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The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death

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Abstract

The endoplasmic reticulum is one of the largest intracellular organelles represented by continuous network of cisternae and tubules, which occupies the substantial part of neuronal somatas and extends into finest neuronal processes. The endoplasmic reticulum controls protein synthesis as well as their post-translational processing, and generates variety of nucleus-targeted signals through Ca^{2+}-binding chaperones. The normal functioning of the endoplasmic reticulum signalling cascades requires high concentrations of free calcium ions within the endoplasmic reticulum lumen ($[Ca^{2+}]_L$), and severe alterations in $[Ca^{2+}]_L$ trigger endoplasmic reticulum stress response, manifested by either unfolded protein response (UPR) or endoplasmic reticulum overload response (EOR). At the same time, the endoplasmic reticulum is critically involved in fast neuronal signalling, by producing local or global cytosolic calcium signals via Ca^{2+}-induced Ca^{2+} release (CICR) or inositol-1,4,5-trisphosphate-induced Ca^{2+} release (IICR). Both CICR and IICR are important for synaptic transmission and synaptic plasticity. Several special techniques allowing real-time $[Ca^{2+}]_L$ monitoring were developed recently. Video-imaging of $[Ca^{2+}]_L$ in neurones demonstrates that physiological signalling triggers minor decreases in overall intraluminal Ca^{2+} concentration due to strong activation of Ca^{2+} uptake, which prevents severe $[Ca^{2+}]_L$ alterations. The endoplasmic reticulum lumen also serves as a "tunnel" which allows rapid transport of Ca^{2+} ions within highly polarised nerve cells. Fluctuations of intraluminal free Ca^{2+} concentration represent a universal mechanism, which integrates physiological cellular signalling with protein synthesis and processing. In pathological conditions, fluctuations in $[Ca^{2+}]_L$ may initiate either adaptive or fatal stress responses.

Keywords: Endoplasmic reticulum; Ca^{2+} signalling; Ca^{2+} binding protein; Unfolded protein response; Endoplasmic reticulum overload response; Neuronal survival; Chaperone

1. Introduction

The endoplasmic reticulum is an intracellular organelle of fundamental importance present in all types of eucariotic cells. The endoplasmic reticulum is most likely the largest organelle, with endomembrane accounting for more than 50% of all the cellular membranes, and occupies a substantial part (>10%) of the cell volume. The endoplasmic reticulum lumen is densely packed with numerous enzymatic systems that allow protein synthesis in the rough endoplasmic reticulum and correct post-translational "folding" of these proteins. Any malfunctions in the latter process result in accumulation of unfolded proteins, which in turn activates several signalling systems aimed at appropriate compensatory responses. At the same time, the endoplasmic reticulum is recognised as an important component of a different signalling system, which is the cytosolic calcium signalling cascade. Within a framework of this cascade, the endoplasmic reticulum serves as a rapidly exchanging Ca^{2+} store, able to release Ca^{2+} ions upon appropriate physiological stimulation. In order for the endoplasmic reticulum to work as a dynamic Ca^{2+} store, a high concentration of free Ca^{2+} has to be maintained within its lumen, where $[Ca^{2+}]$ varies between 0.2 and 2 mM. Simultaneously, high intraluminal free Ca^{2+} concentration appears to be a key factor determining the activity of synthesis and processing of proteins within the endoplasmic reticulum, and disruption of endoplasmic reticulum Ca^{2+} homeostasis triggers endoplasmic reticulum stress response.

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When deficits of endoplasmic reticulum Ca^{2+} handling are severe and persisting, the endoplasmic reticulum becomes a source of cell death signals.

The Ca^{2+} ion emerges as a messenger molecule which integrates various signals within the endoplasmic reticulum: the fluctuations of $[Ca^{2+}]_L$ induced by signals originating at the level of the plasmalemma (i.e. Ca^{2+} entry or activation of metabotropic receptors) regulate in turn protein synthesis and processing via generating secondary signalling events between the endoplasmic reticulum and the nucleus. This view of the endoplasmic reticulum as a complex and universal signalling organelle is yet in statu nascendi (see e.g. Corbett and Michalak, 2000; Paschen and Frandsen, 2001); in this essay we shall focus on endoplasmic reticulum signalling functions in neurones. Several abbreviations used throughout the text are worth deciphering at this stage. The concentration of free Ca^{2+} within two compartments, the cytosol and the endoplasmic reticulum are abbreviated as $[Ca^{2+}]_i$ and $[Ca^{2+}]_L$, respectively; SERCA stands for Sarco(Endo)plasmic Reticulum Calcium ATPase, $InsP_3$ for inositol-1,4,5-trisphosphate and *grp* stands for glucose-regulated protein.

2. The endoplasmic reticulum Ca^{2+} store in neurones

Our knowledge about the distribution and the morphological properties of the endoplasmic reticulum in neurones is surprisingly limited, e.g. we do not have data about such an important parameter as the fraction of the cell volume occupied by the endoplasmic reticulum. Yet, based on electron microscopical studies and video-imaging using endoplasmic reticulum-specific markers (Fig. 1), we can define the neuronal endoplasmic reticulum as an extended system, which occupies both soma and processes of the nerve cells. At the ultrastructural level, the endoplasmic reticulum is represented by a complex network of tubules and cisternae (Martone et al., 1993; Meldolesi, 2001; Meldolesi and Grohovaz, 2001; Spacek and Harris, 1997). Importantly, the endoplasmic reticulum system protrudes into both axons and dendrites, being morphologically identified in synaptic terminals (Broadwell and Cataldo, 1983; Lindsey and Ellisman, 1985) as well as in postsynaptic densities.

2.1. Endoplasmic reticulum Ca^{2+} handling: pumps, Ca^{2+} buffers and Ca^{2+} channels

Maintenance of the steep concentration gradient between the endoplasmic reticulum lumen and the cytosol arises from energy-dependent Ca^{2+} pumping by ATPases of the SERCA family. Two types of SERCA pumps, the 2b and 3, are expressed in central neurones, the identity of endoplasmic reticulum Ca^{2+} pumps in peripheral nerve cells remains unknown (Meldolesi, 2001). An important feature of the SERCA pumps is the steep dependence of the Ca^{2+} transport rate on $[Ca^{2+}]_L$: a reduction in the luminal Ca^{2+}

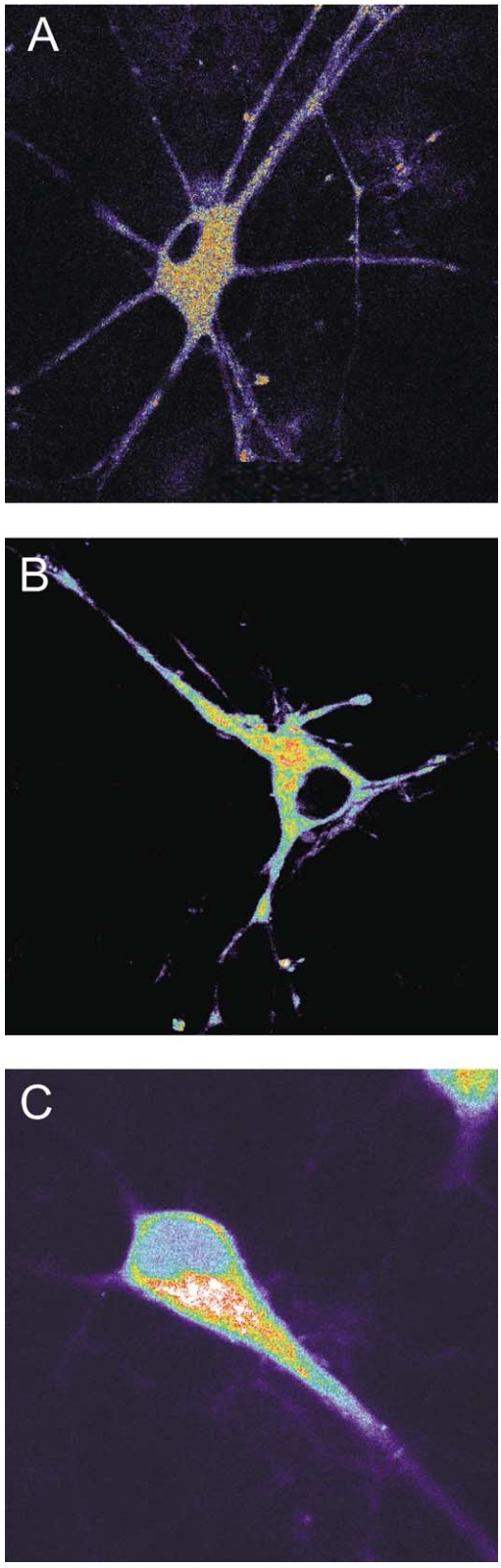

Fig. 1. Visualisation of endoplasmic reticulum calcium store in central nervous system neurones. The confocal images represent hippocampal cultured neurones stained with fluorescent thapsigargin (A), fluorescent ryanodine (B) and low-affinity Ca^{2+} indicator, Fluo-2FF (C).

concentration profoundly increases the activity of Ca^{2+} uptake. This regulation, at least in part, is mediated by calreticulin, which controls SERCA from the luminal side (John et al., 1998). The relationship between $[Ca^{2+}]_L$ and the rate of SERCA-mediated Ca^{2+} uptake was determined directly in mammalian sensory neurones (Solovyova et al., 2002). The emptying of the stores by caffeine resulted in a 4-fold increase in uptake rate, from 1.5 μM/s at rest to 6–7 μM/s when the stores were maximally depleted.

The main feature of the intra-endoplasmic reticulum Ca^{2+} binding proteins is their low Ca^{2+} affinity (in the range of 1 mM) which allows high resting $[Ca^{2+}]_L$ values. The overall concentration of Ca^{2+} binding proteins in the endoplasmic reticulum lumen is very high (in the mM range) hence determining the high capacity of intra-endoplasmic reticulum Ca^{2+} binding. The most important luminal Ca^{2+} binding protein in neurones is calreticulin, which has 20–50 low-affinity (K_d about 1 mM) Ca^{2+} binding sites. Notably, calreticulin is an important chaperone, together with two other endoplasmic reticulum Ca^{2+} buffers the binding protein (BiP or *grp78*), and endoplasmin (*grp94*). In addition, intra-endoplasmic reticulum Ca^{2+} binding is executed by numerous proteins of the CREC family (reticulocalbin, calumenin, Cab55, etc.; Honore and Vorum, 2000) with K_d's in a range of 0.1–1 mM.

The ability of the endoplasmic reticulum to release calcium in response to physiological stimulation is determined by the existence of specific Ca^{2+} release channels residing in the endoplasmic reticulum membrane. Three major types of such channels have been described so far; these are ryanodine and $InsP_3$ receptors (RyR and $InsP_3R$) and receptors activated by NAADP (Fig. 2). The molecular and biophysical properties of the Ca^{2+} release channels are subjects of numerous reviews (see e.g. Patel et al., 1999, 2001; Sitsapesan et al., 1995; Sutko and Airey, 1996; Thrower et al., 2001; Williams et al., 2001) and will not be discussed here in details. As far as neurones are concerned, expression of both RyRs and $InsP_3Rs$ has been demonstrated in CNS as well as in PNS (Meldolesi, 2001). The NAADPR has been functionally demonstrated in brain microsomes (Bak et al., 1999), but its importance in neuronal calcium signalling remains to be explored. In pancreatic acinar cells, a clear role for this receptor in the Ca^{2+} signalling response elicited by the hormone and neuropeptide cholecystokinin has been established. Furthermore, important functional interactions between $InsP_3$, cyclic-ADP-ribose and NAADP receptors have been demonstrated (Cancela et al., 2000, 2002). The density and subcellular distribution of RyRs and $InsP_3Rs$ varies profoundly between different types of neurones. RyRs are predominantly concentrated within axons and presynaptic terminals in cerebellar basket cells (Llano et al., 2000), whereas in hippocampal CA1 pyramidal neurones RyRs are concentrated in dendrites and postsynaptic zones (Martone et al., 1997). A high density of RyRs is found in somatas of both peripheral (Solovyova et al., 2002) and central neurones (Fig. 1). Very high levels of expression of $InsP_3$ receptors have been detected in cerebellar Purkinje neurones, with specific concentration in dendrites and spines (Satoh et al., 1990).

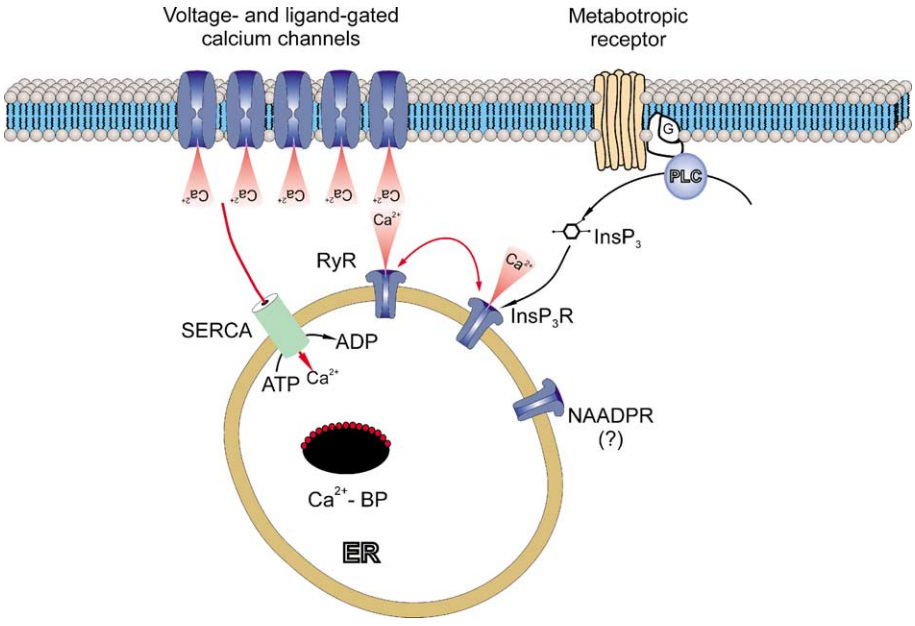

Fig. 2. General principle of organisation of neuronal endoplasmic reticulum calcium store. Signalling between the plasmalemma and endoplasmic reticulum Ca^{2+} store is achieved through (i) metabotropic receptors, which controls synthesis of $InsP_3$ and (ii) voltage- and ligand-gated plasmalemmal calcium channels, which provide for stimulation-dependent calcium influx. Both Ca^{2+} and $InsP_3$ control gating of intracellular Ca^{2+} release channels. Abbreviations: G—G-proteins; PLC—phospholipase C, which controls synthesis of $InsP_3$; ER—endoplasmic reticulum; RyR—ryanodine receptors; $InsP_3R$—$InsP_3$ receptors, NAADPR—NAADP receptors; SERCA—Sarco(endo)plasmic reticulum calcium ATPase; Ca^{2+}-BP—Ca^{2+} binding proteins.

2.2. Endoplasmic reticulum Ca^{2+} release

The importance of the endoplasmic reticulum as a dynamic calcium pool in nerve cells was first appreciated in the late 1980s and early 1990s when several groups reported stimulation-induced cytosolic Ca^{2+} signals recorded from cultured neurones, which were not affected by Ca^{2+} removal from the extracellular media. These $[Ca^{2+}]_i$ signals were triggered by either caffeine (potent activator of RyRs) in 10–30 mM concentrations (Friel, 1995; Friel and Tsien, 1992; Garaschuk et al., 1997; Kano et al., 1995; Lipscombe et al., 1988; Thayer et al., 1988; Usachev et al., 1993; Usachev and Verkhratsky, 1995) or by neurotransmitters (glutamate or ATP) stimulating metabotropic (i.e. $InsP_3$ producing) receptors (Brorson et al., 1991; Irving et al., 1992a,b; Khodakhah and Ogden, 1993; Llano et al., 1991; Svichar et al., 1997a,b). The discovery of the caffeine-induced Ca^{2+} release from the endoplasmic reticulum initiated a search for genuine Ca^{2+}-induced Ca^{2+} release (CICR) (i.e. Ca^{2+} release activated by Ca^{2+} entry through plasmalemmal channels upon electrical/chemical stimulation) in nerve cells. At that time the CICR mechanism had already been characterised in cardiac muscle, where it accounted for approximately 95% of the Ca^{2+} entry into the cytosol during the contraction cycle. In neurones, however, the involvement of CICR in shaping of $[Ca^{2+}]_i$ transients appeared to be less obvious. Numerous indirect approaches, undertaken by several groups, resulted in several observations suggesting a role of CICR in the amplification of depolarisation-induced $[Ca^{2+}]_i$ elevations. The first group of findings demonstrated that pharmacological manipulation with RyRs modified $[Ca^{2+}]_i$ elevations triggered by depolarisation. That is, treatment with ryanodine decreased the rate of rise and suppressed the amplitude of depolarisation-evoked $[Ca^{2+}]_i$ transients (Friel and Tsien, 1992; Usachev et al., 1993). Conversely, incubation with low concentrations of caffeine (1–2 mM which are believed to facilitate Ca^{2+}-dependent gating of RyRs) accelerated Ca^{2+} responses evoked by membrane depolarisation (Friel and Tsien, 1992; Usachev et al., 1993; Usachev and Thayer, 1997). The second group of experimental data established a direct link between Ca^{2+} entry and Ca^{2+} release from the endoplasmic reticulum by (i) demonstrating a facilitation of depolarisation-evoked $[Ca^{2+}]_i$ transients by elevation of extracellular Ca^{2+} (Hua et al., 1993; Llano et al., 1994; Shmigol et al., 1995) and (ii) by discovering a supralinear relationship between the amount of Ca^{2+} entry via voltage-gated channels and the amplitude of the corresponding $[Ca^{2+}]_i$ elevation (Hua et al., 1993; Llano et al., 1994; Messutat et al., 2001; Shmigol et al., 1995). Although these observations provided strong clues for the existence of genuine CICR in neurones, they also very much questioned its physiological relevance as CICR became apparent only at very long depolarisations (~ 60 ms for Purkinje neurones and about 200–500 ms for sympathetic and sensory neurones), which are incompatible with the usual duration of neuronal action potentials. Moreover, these experiments suggested a non-linear nature of CICR in nerve cells, implying the necessity of attaining a relatively high level of "threshold" $[Ca^{2+}]_i$.

The matter is, however, more complicated. The endoplasmic reticulum store does not serve only as a source of Ca^{2+}, it also contributes to Ca^{2+} buffering (endoplasmic reticulum as Ca^{2+} sink; Friel and Tsien, 1992). That is Ca^{2+} release and Ca^{2+} uptake may counterbalance each other, and the net Ca^{2+} translocation depends on the ratio of Ca^{2+} uptake/Ca^{2+} release rates. Indeed, as has been recently demonstrated by D. Friel and colleagues (Albrecht et al., 2001; Hongpaisan et al., 2001) the endoplasmic reticulum in sympathetic neurones works in two different modes accounting for net Ca^{2+} uptake at weak stimulation, which turns into net Ca^{2+} release at high levels of stimulation.

Our knowledge about the second type of Ca^{2+} release, the inositol-1,4,5-trisphosphate-induced Ca^{2+} release (IICR), in nerve cells is even less detailed. Several studies have demonstrated that metabotropic agonists do trigger Ca^{2+} release in various types of neurones (e.g. glutamate and acetylcholine in central neurones (Brorson et al., 1991; Irving and Collingridge, 1998; Llano et al., 1991), ATP in both central (Lalo et al., 1998; Nishizaki and Mori, 1998) and peripheral (Liu et al., 2000; Svichar et al., 1997b) neurones). In many cases, this Ca^{2+} release was inhibited by intracellular administration of the $InsP_3R$ antagonist heparin. Presumed $InsP_3$-driven Ca^{2+} release was also observed in Purkinje neurones in brain slices in response to synaptic stimulation (Finch and Augustine, 1998; Takechi et al., 1998) suggesting its functional importance. Finally, direct injection of $InsP_3$ into Purkinje neurones also triggered elevations in $[Ca^{2+}]_i$, however, only at very high (20 μM) concentrations (Khodakhah and Ogden, 1995). Yet, beside its proven existence, the parameters of IICR in nerve cells remain unknown. Even the fundamental question about whether CICR and IICR share the same endoplasmic reticulum calcium pool or whether they are coupled with two distinct subcompartments (as was suggested by some investigators; Blaustein and Golovina, 2001) has not been experimentally addressed. The way forward necessitated new approaches, in particular, it required the possibility of real-time monitoring of $[Ca^{2+}]_L$ dynamics.

2.3. Endoplasmic reticulum Ca^{2+} dynamics

Several experimental techniques for visualisation of endoplasmic reticulum and $[Ca^{2+}]_L$ monitoring were developed and refined in recent years (Fig. 3). In essence, all these techniques relied on Ca^{2+} indicators with sufficiently low affinity to report high free Ca^{2+} levels present in the endoplasmic reticulum lumen. An important technical problem was to ensure that the signal acquired originated solely from the endoplasmic reticulum compartment. Two strategic approaches were applied so far. The first approach

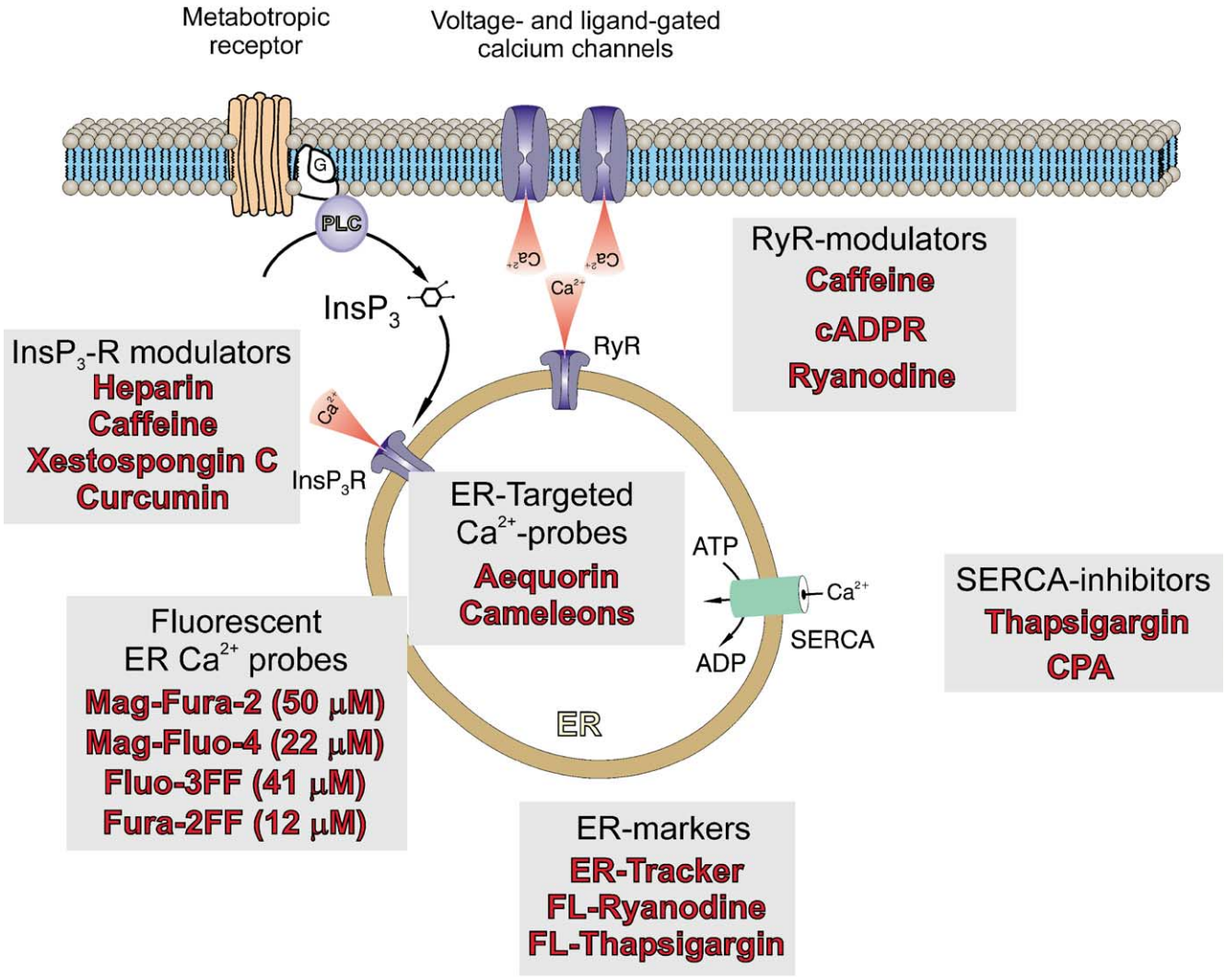

Fig. 3. Probing and visualisation of endoplasmic reticulum calcium stores—an overview of contemporary tools. The endoplasmic reticulum could be visualised with ER-Tracker, or fluorescent thapsigargin or ryanodine (both available in green and red versions). Fluctuations of free Ca^{2+} concentration within the ER lumen are assessed either with low-affinity fluorescent Ca^{2+} probes or with ER-targeted Ca^{2+} sensitive luminescent (aequorin) or fluorescent (chameleons) proteins. Ca^{2+} accumulation into the ER is blocked by SERCA inhibitors thapsigargin or cyclopiazonic acid, CPA. Pharmacological modulators for ryanodine receptors, RyR's are caffeine, ryanodine and cyclic ADP ribose, cADPR. Pharmacology of $InsP_3$ receptors, $InsP_3R$'s is complicated. Heparin blocks $InsP_3R$'s in 100 μM concentrations, however, it also activates RyRs (Ehrilch et al., 1994); caffeine inhibits $InsP_3R$'s in millimolar concentrations (Ehrilch et al., 1994; Petersen and Cancela, 1999; Wakui et al., 1990). Recently discovered presumed selective blocker of $InsP_3R$'s xestospondin C (Gafni et al., 1997) also inhibits SERCA pumps (De Smet et al., 1999). Finally, curcumin, an active component of the food flavour turmeric, also was recently put forward as a membrane-permeable $InsP_3R$ inhibitor (Dyer et al., 2002). Other abbreviations are the same as in Fig. 2.

employed cell transfection with an endoplasmic reticulum-targeted Ca^{2+} probe, such as the luminescent indicator aequorin (Alonso et al., 1998; Montero et al., 1995, 2001) or fluorescent indicators based on fluorescence resonance energy transfer between two modified fluorescent proteins (e.g. green and blue) known as "cameleon" probes (Miyawaki et al., 1997; Yu and Hinkle, 2000). The alternative approach used "conventional" low-affinity Ca^{2+}-sensitive fluorescent probes such as Mag-Fura-2, Mag-Fluo-4, Fluo-3FF, etc., with K_d's in the range of 20–60 μM (Golovina and Blaustein, 1997; Hofer and Schulz, 1996; Mogami et al., 1998; Park et al., 1999, 2000; Solovyova et al., 2002; Tse et al., 1994). Cells were stained with a membrane-permeable form of the dye, so that the indicator was trapped within both the cytosol and the endoplasmic reticulum lumen. The cytosolic portion of the dye was then removed by either intracellular dialysis (Park et al., 1999; Solovyova et al., 2002) or by permeabilisation of the plasma membrane with various detergents, such as digitonin or saponin (Tengholm et al., 2001). In certain cell types such a permeabilisation is not necessary as the dye is either highly compartmentalised within the endoplasmic reticulum (Golovina and Blaustein, 2000; Hofer et al., 1998; Thomas et al., 2000) or the cytosolic Ca^{2+} signals are small enough in order not to be detected by a low-affinity probe (Park et al., 2000). Both strategies were successfully employed for

studying endoplasmic reticulum calcium dynamics in various non-excitable cells.

In neurones, similar attempts were performed only very recently. The endoplasmic reticulum-targeted aequorin technique was applied to a chromaffin cells (Alonso et al., 1999), which retains some neurone-like properties. The low-affinity fluorescent probe (Mag-Fura-2) method was applied to primary cultured dorsal root ganglia (DRG) neurones (Solovyova et al., 2002). The $[Ca^{2+}]_L$ in chromaffin cells cells varied between 300 and 600 μM, in DRG neurones between 100 and 300 μM. Importantly, experiments using Mag-Fura-2 were combined with monitoring of membrane currents in the whole-cell mode, thus permitting direct correlation between Ca^{2+} entry and Ca^{2+} liberation from the store. Using this approach, genuine CICR was directly demonstrated, and moreover, a linear relationship between Ca^{2+} entry and Ca^{2+} release was found. Interestingly, the drop in $[Ca^{2+}]_L$ even upon maximal CICR corresponded to a reduction of less than 10% of the resting level. Furthermore, even maximal stimulation of RyRs with caffeine, or inhibition of SERCA pumping with thapsigargin failed to decrease $[Ca^{2+}]_L$ to less that 50% of the resting level.

2.4. Endoplasmic reticulum and synaptic transmission

Whether functionally relevant endoplasmic reticulum-dependent Ca^{2+} signalling exists in neurones is an obvious and highly important question. Experimental data gathered during the last couple of years have clearly demonstrated the fundamental importance of Ca^{2+} release from endoplasmic reticulum for synaptic plasticity in several regions of the brain. These studies revealed a critical importance of both $InsP_3Rs$ and RyRs and highlighted several molecular cascades which recruit Ca^{2+} release upon synaptic transmission and couple it with regulation of synaptic plasticity in the form of long-term potentiation (LTP) or long-term depression (LTD) (see Pozzo-Miller et al., 2000; Rose and Konnerth, 2001; Svoboda and Mainen, 1999 for review).

First, it was demonstrated that Ca^{2+} release via $InsP_3Rs$ and RyRs differentially regulates the mode (LTD vs. LTP) of synaptic modification in the CA1 region of the hippocampus. That is, inhibition or genetic deletion of $InsP_3Rs$ led to a conversion of LTD to LTP, whereas inhibition of RyRs eliminated specifically homosynaptic LTD (Nishiyama et al., 2000). More precisely, genetic elimination of RyR type 3 significantly facilitated LTP, without any effects of LTD (Futatsugi et al., 1999). This functional difference in IICR and CICR effects most likely reflects the specific distribution of Ca^{2+} release channels in hippocampal neurones, where intra-spine endoplasmic reticulum compartments contains only RyRs type 3, whereas endoplasmic reticulum membrane in the dendritic shaft is endowed with both $InsP_3Rs$ and type 1 RyRs (Svoboda and Mainen, 1999). Thus, both CICR and IICR mechanisms could be activated upon synaptic transmission in CA1 neurones spines and/or dendrites, and moreover, the site of release determines the final effect on synaptic modification. CICR, activated by either caffeine or synaptically activated Ca^{2+} entry was demonstrated in both cultured hippocampal neurones and organotypic hippocampal slices (Emptage et al., 1999; Korkotian and Segal, 1998), although in the acute hippocampal slice preparation the contribution of CICR to $[Ca^{2+}]_i$ transients in single spines was negligible (Kovalchuk et al., 2000). The IICR in hippocampal neurones was rather peculiar: it could be activated by synergistic action of Ca^{2+} ions entering through plasmalemmal channels and metabotropic glutamate receptor (mGluR)-mediated mobilisation of $InsP_3$ (Nakamura et al., 1999, 2000). This mechanism might serve as an important coincidence detector for determining the mode of synaptic modification.

Synaptic plasticity in cerebellum appeared to be exclusively controlled by the mGluR1/$InsP_3Rs$ signalling cascade. The LTD at cerebellar parallel fibres–Purkinje neurone synapses was severely altered in mice lacking mGluR type 1 (Aiba et al., 1994). Selective reintroduction of mGluR1 into Purkinje neurones of otherwise mGluR1 deficient mice rescued the LTD to a full extent (Ichise et al., 2000). A similar complete disappearance of LTD was observed in mice with $InsP_3R$ type1 altered by either genetic modification or by introducing a specific antibody (Inoue et al., 1998).

All these experiments undeniably prove the functional importance of endoplasmic reticulum calcium stores in the regulation of the most visible neuronal function, synaptic transmission. This regulation relies upon highly localised Ca^{2+} release events, co-ordinated in time with synaptic excitation.

3. The endoplasmic reticulum as a Ca^{2+} signalling highway

Besides controlling local Ca^{2+} transients, the endoplasmic reticulum has recently emerged as an important mechanism for globalisation of calcium signals, providing a specific route for calcium transportation within highly polarised cells (Cancela et al., 2002) (Fig. 4). To appreciate the importance of specific intracellular Ca^{2+} transport systems, one has to bear in mind a specific condition for Ca^{2+} diffusion in the cytosolic compartment. This specificity is determined by the extensive Ca^{2+} buffering provided by numerous high-affinity Ca^{2+} binding proteins (Kasai and Petersen, 1994), with K_d's in a range of hundreds to thousands of nM (Burgoyne and Weiss, 2001). Therefore, only a tiny fraction of Ca^{2+} ions entering the cytosol appears in a free form, cytosolic buffers almost immediately scavenge most of Ca^{2+}. In neurones, Ca^{2+} buffering varies greatly between different cells: e.g. in chromaffine cells the ratio between free/bound Ca^{2+} entering the cytosol is 1:50; in hippocampal neurones it varies between 1:100 and 1:150 and in Purkinje neurones only 1 out of 2000 Ca^{2+} ions reaching the cytosol remains free (Fierro and Llano, 1996;

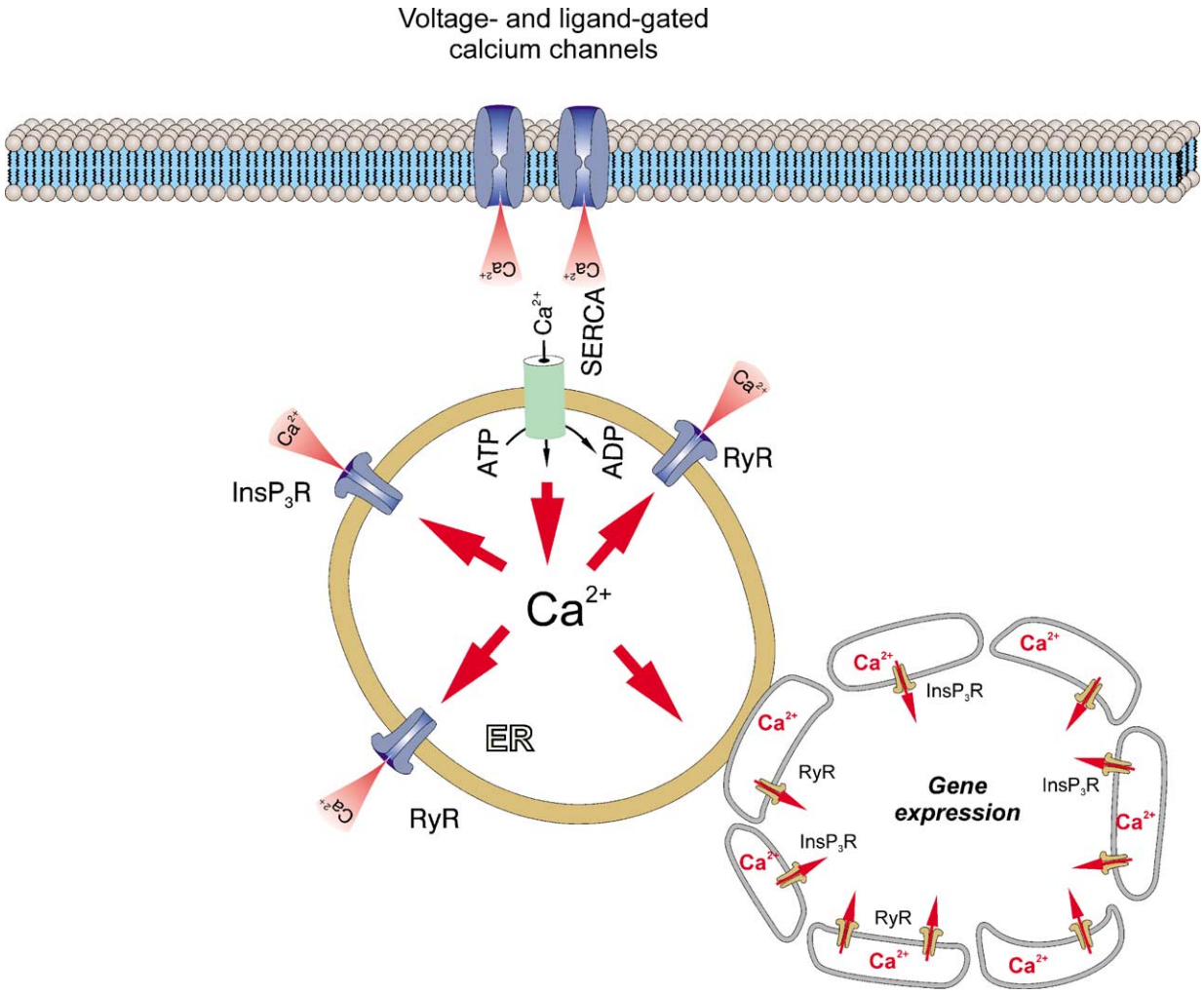

Fig. 4. Endoplasmic reticulum as a calcium tunnel. This scheme illustrates the possible role of endoplasmic reticulum in conveying Ca^{2+} signals from plasmalemma towards cell interior and nucleus. Within the framework of this hypothesis Ca^{2+} ions entering the cytosol are pumped into the endoplasmic reticulum lumen, where they rapidly diffuse and are being released in a proximity of their intracellular targets. Other abbreviations are the same as in Fig. 2.

Lee et al., 2000a,b). This extensive buffering in essence forms the basis for cytosolic calcium signalling and creates a specific condition that favours localisation of cytosolic calcium elevations, as the rate of Ca^{2+} diffusion within the cytosol does not exceed several μm/s. Consequently, physiologically relevant cytosolic calcium signalling occurs in microdomains, which is very important for triggering/regulation of various cellular functions such as neurotransmitter release or regulation of plasmalemmal excitability. Yet, it comes at a price of great difficulties in conveying Ca^{2+} signals through the cytosolic phase into the cell interior and into the nucleus, where Ca^{2+} ions also serve crucial regulatory functions.

In this respect, the endoplasmic reticulum appears to be a perfect environment for an intracellular Ca^{2+} transporting highway. Indeed, the much lower affinity of intra-endoplasmic reticulum Ca^{2+} buffers in conjunction with a constant high $[Ca^{2+}]_L$ results in substantially higher Ca^{2+} diffusion rates. Thus, the endoplasmic reticulum lumen may provide the means for rapid Ca^{2+} equilibration after localised entry. However, for this scheme to be operational, the endoplasmic reticulum lumen should be constituted as one continuous space. This particular issue still remains quite controversial, with some investigators insisting on the existence of several separate Ca^{2+} storing compartments (Blaustein and Golovina, 2001). Yet, direct imaging of the endoplasmic reticulum lumen using various fluorescent probes has demonstrated the continuity of the endoplasmic reticulum in various types of cells. Not only fluorescent calcium probes (Park et al., 2000) can diffuse freely within the endoplasmic reticulum lumen, but a much larger molecule, endoplasmic reticulum-targeted green fluorescent protein (GFP), does not experience difficulties in travelling throughout the endoplasmic reticulum (Dayel et al., 1999; Subramanian and Meyer, 1997). Finally, experiments on pancreatic acinar cells demonstrated that Ca^{2+} rapidly equilibrates within the endoplasmic reticulum lumen following local uncaging of caged calcium (Mogami et al., 1997; Park et al., 2000; Petersen

et al., 2001). In neurones this question remains open, but for these cells in particular the need for a Ca^{2+} transporting organelle is great, since sites of Ca^{2+} entry may be millimetres and even centimetres away from the cell body.

The endoplasmic reticulum Ca^{2+} tunnel hypothesis has been directly tested in only one type of cell—namely the pancreatic acinar cell. In this preparation, the endoplasmic reticulum stores could be completely replenished after maximal stimulation under conditions when the area of Ca^{2+} entry was limited by a cell-attached pipette (Mogami et al., 1997; Petersen et al., 2001). There is also indirect evidence for the importance of an endoplasmic reticulum Ca^{2+} tunnel for transcellular Ca^{2+} transport in dental enamel cells (Franklin et al., 2001).

In neurones this very attractive hypothesis has not yet been tested, however, experiments in cultured central neurones have clearly shown that nuclear calcium signalling following peripheral Ca^{2+} entry is significantly decreased after inhibition of SERCA pumps (Hardingham et al., 2001), obviously implying the importance of the endoplasmic reticulum in coupling Ca^{2+} entry sites with nuclear Ca^{2+} signalling.

4. Disruption of endoplasmic reticulum Ca^{2+} homeostasis and neurodegeneration

Besides being a Ca^{2+} signalling organelle, the endoplasmic reticulum lumen sets the stage for post-translational modification of proteins, notably in their folding. The proper folding of proteins, during which they acquire their tertiary and quaternary structures, is controlled by several enzymatic systems including peptydil prolyl isomerases and glycosylation enzymes (glycosidases and mannosidases—see Chevet et al., 2001; Zapun et al., 1999 for review). All this complicated machinery is regulated by several families of chaperones, such as glucose regulated proteins (*grp78*, *grp94*, etc.), lectin-like chaperones (calreticulin, calnexin and calmegin) and protein disulfide isomerases (PDI, ERp57). Any disturbances in correct protein folding leads to accumulation of unfolded proteins within the endoplasmic reticulum inducing a condition generally referred to as the endoplasmic reticulum stress response. This may happen under many conditions, e.g. when too many newly synthesised proteins enter the endoplasmic reticulum lumen, or when limited glucose supply undermines glycosylation (Pahl, 1999). Whatever the reason, the cell adaptation to these conditions requires modulation of protein synthesis, i.e. up-regulation of beneficial enzymes and limiting the excessive synthesis of other proteins. This implies a necessity for a specific signalling system, which would send a signal down to the nucleus in order to affect transcriptional processes. Indeed such a signalling system has been discovered and not surprisingly, it originates from the endoplasmic reticulum where actual protein processing takes place.

The endoplasmic reticulum stress response has been described in two forms, the unfolded protein response (UPR) and the endoplasmic reticulum overload response (EOR) (see (Pahl, 1999; Patil and Walter, 2001) for review). The UPR was characterised in detail in yeast and subsequently many of its components were found in other types of cells (Cox et al., 1997; Sidrauski et al., 1998). Overall, the UPR is characterised by an activation of the expression of endoplasmic reticulum resident chaperones (mainly *grp*'s) and by overall suppression of protein synthesis. The UPR may also up-regulate the synthesis of pro-apoptotic proteins *gadd34* and *gadd153*, opening thus a way to cell death. The second type of endoplasmic reticulum stress response, the EOR is manifested by an activation of the transcription factor NF-κB, which in turn induces transcription of numerous pro-inflammatory proteins, and cell-adhesion molecules (Pahl and Baeuerle, 1995). Both types of endoplasmic reticulum stress responses are highly conserved, representing a fundamental adaptive cell reaction which has two faces: one is cytoprotective when signalling systems try to overcome the endoplasmic reticulum stress by overexpression of relevant chaperones and tuning the overall protein synthesis. Another face is cytotoxic—when the cell could not cope with the stress; the endoplasmic reticulum releases pro-apoptotic factors therefore eliminating the cell in the least harmful manner for the neighbours.

A very important next step was the discovery that both UPR and EOR may be triggered not only by biochemical stress, but also by disturbance in the endoplasmic reticulum Ca^{2+} homeostasis. Even more important, this pathway was found to be operative in various types of neurones. The irreversible inhibition of SERCA by thapsigargin and hence constant depletion of the endoplasmic reticulum Ca^{2+} content triggers a 200-fold increase in *grp78*, *grp94*, *gadd34* and *gadd153* in cultured cortical neurones (Mengesdorf et al., 2001), whereas the overall protein synthesis was inhibited (Doutheil et al., 1999) thus very much resembling the UPR. The EOR like response with a significant NF-κB activation was also discovered in cortical neuronal cultures in response to endoplasmic reticulum depletion; and even more importantly inhibition of $InsP_3R$'s with xestospongin C prevented NF-κB activation (Glazner et al., 2001). These observations fit together with the firmly established fact that treatment of neurones with thapsigargin triggers cell death by apoptotic mechanisms (Bian et al., 1997; Nath et al., 1997; Silverstein and Nelson, 1992; Takei and Endo, 1994; Tsukamoto and Kaneko, 1993; Wei et al., 1998). These observations naturally initiated the hypothesis that intra-endoplasmic reticulum Ca^{2+} content plays a critical role in the instigation of the endoplasmic reticulum stress response.

Indeed this hypothesis seems to be quite logical, especially remembering that at least one class of chaperones, the lectin-like ones, such as calreticulin, are the major Ca^{2+} binding proteins within the endoplasmic reticulum lumen, and $[Ca^{2+}]_L$ fluctuations inevitably affect them. Interestingly also, the *grp78*, the chaperone which is up-regulated

upon URP and plays a defensive role in fighting against the excess of unfolded proteins, also acts as a stabiliser of the endoplasmic reticulum Ca^{2+} content (Lee et al., 1999; Yu et al., 1999). Moreover, as the endoplasmic reticulum stress response induces expression not only of chaperones, it also up-regulates the expression of SERCA pumps (Thuerauf et al., 2001) with the obvious aim of restoring the disturbed endoplasmic reticulum Ca^{2+} homeostasis.

Yet, calreticulin remains the likeliest chaperone which activity may be directly regulated by intraluminal Ca^{2+} levels (Corbett and Michalak, 2000). Indeed calreticulin chaperone activity and its ability to interact with other chaperones such as protein disulfate isomerase and ERp57 are strictly calcium-dependent. Typical resting $[Ca^{2+}]_L$ levels of 300–400 μM favour full activation of chaperone function and therefore effective interactions with unfolded proteins. Upon store depletion, however, (when $[Ca^{2+}]_L$ falls to 50–100 μM) these interactions and chaperone activity are severely impaired (Oliver et al., 1999; Primm et al., 1996). Therefore, the mechanism, which directly couples the endoplasmic reticulum Ca^{2+} content and protein processing in the endoplasmic reticulum, is already in place. Furthermore, by regulating chaperone activity $[Ca^{2+}]_L$ may affect the levels of unfolded proteins and therefore initiate signalling between the endoplasmic reticulum and the nucleus.

An important part of these interactions also concerns neuronal pathology. As we have already mentioned, the irreversible depletion of the endoplasmic reticulum Ca^{2+} triggers apoptosis and neuronal death. The same processes are likely to occur under various brain pathological conditions. That is, the pathogenesis of Alzheimer disease may well include the disturbances of endoplasmic reticulum Ca^{2+} homeostasis as a key process. Indeed, the synthesis of a highly toxic isoform of amiloid beta peptide, Aβ(1–42) is controlled by presenilins, which are endoplasmic retic-

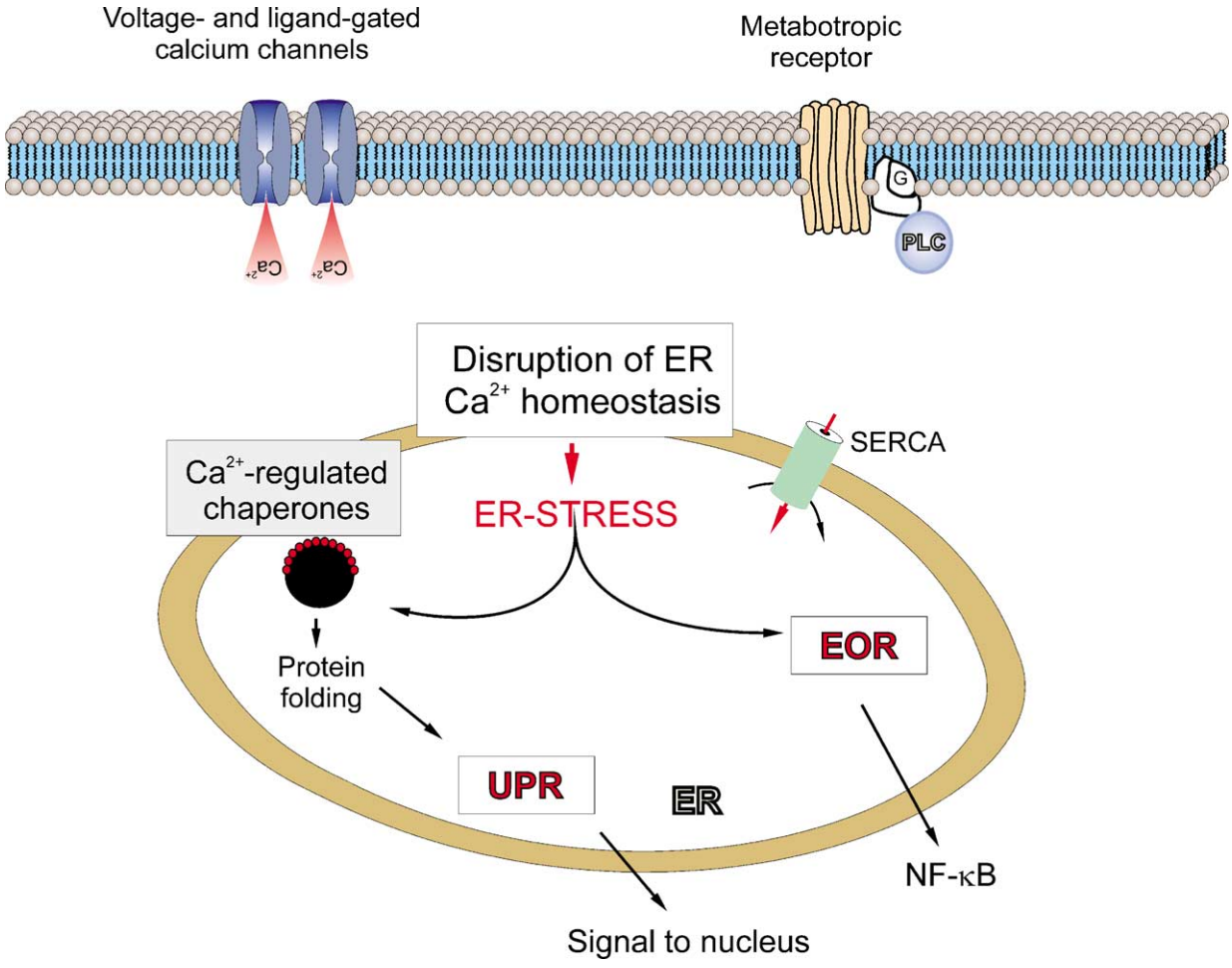

Fig. 5. Endoplasmic reticulum calcium content integrates rapid neuronal signals and long-term adaptive responses. Here the possible integrating role of Ca^{2+} level within the endoplasmic reticulum lumen is shown in a schematic form. The actual Ca^{2+} concentrations within the endoplasmic reticulum regulates activity of numerous chaperones, which in turn control post-translational processing of proteins. Disruption of endoplasmic reticulum Ca^{2+} homeostasis induces stress response manifested by unfolded protein response (UFP) or endoplasmic reticulum overload response (EOR). These stress responses are part of a signalling chain between endoplasmic reticulum and nucleus (executed e.g. via NF-κB), which controls cellular adaptive/survival/death responses (see text for further details). Other abbreviations are the same as in Fig. 2.

ulum resident proteins (Refolo et al., 1999; Weidemann et al., 1997); and moreover, the endoplasmic reticulum has been identified as a site of Aβ(1–42) synthesis. Aβ(1–42) has well documented ionophore-like properties (Engstrom et al., 1995) and therefore its accumulation within the endoplasmic reticulum may increase Ca^{2+} leakage from the lumen, leading thus to endoplasmic reticulum Ca^{2+} depletion and endoplasmic reticulum stress. Endoplasmic reticulum Ca^{2+} depletion in Alzheimer disease may be even further exaggerated by additional Ca^{2+} leaks through RyR's. The expression of these receptors was significantly increased in cells expressing mutant presenilins (Chan et al., 2000).

A direct role of the endoplasmic reticulum stress response in the pathogenesis of Alzheimer disease has been further substantiated by the recent observation that artificial expression of familial Alzheimer disease-specific mutations of presenelein-1 gene down-regulate the adaptive parts of UPR, most notably by decreasing the levels of *grp78* (Imaizumi et al., 2001; Katayama et al., 1999, 2001). These observations gained pathophysiological significance after reduced levels of *grp78* were detected in the brains of familial Alzheimer disease patients (Katayama et al., 1999). Later on, that alternative splices form of presenilin 2 expressed in sporadic Alzheimer disease has the very same effect on endoplasmic reticulum stress response (Sato et al., 2001).

Similarly, disturbances in endoplasmic reticulum Ca^{2+} homeostasis may account for cell damage upon brain ischemia and excitotoxicity. This, rather surprising, suggestion stems from an interesting series of experiments performed by Japanese investigators (Kitao et al., 2001; Kuwabara et al., 1996; Ozawa et al., 1999). They cloned another endoplasmic reticulum resident chaperone, a 150-kDa oxygen-regulated protein (*orp150*) from astrocytes subjected to ischemic stress. The limited susceptibility of astrocytes to excitotoxicity and oxygen deprivation is a well-known phenomenon, and it was suggested that *orp150* might be responsible for it. Further experiments found an up-regulation of *orp150* in human brain after a seizure attack and in mice hippocampus subjected to kainate lesion. Moreover, hippocampal neurones isolated from *orp150* deficient mice demonstrated higher $[Ca^{2+}]_i$ increases in response to glutamate or NMDA and exaggerated glutamate-induced cell death; similarly the *orp150* deficient animals demonstrated decreased survival after kainate-induced brain lesions. Conversely, when the levels of *orp150* were selectively increased in nerve cells by targeted overexpression, both neuronal in vitro and animal in vivo survival after excitotoxic shock was increased (Kitao et al., 2001). Interestingly, in cultured neurones with an increased level of *orp150*, glutamate-induced $[Ca^{2+}]_i$ loads were substantially reduced, implying thus that this chaperon may also stabilise cellular Ca^{2+} homeostasis, most likely by limiting Ca^{2+} release from the endoplasmic reticulum occurring upon excessive glutamate stimulation (Kitao et al., 2001). Finally, depletion of endoplasmic reticulum Ca^{2+} and consequent endoplasmic reticulum stress may participate in neurodegeneration during epileptic seizures, as the RyR blocker dantrolene had a significant neuroprotective action in various epilepsy models (Niebauer and Gruenthal, 1999).

5. Conclusion—the endoplasmic reticulum as an integrating signalling organelle

Several signalling systems operating within different temporal and spatial domains originate from the endoplasmic reticulum. A wealth of evidence accumulated to date supports the notion that all these very different signalling systems have a common denominator. This common denominator is represented by the concentration of free Ca^{2+} within the lumen of the endoplasmic reticulum (Fig. 5). The intra-endoplasmic reticulum Ca^{2+} controls rapid local signalling, which occurs during synaptic transmission. These local signals regulate long-term synaptic plasticity, being thus involved in such processes as learning and memory. At the same time, Ca^{2+} concentration within the endoplasmic reticulum lumen governs intra-endoplasmic reticulum chaperones which police proper protein folding. When intra-endoplasmic reticulum Ca^{2+} undergoes significant fluctuations, the protein folding becomes affected, triggering several specific signals, which link endoplasmic reticulum processes with the nucleus and constitute appropriate changes in gene expression. Finally, when endoplasmic reticulum Ca^{2+} homeostasis is disturbed irreversibly, the endoplasmic reticulum releases death signals, which underlie neurodegenerative processes driving many severe brain diseases.

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