a multitude of morphological evidence which describes neuronal ER as a continuous interconnected network [44]. Furthermore, a direct approach aimed at investigating the continuity of the ER in Purkinje neurones with a lypophylic fluorescent dye travelling exclusively in ER membranes [48] has clearly demonstrated the continuity of the ER. Similarly, a wealth of experimental data obtained in non-neuronal cells favours, to a very large extent, the idea of ER continuity. For instance, relatively large molecules, such as ER-targeted GFP, were reported to rapidly diffuse within the ER luminal space [10, 45]. In addition, a series of refined experiments on pancreatic acinar cells [30] have convincingly demonstrated that (1) fluorescent Ca^{2+} probes can diffuse freely within the ER lumen, and (2) even more importantly that $[Ca^{2+}]_L$ rapidly equilibrates within the ER lumen following local photorelease of caged calcium.

The existence of a continuous Ca^{2+} pool connected through the ER lumen could be very important for neuronal function. First, rapid Ca^{2+} diffusion through the ER "Ca^{2+} tunnels" [29] supports Ca^{2+} release in cell subcompartments by preventing severe store depletion following intensive local stimulation. Second, the same intraluminal Ca^{2+} diffusion may be instrumental in conveying Ca^{2+} signals from distal neuronal processes toward the nucleus [34], as was suggested by recent findings showing the importance of ER Ca^{2+} uptake in nuclear Ca^{2+} signalling [18, 35]. Third, intra-ER Ca^{2+} equilibration could facilitate clearance of local excessive Ca^{2+} loads. Finally, the existence of a continuous ER Ca^{2+} store can be very important in guarding against profound store depletion (which may happen more easily in small, separated Ca^{2+} pools), thus protecting normal functioning of intraluminal chaperones and therefore supporting cell functioning.

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References

1. Alford S, Frenguelli BG, Schofield JG, Collingridge GL (1993) Characterization of Ca^{2+} signals induced in hippocampal CA1 neurones by the synaptic activation of NMDA receptors. J Physiol (Lond) 469:693–716
2. Aridor M, Balch WE (1999) Integration of endoplasmic reticulum signaling in health and disease. Nat Med 5:745–751
3. Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13–26
4. Berridge MJ (2002) The endoplasmic reticulum: a multifunctional signalling organelle. Cell Calcium 32:235–249
5. Blaustein MP, Golovina VA (2001) Structural complexity and functional diversity of endoplasmic reticulum Ca^{2+} stores. Trends Neurosci 24:602–608
6. Bootman MD Petersen OH, Verkhratsky A (2002) The endoplasmic reticulum is a focal point for co-ordination of cellular activity. Cell Calcium 32:231–234
7. Camello C, Lomax R, Petersen OH, Tepikin AV (2002) Calcium leak from intracellular stores-the enigma of calcium signalling. Cell Calcium 32:355–361
8. Cheek TR, Barry VA, Berridge MJ, Missiaen L (1991) Bovine adrenal chromaffin cells contain an inositol 1,4,5-trisphosphate-insensitive but caffeine-sensitive Ca^{2+} store that can be

regulated by intraluminal free Ca^{2+}. Biochem J 275:697–701
9. Corbett EF, Michalak M (2000) Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem Sci 25:307–311
10. Dayel MJ, Hom EF, Verkman AS (1999) Diffusion of green fluorescent protein in the aqueous-phase lumen of endoplasmic reticulum Biophys J 76:2843–2851
11. Ehrilch BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I (1994) The pharmacology of intracellular Ca^{2+} release channels. Trends Pharmacol Sci 15:145–149
12. Emptage N, Bliss TV, Fine A (1999) Single synaptic events evoke NMDA receptor-mediated release of calcium from internal stores in hippocampal dendritic spines. Neuron 22:115–124
13. Finch EA, Augustine GJ (1998) Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396:753–756
14. Glazner G, Fernyhough P (2002) Neuronal survival in the balance: are endoplasmic reticulum membrane proteins the fulcrum? Cell Calcium 32:421–433
15. Golovina VA, Blaustein MP (1997) Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. Science 275:1643–1648
16. Golovina VA, Blaustein MP (2000) Unloading and refilling of two classes of spatially resolved endoplasmic reticulum Ca^{2+} stores in astrocytes. Glia 31:15–28
17. Golovina VA, Bambrick LL, Yarowsky PJ, Krueger BK, Blaustein MP (1996) Modulation of two functionally distinct Ca^{2+} stores in astrocytes: role of the plasmalemma Na/Ca exchanger. Glia 16:296–305
18. Hardingham GE, Arnold FJ, Bading H (2001) Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. Nat Neurosci 4:261–267
19. Imanishi T, Yamanaka H, Rhee JS, Akaike N (1996) Interaction between the intracellular Ca^{2+} stores in rat dissociated hippocampal neurones. Neuroreport 7:1421–1426
20. Irving AJ, Collingridge GL, Schofield JG (1992)L-Glutamate and acetylcholine mobilise Ca^{2+} from the same intracellular pool in cerebellar granule cells using transduction mechanisms with different Ca^{2+} sensitivities. Cell Calcium 13:293–301
21. Khodakhah K, Armstrong CM (1997) Inositol trisphosphate and ryanodine receptors share a common functional Ca^{2+} pool in cerebellar Purkinje neurons. Biophys J 73:3349–3357
22. Khodakhah K, Ogden D (1993) Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurones, cultured cerebellar astrocytes and peripheral tissues. Proc Natl Acad Sci USA 90:4976–4980
23. Kimball BC, Yule DI, Mulholland MW (1996) Caffeine- and ryanodine-sensitive Ca^{2+} stores in cultured guinea pig myenteric neurons. Am J Physiol 270:G594–603
24. Kovalchuk Y, Eilers J, Lisman J, Konnerth A (2000) NMDA receptor-mediated subthreshold Ca(2+) signals in spines of hippocampal neurons. J Neurosci 20:1791–1799
25. Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256–1265
26. Masumiya H, Li P, Zhang L, Chen SR (2001) Ryanodine sensitizes the Ca^{2+} release channel (ryanodine receptor) to Ca^{2+} activation. J Biol Chem 276:39727–39735
27. Meldolesi J (2001) Rapidly exchanging Ca^{2+} stores in neurons: molecular, structural and functional properties. Prog Neurobiol 65:309–338
28. Michalak M, Robert-Parker JM, Opas M (2002) Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium 32:269–278
29. Mogami H, Nakano K, Tepikin AV, Petersen OH (1997) Ca^{2+} flow via tunnels in polarized cells: recharging of apical Ca^{2+} stores by focal Ca^{2+} entry through basal membrane patch. Cell 88:49–55
30. Park MK, Petersen OH, Tepikin AV (2000) The endoplasmic reticulum as one continuous Ca^{2+} pool: visualization of rapid Ca^{2+} movements and equilibration. EMBO J 19:5729–5739
31. Park MK, Tepikin AV, Petersen OH (2002) What can we learn about cell signaling by combining optical imaging and patch clamp techniques? Pflugers Arch 444:305–316
32. Patel S, Joseph SK, Thomas AP (1999) Molecular properties of inositol 1,4,5-trisphosphate receptors. Cell Calcium 25:247–264
33. Petersen OH, Cancela JM (1999) New Ca^{2+}-releasing messengers: are they important in the nervous system? Trends Neurosci 22:488–495
34. Petersen OH, Tepikin A, Park MK (2001) The endoplasmic reticulum: one continuous or several separate Ca^{2+} stores? Trends Neurosci 24:271–276
35. Power JM, Sah P (2002) Nuclear calcium signaling evoked by cholinergic stimulation in hippocampal CA1 pyramidal neurons. J Neurosci 22:3454–3462
36. Rossi D, Sorrentino V (2002) Molecular genetics of ryanodine receptors Ca^{2+} release channels. Cell Calcium 32:307–319
37. Satoh T, Ross CA, Villa A, Supattapone S, Pozzan T, Snyder SH, Meldolesi J (1990) The inositol 1,4,5,-trisphosphate receptor in cerebellar Purkinje cells: quantitative immunogold labeling reveals concentration in an ER subcompartment. J Cell Biol 111:615–624
38. Sharp AH, McPherson PS, Dawson TM, Aoki C, Campbell KP, Snyder SH (1993) Differential immunohistochemical localization of inositol 1,4,5,-trisphosphate- and ryanodine-sensitive Ca^{2+} release channels in rat brain. J Neurosci 13:3051–3063
39. Simpson PB, Nahorski SR, Challiss RA (1996) Agonist-evoked Ca^{2+} mobilization from stores expressing inositol 1,4,5-trisphosphate receptors and ryanodine receptors in cerebellar granule neurones. J Neurochem 67:364–373
40. Sitsapesan R, McGarry SJ, Williams AJ (1995) Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. Trends Pharmacol Sci 16:386–391
41. Solovyova N, Verkhratsky A (2002) Monitoring of free calcium in the neuronal endoplasmic reticulum: an overview of modern approaches. J Neurosci Methods 122:1–12
42. Solovyova N, Fernyhough P, Glazner G, Verkhratsky A (2002) Xestospongin C empties the ER calcium store but does not inhibit InsP(3)-induced Ca^{2+} release in cultured dorsal root ganglia neurones. Cell Calcium 32:49–52
43. Solovyova N, Veselovsky N, Toescu EC, Verkhratsky A (2002) Ca^{2+} dynamics in the lumen of the endoplasmic reticulum in sensory neurones: direct visualisation of Ca^{2+}-induced Ca^{2+} release triggered by physiological Ca^{2+} entry. EMBO J 21:622–630
44. Spacek J, Harris KM (1997) Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J Neurosci 17:190–203
45. Subramanian K, Meyer T (1997) Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. Cell 89:963–971
46. Takechi H, Eilers J, Konnerth A (1998) A new class of synaptic response involving calcium release in dendritic spines. Nature 396:757–760
47. Taylor CW, Laude AJ (2002) IP3 receptors and their regulation by calmodulin and cytosolic Ca^{2+} Cell Calcium 32:321–334
48. Terasaki M, Slater NT, Fein A, Schmidek A, Reese TS (1994) Continuous network of endoplasmic reticulum in cerebellar Purkinje neurons. Proc Natl Acad Sci USA 91:7510–7514
49. Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P (2000) A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. Cell Calcium 28:213–223
50. Verkhratsky A (2002) The endoplasmic reticulum and neuronal calcium signalling. Cell Calcium 32:393–404

51. Verkhratsky A, Petersen OH (2002) The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death. Eur J Pharmacol 447:141–154
52. Verkhratsky A, Shmigol A (1996) Calcium-induced calcium release in neurones. Cell Calcium 19:1–14
53. Veselovsky NS, Engert F, Lux HD (1996) Fast local superfusion technique. Pflugers Arch 432:351–354
54. Wakui M, Osipchuk YV, Petersen OH (1990) Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+} release. Cell 63:1025–1032