The location of InsP3 receptors in Purkinje cells of murine cerebellum does not support a direct interaction in the transfer of calcium ions between ER and mitochondria

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Abstract

The inositol-3-phosphate receptors (IP3Rs) of cerebellar Purkinje cells are located in abundant, large stacks of endoplasmic reticulum (ER) cisternae. Using thin section electron microscopy, we identify very frequent associations of the ER stacks with mitochondria. The associations have two components: a single, close ER-mitochondria contact on one side to the stack, and multiple layers of ER cisternae decorated by IP3Rs receptors on the side away from the mitochondria. Due to their location in the stacks, IP3Rs are never in contact with the mitochondria, although they are in their vicinity. We conclude that transfer of Ca2+ between ER and mitochondria is not directly mediated by IP3Rs, but is based on mitochondrial Ca2+ uptake from the local cytoplasmic spikes during IP3Rs’ activity.

Key Words: IP3 receptors; mitochondria; cerebellum Purkinje cells.

Ca2+ are universal intracellular messengers by virtue of the fact that the ions are segregated within membrane-limited compartments, mostly endo- and sarcoplasmic reticulum, (ER and SR), and are maintained at extremely low levels in the cytoplasm. Ca2+ is released on demand into the cytoplasm through multiple isoforms of high conductance channels that are part of two analogous families: the inositol 1,4,5-trisphosphate receptors (IP3Rs) and the ryanodine receptors (RyRs). IP3Rs and RyRs are intrinsic components of ER and SR membranes, including the cytoplasmic layer of the nuclear envelope. Both RyRs and IP3Rs are present in the majority of cells, but at different levels. For example, skeletal muscle is rich in RyRs and relative poor in IP3Rs, cardiac muscle, is also rich in RyRs, but has a slightly higher level of IP3Rs and most neurons are very rich in IP3Rs and fairly poor in RyRs.

Mitochondrial respiration is activated by a low concentration of Ca2+ in the matrix. It is well established that mitochondria take up a small amount of Ca2+ following its release from ER and SR and that this uptake is very fast after a release. Uptake occurs during the relative short events of E-C coupling and contraction in skeletal and cardiac muscles. Thus mitochondria are involved to some extent in the cell Ca2+ homeostasis, most significantly in cells rich of the organelles.

The functional relationship between ER/SR (the Ca2+ sources), the clusters of RyR and IP3Rs release sites (the release valves) and mitochondria (the Ca2+ sinks) is facilitated by the close proximity between the organelles and their connection by tethers, most obvious in liver cells, and striated muscles, leading to the concept of a mitochondria associated membrane (MAM) system. Although direct molecular exchanges between ER/SR and mitochondrion membranes are demonstrated, this may not involve a direct transfer of Ca2+ mediated by the two types of release channels. Indeed, the RyR Ca2+ release sites in skeletal and cardiac muscles are located in proximity of mitochondria but there is no direct contact between RyRs and the mitochondrial membrane. In the case of IPR3s, the structural evidence is lacking. Due to the relative scarcity of IP3Rs, the exact position of the channels in MAMs in relation to the mitochondrial membrane has not been established by ultrastructure, and a close contact between the channels and the outer mitochondrial has been assumed, but not demonstrated.

Prominent and frequent arrays of ER stacks of Purkinje cells of the cerebellum are a rich source of IP3Rs.

The electron dense particles between the ER membranes of stacks have been clearly identified as IP3Rs on the basis of immunolabeling and ultrastructure.
molecules are arranged in the ER with the cytoplasmic domains extending on the cytoplasmic side of the membranes and are natively organized in semicrystalline arrays with parameters very similar to those of RyRs, despite the difference in size of the two channels (compare Katayama E, et al. 1996; Paolini C, et al.

**Fig 1.** Selection of images from the soma of a rat cerebellar Purkinje cell showing a variety of associations between ER membranes stacks and mitochondria. One to two stacks are closely apposed to the mitochondria and in all cases the distances between the membranes of mitochondrion and of the stack facing it are narrow. Figure 1 A and D are shown at higher magnification in Figures 3 and 2 respectively.

**Fig 2.** A) Two associations between ER stacks and a mitochondrion, a brief and a prologued one. B) In the same image, some of the stack membranes and the lumen of the ER cisternae are colored in green The inner and the outer mitochondrial membranes are in orange. The green-to-orange space is narrow, while the space between facing stack cisternae is wider and occupied by small densities.
Release of caged Ins3P in cultured Purkinje cells results in a robust release of Ca²⁺ into the cytoplasm of both dendrites and soma. The abundance and clustering of Ip3Rs in these cells offers a unique opportunity for exploring the spatial relationship between IP3Rs and the mitochondrial membrane.

Materials and Methods
A single adult rat was euthanized by CO₂ anesthesia. The brain was fixed by perfusion through the carotid artery with 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. After removing from the skull, small sections of the cerebellum were post fixed for 1hr in 2% OsO₄ in the same buffer, stained en-bloc with saturated uranyl acetate for 3 hrs, dehydrated and embedded in Epon. Thin sections were imaged after staining with uranyl acetate and lead solution, as detailed in Lavorato M, Franzini-Armstrong C. (2017).

Results
The murine Purkinje cell soma is occupied by a high density of randomly distributed mitochondria, ER stacks, and mitochondria-associated stacks (Figure 1). While the ER stacks and their ultrastructure have been illustrated in great detail and their content of IP3Rs has been identified, the frequent close positioning of ER stacks and mitochondria in these cells has escaped attention. ER stacks are apposed to mitochondria at a frequency that indicates a specific binding between the two organelles, rather than the random effect of proximity within a limited space. Of 121 total profiles examined, 35% are associations between 1-2 stacks and mitochondria, 25% are stacks only and 40% are mitochondria only.

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**Fig 3.** The average distance between proximal stack membrane (yellow) and mitochondrial membrane (blue) is 8.7 nm. The distance between facing cisternae membranes in the stack (orange markers) is 14.8 nm. The cytoplasmic domains of IP3 receptors do not fit into the narrower gap.
The frequency of associations is likely to be higher than these figures, because many of them are simply not included in the section thickness. The stack-mitochondrion association is quite specific. In all cases a peripheral, smooth surface of the stack faces the mitochondrion and is closely apposed to it, while the rest of the stack, occupied by periodic densities between cisternae, earlier identified as IP3Rs, is on the opposite side. Coloring of the stacks’ membranes and the lumen of each cisterna (Figure 2) is helpful in identifying the various components. The difference between the surfaces of ER cisternae closely opposed to mitochondria and those opposed to each other in the stacks is immediately apparent in a comparison between the stack-to-mitochondrion distance (between yellow blue line) and inter-cisternae distance (yellow to yellow line, orange markers) in Figure 3. For accuracy, the distances were measured between the translucent central membrane layers and adjusted by subtracting the membrane thickness. The mean stack-mitochondrion intermembrane distance is 8.7 +/- 2.4 nm (n=22 measurements), while the distance been cisternae membranes is 14.8 +/- 2.2 nm (n=20). Inter membrane distances in conventional electron micrographs are affected by artifacts during fixation and dehydration that result in shrinkages and in wrinkling of the membrane profiles. For that reason the measured values are not absolute. Despite these reservations, it is clear that the space between the cisternae occupied by evenly spaced densities is wider and most likely protected from shrinkage by the very presence of the IP3Rs. The former space is wide enough to accommodate the cytoplasmic domains of a single IP3Rs layer with the channel domain contained within the membrane, as in Baker MR, Fan G, Serysheva I. (2017). By contrast, the stack-to-mitochondrion gap is too narrow to allow space for IP3Rs (Figure 4).
Discussion
The association of Ip3R-rich ER stacks with mitochondria in cerebellar Purkinje cells is a striking example of mitochondria-associated ER membrane MAM.17 It is logical to assume that a tethering association exists between stacks and mitochondria such as illustrated for other ER/SR-mitochondria doublets.13,14,24 Within this association we can distinguish two components. The stacks’ membrane facing the mitochondrion is separated from it by a narrow gap. These sites fall into the category of “contacts”, where the membrane proximity is of the type that permits direct communication between membrane components.13,14,25,26
Specific tethering of the SR stacks to the mitochondrion is suggested by the presence of small connecting structures and more significantly demonstrated by the frequency of stereospecific associations between mitochondria and SR. The intermembrane distance at the contact sites is too narrow to accommodate the cytoplasmic domains of IP3Rs, so the channels are excluded from the contact sites. The size of the ER-mitochondria gap in other cases of close associations has not been systematically studied in the literature, but in general it seems to be narrow. The cytoplasmic domains of IP3Rs are located in the wide inter-lamellar spaces of mitochondria associated stacks and are thus at some, if small, distance from the mitochondrion. Under these conditions, uptake of Ca2+ by the mitochondrion takes advantage of the relatively large, localized and brief increase in cytoplasmic Ca2+ created by the release from ER. A small portion of the Ca2+ liberated into this microdomain site is rapidly and effectively taken up by the mitochondria located within a short distance from the release sites.27-30 Reuptake of Ca2+, by the Ca2+ ATPase located in the stacks,1,31 reduces the duration the Ca2+ spikes, thus providing the basis for short oscillatory responses that are followed by the mitochondrion.9
The frequency of IP3R-rich stacks in Purkinje cells indicates requirement for a rapid, large Ca2+ release in response to IP3R activation (e.g., see Gomez LC, et al. 2020.)32 and the frequent stack-mito associations suggest that the mitochondria, despite their limited Ca2+ uptake, are designed to mitigate the release effect perhaps by reducing the Ca2+ transient duration. The relevance of location to the effect of mitochondria in Ca2+ distribution is beautifully illustrated by the example of exocrine cells in the pancreas.33
The concept of exchange of Ca2+ between ER/SR and mitochondria requiring proximity, but not an actual positioning of the Ca2+ release channels at the contact sites, was first emphasized, in the case of RyRs, for cardiac muscle,15 and is true for all varieties of skeletal muscles, e.g., see Franzini-Armstrong C, et al. (2011).18 In that respect cerebellar stacks and muscles dyads are analogous. It is not clear whether the IP3R exclusion from all mitochondrial contact sites detected in Purkinje cells applies to other extensive ER-mitochondria associations, such as those in liver cells. The relative scarcity of IP3Rs at these sites makes it basically impossible to reliably identify the IP3R channels in electron micrographs and the question remains open to further inquiry.

List of acronyms
E-C coupling - Excitation-Contraction coupling
IP3R - inositol 3 phosphate receptor
MAM - mitochondria associated membrane
RyR - ryanodine receptor

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RI analyzed data and wrote manuscript. CF-A performed electron microscopy and wrote manuscript. Both Authors approved the final typescript.

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