Cross talk between Ca^{2+} and redox signalling cascades in muscle and neurons through the combined activation of ryanodine receptors/ Ca^{2+} release channels

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Calcium release mediated by the ryanodine receptors (RyR) Ca^{2+} release channels is required for muscle contraction and contributes to neuronal plasticity. In particular, Ca^{2+} activation of RyR-mediated Ca^{2+} release can amplify and propagate Ca^{2+} signals initially generated by Ca^{2+} entry into cells. Redox modulation of RyR function by a variety of non-physiological or endogenous redox molecules has been reported. The effects of RyR redox modification on Ca^{2+} release in skeletal muscle as well as the activation of signalling cascades and transcription factors in neurons will be reviewed here. Specifically, the different effects of S-nitrosylation or S-glutathionylation of RyR cysteines by endogenous redox-active agents on the properties of skeletal muscle RyRs will be discussed. Results will be presented indicating that these cysteine modifications change the activity of skeletal muscle RyRs, modify their behaviour towards both activators and inhibitors and affect their interactions with FKBP12 and calmodulin. In the hippocampus, sequential activation of ERK1/2 and CREB is a requisite for Ca^{2+} -dependent gene expression associated with long-lasting synaptic plasticity. The effects of reactive oxygen/nitrogen species on RyR channels from neurons and RyR-mediated sequential activation of neuronal ERK1/2 and CREB produced by hydrogen peroxide and other stimuli will be discussed as well.

Keywords: hydrogen peroxide; S-nitrosylation; S-glutathionylation; skeletal muscle; neuronal function; redox regulation

1. INTRODUCTION

The cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which cells at rest maintain at levels less than or equal to 100 nM, can increase rapidly and transiently in response to various types of stimuli (Berridge *et al.* 2000). Increases in $[\text{Ca}^{2+}]_i$ can initiate and modulate different cellular events; these include short-term responses, such as muscle contraction and secretion, as well as long-term responses including cell growth and proliferation and the expression of genes required for memory acquisition and learning (Berridge *et al.* 2003). Furthermore, an uncontrolled increase in $[\text{Ca}^{2+}]_i$ can lead to an alteration of intracellular Ca^{2+} homeostasis that may trigger apoptosis or cause irreversible cell injury and necrosis.

Two different Ca^{2+} channels mediate the fast release of Ca^{2+} from intracellular stores, the ryanodine receptors (RyR) and the inositol trisphosphate receptors. Both Ca^{2+} release channels can increase their activity in response to an increase in $[\text{Ca}^{2+}]_i$. This response generates Ca^{2+} -induced Ca^{2+} release (CICR), a universal cellular mechanism that allows amplification and propagation of the Ca^{2+} signals

initially created by Ca^{2+} entry. In particular, ryanodine receptors (RyR)-mediated CICR is required for cardiac muscle contraction and has a central role in secretion and neuronal function, as discussed below.

There are three mammalian RyR isoforms (RyR1, RyR2 and RyR3), which are codified by three different genes localized in three different chromosomes in humans. These proteins were originally identified in skeletal muscle (RyR1), in heart muscle (RyR2) and in brain (RyR3), although it is now known that brain and other tissues express all three mammalian RyR isoforms (Coronado *et al.* 1994; Furuichi *et al.* 1994; Mori *et al.* 2000). Multiple cellular components—including ATP, H^+ , Ca^{2+} and Mg^{2+} —and specific proteins, including kinases and phosphatases, as well as endogenous oxidative/nitrosative species, regulate RyR channels (Fill & Copello 2002). In particular, many studies have reported modifications of RyR cysteines by non-physiological redox compounds (for reviews, see Hamilton & Reid 2000; Pessah *et al.* 2002; Hidalgo *et al.* 2002). These studies have contributed to the current understanding of how these cysteine modifications change RyR function and affect Ca^{2+} release; they have also promoted active research into how redox compounds of physiological significance affect RyR activity. These endogenous redox components include the free radicals nitric oxide (NO) and superoxide anion, which through enzymatic or non-enzymatic

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chemical reactions can be readily converted into non-radical species of lower reactivity but longer half-life such as *S*-nitrosoglutathione (GSNO) or hydrogen peroxide (H_2O_2). All these endogenous redox components can modify RyR function (Hidalgo *et al.* 2004). Additionally, RyR channels are highly susceptible to modification by other endogenous redox agents, including glutathione (GSH), glutathione disulphide (GSSG), NADH, and by changes in the GSH/GSSG ratio (reviewed in Hidalgo *et al.* 2004).

RyR channels have been proposed to act as intracellular redox sensors (Eu *et al.* 2000; Hidalgo *et al.* 2005). The redox sensitivity of skeletal RyR1 is due to the presence of highly reactive cysteine residues in the RyR1 molecule, which have a pK value of 7.4 (Liu *et al.* 1994; Pessah *et al.* 2002) and allow RyR1 redox modification at physiological pH. Additionally, RyR1 exhibit a well-defined redox potential (Feng *et al.* 2000; Xia *et al.* 2000); through this property they may be susceptible to changes in cellular redox potential. Oxidative/nitrosative modifications that affect RyR function are likely to modify RyR-mediated Ca^{2+} release from intracellular stores. Hence, changes in cellular redox state or generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) may result in inhibition or activation of Ca^{2+} signalling cascades. A review of recent data, showing that redox modifications of the RyR channels present in skeletal muscle cells or neurons exert a central role in regulating single channel properties and CICR from vesicles and cells, will be presented next. A discussion of the effects of redox modifications on the function of cardiac RyR2 channels has been presented elsewhere (Hidalgo *et al.* 2004).

2. SKELETAL MUSCLE

(a) *Functional effects of modifications of skeletal muscle RyR1 by ROS/RNS*

Physiological activation of RyR1-mediated Ca^{2+} release in response to muscle depolarization is a very fast process (ms range) which does not require Ca^{2+} entry into the muscle cells. RyR1 channels open following membrane depolarization, presumably by direct coupling with plasma membrane voltage sensors (Rios & Pizarro 1991). Each of the four homologous 565 kDa RyR1 protein subunits contains approximately 100 cysteine residues (Takeshima *et al.* 1989). In native RyR1, approximately 50 of these residues appear to be in the reduced state; of these, approximately 10–12 are highly susceptible to oxidation/modification by exogenous sulphhydryl (SH) reagents (Sun *et al.* 2001a). Endogenous redox-active molecules, including molecular oxygen (Eu *et al.* 2000) and GSSG (Zable *et al.* 1997; Sun *et al.* 2001a,b; Aracena *et al.* 2003), enhance RyR activity. Likewise, H_2O_2 markedly activates single RyR2 channels (Boraso & Williams 1994) or RyR1 channels maintained under redox control (provided by controlled cytoplasmic and luminal GSH/GSSG ratios; Oba *et al.* 2002a). Furthermore, superoxide anion generation by a NADH oxidase activity that is present in heavy sarcoplasmic reticulum (SR) vesicles and which copurifies with RyR1 channels is presumably

responsible for the activation of [^3H]-ryanodine binding (a reflection of increased RyR1 activity) produced by 1 mM NADH (Xia *et al.* 2003).

In certain conditions, NO or NO donors enhance RyR1 activity, while in others they exert an inhibitory effect (Stoyanovsky *et al.* 1997; Suko *et al.* 1999; Sun *et al.* 2001a,b, 2003; Eu *et al.* 2003). In particular, submicromolar NO concentrations activate RyR1 by *S*-nitrosylation of a single cysteine residue (Cys-3635); this reaction occurs only at low (physiological) $p\text{O}_2$ but not at ambient $p\text{O}_2$ (Sun *et al.* 2003). Likewise, *in situ* generation of NO stimulates whole muscle contractility at low $p\text{O}_2$, but inhibits contractility at higher $p\text{O}_2$ (Eu *et al.* 2003). Contrary to NO, GSNO stimulates RyR1 channel activity independently of $p\text{O}_2$ through *S*-nitrosylation of a few critical SH residues other than Cys-3635 (Sun *et al.* 2003). We have shown that *S*-nitrosylation of RyR1 enhances its activation by Ca^{2+} , but does not affect the strong inhibitory effect of Mg^{2+} on CICR (Aracena *et al.* 2003). Both GSNO and the NO donor NOR-3 produce a significant increase in the *S*-nitrosylation of RyR1 over the endogenous levels and affect the interactions of RyR1 with FKBP12 and calmodulin, as detailed below. In addition, GSNO or the combination of millimolar GHS plus micromolar H_2O_2 enhance significantly the endogenous *S*-glutathionylation of RyR1 (Aracena *et al.* 2005), a modification that decreases specifically RyR1 inhibition by Mg^{2+} without affecting its activation by Ca^{2+} (Aracena *et al.* 2003). These findings suggest that RyR1 possesses cysteine residues with different susceptibilities to *S*-glutathionylation or *S*-nitrosylation, and that these cysteine modifications cause independent effects on RyR1 channel activation by Ca^{2+} or inhibition by millimolar Mg^{2+} or Ca^{2+} (figure 1). The implications of these findings for redox enhancement of CICR in skeletal muscle, as well the possible sources and the activation mechanisms of ROS/RNS generation in living skeletal muscle fibres are discussed in §2b.

In mammalian skeletal muscle, RyR1 channels interact with several different accessory proteins, which contribute to tightly regulate their activity. These proteins include the skeletal L-type Ca^{2+} channel dihydropyridine receptor, which acts as voltage sensor during excitation–contraction coupling, triadin, calmodulin, FKBP12 (a 12 kDa FK506 binding protein) and the SR luminal protein calsequestrin (MacKrell 1999; Fill & Copello 2002). Calmodulin in its apocalmodulin (Ca^{2+} -free) form enhances RyR1 channel activity, whereas when present as Ca^{2+} -calmodulin it behaves as a channel inhibitor (Tripathy *et al.* 1995; Rodney *et al.* 2000, 2001). At physiological tissue $p\text{O}_2$ (approx. 10 mmHg), submicromolar NO activates RyR1 by *S*-nitrosylation of a single cysteine residue (Cys-3635) (Sun *et al.* 2003) without affecting Ca^{2+} -calmodulin binding to RyR1 (Eu *et al.* 2000). The specific *S*-nitrosylation of Cys-3635, which resides within a calmodulin binding domain, by NO or the NO donor NOC-12 does not take place at ambient $p\text{O}_2$; *S*-nitrosylation of Cys-3635 activates RyR1 and reverses its inhibition by Ca^{2+} -calmodulin (Sun *et al.* 2003). In contrast, GSNO activates RyR1 by oxidation and *S*-nitrosylation of thiols other than Cys-3635, and

Figure 1. Calcium dependence of the activity of single RyR1 channels. The theoretical curves were obtained as described (Marengo *et al.* 1998), except that the maximal value of open probability was taken as 1.0 and both the activation and inactivation functions were considered non-cooperative. The solid black curve represents the response to cytoplasmic (*cis*) $[Ca^{2+}]$ of native RyR channels, which is obtained as the combination of two independent functions (dotted lines), an activation function with $K_a = 5 \mu M$ and an inactivation function with $K_i = 41 \mu M$. The activation function depicts the channel activation produced by Ca^{2+} binding to the high-affinity site(s), while the inactivation function describes binding of Ca^{2+} or Mg^{2+} to the low-affinity divalent cation inhibitory site(s). Incubation of RyR1 channels with reducing agents increases K_a from 5 to 50 μM and decreases K_i from 41 to 2.2 μM (grey dashed lines), yielding the bell-shaped function represented by the solid light grey line. In contrast, oxidation/alkylation of free sulphhydryl residues present in native RyR1 channels decreases K_a from 5 to 0.6 μM and increases K_i from 41 μM to 5 mM (grey dotted lines); these values yield the bell-shaped function represented by the solid dark grey line. RyR1 S-nitrosylation produces only a decrease in K_a , whereas RyR1 S-glutathionylation produces only an increase in K_i .

calmodulin is not involved (Sun *et al.* 2003). In triad vesicles isolated from skeletal muscle, we have found that S-nitrosylation or S-glutathionylation of triad proteins, including RyR1, increase the K_d of $[^{35}S]$ - Ca^{2+} -calmodulin and of $[^{35}S]$ -apocalmodulin binding to triads without affecting maximal binding capacity (B_{max}). Moreover, the combined S-glutathionylation and S-nitrosylation induced by GSNO increases by fourfold the K_d of $[^{35}S]$ - Ca^{2+} -calmodulin binding to triads and abolishes the binding of $[^{35}S]$ -apocalmodulin (Aracena *et al.* 2005).

FKBP12 is another RyR1 regulatory protein that may specifically coordinate RyR1 channel gating (Gaburjakova *et al.* 2001). Removal of this protein with rapamycin or FK506 leads to RyR1 activation and promotes the emergence of sub-conductance states in single RyR1 channels incorporated in lipid bilayers (Ma *et al.* 1995; Kaftan *et al.* 1996). In addition, tissue-specific FKBP12 knockout mice exhibit altered orthograde and retrograde signalling between the voltage sensor and RyR1, leading to altered L-type currents (Tang *et al.* 2004). We have found that $[^{35}S]$ -FKBP12 binds to RyR1 present in triads or heavy SR vesicles with similar affinity ($K_d = 13.1$ nM) (Aracena *et al.*

in press). These results indicate that RyR1-voltage sensor interactions do not affect the high-affinity binding of FKBP12 to RyR1. RyR1 S-nitrosylation, but not S-glutathionylation, specifically increases fivefold the K_d value of FKBP12 binding to RyR1 without affecting B_{max} (Aracena *et al.* 2005).

Since both FKBP12 and Ca^{2+} -calmodulin inhibit RyR1 activity, decreased binding of these proteins to RyR1 may contribute to the activation of CICR release kinetics induced by S-glutathionylation and S-nitrosylation (Aracena *et al.* 2003). Furthermore, *in vivo* conditions that promote the combined S-nitrosylation and S-glutathionylation of RyR1 are bound to alter both FKBP12 and calmodulin binding to RyR1, and thus may alter excitation-contraction coupling in skeletal muscle fibres.

(b) Relevance of RyR1 modifications induced by ROS/RNS to skeletal muscle function

The results discussed so far indicate that channel S-nitrosylation enhances Ca^{2+} release by increasing the affinity of Ca^{2+} binding to RyR1 (Aracena *et al.* 2003). Mammalian skeletal muscle contains endogenous sources of NO, and it has been reported that endogenous NO generated by nNOS induces a significant enhancement of whole-muscle contractility at low (physiological) pO_2 but not at ambient pO_2 (Eu *et al.* 2003). Moreover, cysteine RyR1 modifications that enhance channel activation by Ca^{2+} and ATP (Marengo *et al.* 1998; Oba *et al.* 2002b; Aracena *et al.* 2003; Bull *et al.* 2003), and especially those that reduce the powerful inhibitory effect of Mg^{2+} on RyR1-mediated Ca^{2+} release (Donoso *et al.* 2000; Aracena *et al.* 2003) may stimulate CICR in living skeletal muscle fibres. *In vitro*, submillimolar $[Mg^{2+}]$ strongly inhibits RyR1 channels (Laver *et al.* 1997) and RyR1-mediated Ca^{2+} release (Meissner *et al.* 1986; Moutin & Dupont 1988; Donoso *et al.* 2000; Aracena *et al.* 2003). Mg^{2+} inhibition may be a contributing factor to the reported lack of sparks due to spontaneous CICR in skeletal muscle fibres (Shirokova *et al.* 1998).

(c) RyR1 S-glutathionylation

Endogenous generation of ROS or GSNO (via NO generation) may enhance RyR S-glutathionylation, and thus may overcome the strong inhibitory effect of Mg^{2+} on CICR. Skeletal muscle fibres generate ROS following physiological contraction (Bejma & Li 1999; Reid & Durham 2002) as well as during heat stress conditions (Stofan *et al.* 2000). Skeletal muscle homogenates contain a constitutively active non-phagocytic NAD(P)H oxidase complex that generates superoxide anion (Javesghani *et al.* 2002). Enzymatic or spontaneous dismutation of superoxide anion generates hydrogen peroxide, which markedly activates RyR1 *in vitro* (Favero *et al.* 1995; Oba *et al.* 2002a) but does not seem to play a significant role in the post fatigue recovery process (Oba *et al.* 2002a). We have found the membrane bound components of NAD(P)H oxidase (gp91^{phox} and p22^{phox}) in skeletal muscle transverse tubules, which are absent from heavy SR vesicles (figure 2). Activation of this transverse tubular NAD(P)H oxidase, which generates superoxide anion and hydrogen peroxide, leads to S-glutathionylation

Figure 2. Western blot analysis to test the presence of NAD(P)H subunits in triads, transverse tubules (TT) and junctional sarcoplasmic reticulum (heavy SR) vesicles devoid of transverse tubules. Isolated triad vesicles contain 10–15% TT still attached to junctional SR membranes. All membrane fractions were isolated from mammalian skeletal muscle as described (Barrientos & Hidalgo 2002). Mouse monoclonal anti-gp91^{phox}, anti-p22^{phox}, anti-p47^{phox} and anti-p67^{phox} antibodies were a kind gift from Dr Mark T. Quinn (Veterinary Molecular Biology, Montana State University, Bozeman, MT).

and stimulation of the neighbouring RyR1 in isolated triad vesicles (Hidalgo *et al.* in preparation). It has been reported that exogenously added hydrogen peroxide stimulates caffeine-induced contractions in skinned skeletal muscle fibres; in contrast, addition of hydrogen peroxide does not affect action potential-generated contractions (Posterino *et al.* 2003). These results support activation by hydrogen peroxide of RyR1-mediated CICR but not of depolarization-induced Ca²⁺ release. Furthermore, in skinned skeletal muscle fibres hydrogen peroxide activates contraction without producing an increase in [Ca²⁺]_i (Andrade *et al.* 1998) or even after causing [Ca²⁺]_i to decrease (Andrade *et al.* 2001). Hence, a definite demonstration of the relevance of RyR1 modifications by hydrogen peroxide during excitation–contraction coupling is still missing (Lamb & Posterino 2003). It is not known, however, if reducing or oxidizing agents externally added to skeletal muscle cells effectively cause RyR1 redox modifications. This issue is important since RyR1 present endogenous levels of S-glutathionylation when isolated (Aracena *et al.* 2005), an indication that RyR1s effectively function *in vivo* with some S-glutathionylated cysteine residues. Although the cellular sources of this RyR1 redox modification are not established, the transverse tubule NAD(P)H oxidase may contribute to this modification. This enzyme may provide direct redox control of RyR1 function, so that increased muscle activity may result in enhanced RyR1 S-glutathionylation and Ca²⁺ release. We have preliminary data indicating that depolarization of muscle cells in culture activates this transverse tubule NAD(P)H oxidase, which in turn stimulates RyR1 activity (Espinosa *et al.* in preparation; P. Aracena, C. Gilman, S. L. Hamilton & C. Hidalgo, unpublished

observations). It remains to be investigated, however, whether RyR1 S-glutathionylation increases following physiological activation of skeletal muscle fibres, and whether this specific RyR1 redox modification affects the physiological excitation–contraction coupling process.

(d) RyR1 S-nitrosylation

There is experimental evidence indicating that NO-induced RyR1 cysteine S-nitrosylation affects muscle function. RyR1 channel S-nitrosylation caused by endogenous NO generation is likely responsible for the reported increase in skeletal muscle contractility observed at physiological pO₂ (Eu *et al.* 2003). In single skeletal muscle fibres from mouse, challenged by short membrane depolarizations under voltage-clamp conditions, NO donors induce a progressive increase in resting [Ca²⁺]_i (Pouvreau *et al.* 2004). High NO levels may produce continuous Ca²⁺ leak from the SR, possibly mediated by redox-modified RyR1s that do not close once they are activated by membrane depolarization. This SR Ca²⁺ leak and the resulting increase in resting [Ca²⁺]_i may be important in mediating the effects of excess NO on voltage-activated Ca²⁺ release.

3. NEURONS

Several neuronal functions, including synaptic plasticity and gene expression, require transient elevation of [Ca²⁺]_i (Berridge 1998; Berridge *et al.* 2000). This increase is initially produced by Ca²⁺ influx through plasma membrane voltage- or receptor-activated Ca²⁺ channels following neuronal stimulation. In addition, Ca²⁺ entry can stimulate Ca²⁺ release from intracellular stores via CICR or depolarization-induced Ca²⁺ release. A variety of experimental findings indicate that Ca²⁺ release from stores elicits diverse neuronal responses (Simpson *et al.* 1995; Chameau *et al.* 2001; Meldolesi 2001; Verkhratsky & Petersen 2002; Bouchard *et al.* 2003; Ouardouz *et al.* 2003; Pape *et al.* 2004; Gafni *et al.* 2004). Through CICR neurons can amplify and propagate the initial Ca²⁺ entry signal, leading to activation of most calcium-regulated transcription factors (Berridge 1998). In particular, RyR-mediated Ca²⁺ release may have a role in synaptic plasticity and gene expression in neurons (Berridge 1998; Futatsugi *et al.* 1999; Berridge *et al.* 2000; Carafoli 2002; Carrasco *et al.* 2004; Verkhratsky 2004). RyR-mediated Ca²⁺ release may also be involved in causing neurodegenerative processes, as discussed below.

Of the three RyR isoforms expressed in different brain regions, RyR2 is the most abundant (Furuichi *et al.* 1994; Giannini *et al.* 1995; Mori *et al.* 2000). Despite the emerging importance of RyR-mediated Ca²⁺ release for brain function, there is limited information on how these different RyR isoforms contribute to the generation and/or regulation of the Ca²⁺ signals that underlie diverse Ca²⁺ dependent neuronal functions. It is known, however, that membrane depolarization activates RyRs in several neuronal types (Chavis *et al.* 1996; Mouton *et al.* 2001; De Crescenzo *et al.* 2004). Yet, the properties and

regulation of RyR channels from brain have been less studied than those of their skeletal or cardiac counterparts.

(a) *Redox modulation of brain RyR*

CICR relies on the fact that RyR are activated by an increase in cytoplasmic Ca^{2+} concentration, from the submicromolar to the micromolar range; in cells this activation occurs in the presence of ATP and Mg^{2+} . We have studied at the single-channel level the activation induced by Ca^{2+} and ATP of native and oxidized RyR channels from rat brain endoplasmic reticulum. Native RyR channels from rat brain cortex incorporated in planar lipid bilayers display three different Ca^{2+} dependencies (Marengo *et al.* 1996), which can be interchanged through modifications of RyR redox state (Marengo *et al.* 1998; Hidalgo *et al.* 2004). In brief, highly reduced channels (a condition in which most RyR1 cysteine residues have free SH groups) respond poorly to Ca^{2+} activation, whereas increasing cysteine oxidation/alkylation increases the channel response to micromolar $[\text{Ca}^{2+}]$ and decreases the inhibitory effect of higher $[\text{Ca}^{2+}]$. Noteworthy, reducing agents reverse all these changes whereas ATP differentially activates RyR channels depending on their Ca^{2+} response, so that more oxidized channels from brain require less ATP to attain maximal activation (Bull *et al.* 2003). We have found that modification of brain RyR cysteine residues with thimerosal decreases the inhibitory effect of Mg^{2+} on RyR activity whereas reducing agents have the opposite effect (R. Bull *et al.* in preparation). Changes in the redox state of RyR channels from brain affect as well RyR modulation by glucosylceramide (Lloyd-Evans *et al.* 2003), a type of glycosphingolipid that may be implicated in the cell death observed in Alzheimer's disease (Cutler *et al.* 2004) and HIV dementia (Haughey *et al.* 2004). Interestingly, the finding that glucosylceramide augments agonist-stimulated Ca^{2+} release via RyR channels (Lloyd-Evans *et al.* 2003) may be relevant to understand the development of pathological conditions that entail a faulty regulation of Ca^{2+} homeostasis in pathological conditions.

(b) *Participation of RyR-mediated Ca^{2+} release in long-term potentiation induction and CREB activation in the hippocampus*

Activation of the nuclear transcription factor CREB (cAMP/calcium response element binding protein) is crucial for several neuronal functions, including activity-dependent synaptic plasticity (Silva *et al.* 1998; Lonze & Ginty 2002). Activation of CREB and the ensuing CRE-mediated transcription are required for induction of long-term potentiation (LTP), a reversible increase in synaptic transmission inducible in several brain regions in response to tetanic stimulation of afferent fibres. LTP is considered a cellular model of synaptic plasticity that may underlie higher brain functions such as learning and memory (Lynch 2004). A number of genes activated by neuronal electrical activity, among them c-fos and BDNF, have functional CRE sequences in their promoters (Sheng *et al.* 1990; Tao *et al.* 1998); in the

last few years these findings have prompted many studies on the cellular factors that regulate CREB activation.

Participation of Ca^{2+} release from intracellular stores in LTP induction has been described (Auerbach & Segal 1994; Wang *et al.* 1996; Reyes-Harde *et al.* 1999; Lu & Hawkins 2002; Lauri *et al.* 2003; Matias *et al.* 2003; Lynch 2004). LTP induction in most hippocampal synapses requires a rise in intracellular postsynaptic $[\text{Ca}^{2+}]$ mediated by N-methyl-D-aspartate (NMDA) receptors, with contributions from L-type channel activation and intracellular Ca^{2+} stores (Matias *et al.* 2003; Lynch 2004). In the CA1 hippocampal region, RyR blockade significantly reduces tetanic-, NO- and cGMP-induced LTP, and decreases P-CREB immunofluorescence in postsynaptic neurons of hippocampal slices (Lu & Hawkins 2002). These results point to a well-defined postsynaptic location of the RyR channels involved in hippocampal LTP induction. A presynaptic effect of NO resulting in induction of long-term depression (LTD) as well as LTP in the same hippocampal region has also been described (Reyes-Harde *et al.* 1999). A contribution of RyR, in this case presynaptic, has been suggested by the marked reduction of LTD produced by ryanodine when used at inhibitory concentrations. Interestingly, a switch from LTD to LTP was induced at the hippocampal dentate gyrus by a low stimulatory ryanodine concentration, suggesting that RyR-mediated Ca^{2+} release plays a significant role in LTP induction (Wang *et al.* 1996).

Presynaptic RyR also contribute to LTP generation in mossy hippocampal fibres (Lauri *et al.* 2003). This form of LTP is independent of NMDA receptors; instead, Ca^{2+} entry is mediated by presynaptic kainate glutamate receptors, which initiate a calcium-signalling cascade involving RyR-mediated Ca^{2+} release that is abolished by inhibitory ryanodine concentrations. In addition, caffeine (10 mM) can evoke LTP in CA1 hippocampal neurons, which is different to the classical LTP since it does not require postsynaptic receptor activation or Ca^{2+} increase in this area (Martin & Buño 2003). This new form of LTP is generated presynaptically, through the interaction of caffeine with presynaptic purinergic receptors and RyR channels. The resulting increased Ca^{2+} signals enhance neurotransmitter release.

In the hippocampus, Ca^{2+} -dependent activation of the Ras/ERK (extracellular signal-regulated kinase) pathway is essential for long-term CREB phosphorylation and CREB-dependent transcription of genes involved in synaptic plasticity (Hardingham & Bading 1999; Impey & Goodman 2001; Thiels & Klann 2001; Wu *et al.* 2001; Adams & Sweatt 2002; Dolmetsch 2003; Lynch 2004). Furthermore, functionally active neurons display increased metabolic activity plus increased oxygen consumption and generation of ROS/RNS (Davis *et al.* 1998; Yermolaieva *et al.* 2000). Several studies indicate that ROS/RNS may regulate synaptic plasticity. Activity-dependent nitric oxide generation has been associated with synaptic plasticity and Ca^{2+} signalling (Peunova & Enikolopov 1993; Yermolaieva *et al.* 2000), including Ca^{2+} -dependent activation of CREB-phosphorylation in

the hippocampus (Lu & Hawkins 2002). Activation of NMDA receptors generates superoxide anion in rat hippocampal pyramidal neurons in culture and in brain hippocampal slices (Bindokas *et al.* 1996), while cell-permeable scavengers of superoxide anion block LTP induction in area CA1 of the hippocampus (Klann 1998). Noteworthy, transgenic mice that overexpress extracellular superoxide dismutase (a superoxide anion scavenger) exhibit impaired LTP in hippocampal area CA1, despite normal LTP in area CA3, and also show defects in memory acquisition (Thiels *et al.* 2000). These findings strongly suggest that superoxide anion (or its derivative, hydrogen peroxide) is a required signalling molecule for LTP induction and normal neuronal function. Yet, dissimilar results on the effects of hydrogen peroxide on hippocampal LTP induction have been reported (Kamsler & Segal 2004). Initial augmentation, and subsequent long-lasting depression, of population spikes and excitatory postsynaptic potentials by hydrogen peroxide (0.3–2.9 mM) has been described in the CA1 region of rat hippocampal slices (Katsuki *et al.* 1997). In contrast, low concentrations of hydrogen peroxide (1 μ M) cause a twofold increase in tetanic LTP and enhance NMDA-independent LTP in the hippocampus compared to controls (Kamsler & Segal 2003).

The preceding discussion of the literature indicates that LTP induction in hippocampal neurons entails a combined increase of $[Ca^{2+}]_i$, ROS and RNS. All these factors can potentially contribute to enhance RyR-mediated Ca^{2+} release; the resulting amplified Ca^{2+} signals may in turn enhance the long-lasting Ca^{2+} -dependent ERK/CREB phosphorylation cascade required for LTP induction. Hence, it is of interest to investigate whether ROS/RNS stimulate the Ca^{2+} -dependent Ras/ERK pathway through stimulation of RyR-mediated CICR. To this aim, we have studied the effects of hydrogen peroxide and membrane depolarization on ERK/CREB phosphorylation in neurons with functional or inhibited RyR. We exposed N2a and hippocampal cells in primary culture cells to hydrogen peroxide (Carrasco *et al.* 2004; Kemmerling *et al.* submitted). In both cell types, exogenous hydrogen peroxide activates CREB and ERKs and promotes RyR-dependent Ca^{2+} release; these effects are independent of extracellular Ca^{2+} and are inhibited significantly by ryanodine concentrations that block RyR-mediated Ca^{2+} release. Furthermore, thapsigargin antagonizes the increase in P-CREB levels induced by hydrogen peroxide, which also promotes RyR *s*-glutathionylation (Kemmerling *et al.* submitted). These results suggest that oxidative stimulation of RyR-mediated Ca^{2+} release is an intermediate step in the activation of the ERK/CREB phosphorylation cascade required for synaptic plasticity (figure 3). We also found that depolarization induced by high K^+ concentration stimulates CREB phosphorylation in the neuroblastoma cell line N2a (Carrasco *et al.* 2004). Inhibitory concentrations of ryanodine partially reduced depolarization-induced CREB phosphorylation, suggesting that RyR-mediated Ca^{2+} release contributes to the observed stimulation.

Figure 3. Proposed model to illustrate the potential role of neuronal RyR as coincidence detectors of a simultaneous increase in $[Ca^{2+}]_i$ and ROS/RNS. As discussed in the text, stimulation of postsynaptic NMDA receptors (NMDAR) in hippocampal neurons elicits Ca^{2+} entry and promotes ROS generation; the resulting localized $[Ca^{2+}]_i$ increase stimulates the Ca^{2+} -dependent neuronal NO synthase that is attached to the postsynaptic membrane, resulting in the generation of NO and possibly of other NO-derived RNS. The model proposes that the concomitant local increases in Ca^{2+} and ROS/RNS activate jointly RyR-mediated Ca^{2+} release from the endoplasmic reticulum (ER), generating larger Ca^{2+} signals. The resulting RyR-mediated Ca^{2+} signals would stimulate Ca^{2+} -dependent signalling cascades, such as the Ras MAP kinase pathway, producing increased ERK1/2 phosphorylation. Translocation of phospho-ERK1/2 (P-ERK1/2) to the nucleus (N) would stimulate the sustained phosphorylation of the transcription factor CREB, which is a requisite for transcription of genes involved in synaptic plasticity. According to this model, cross talk between Ca^{2+} and redox signals through joint stimulation of RyR-mediated Ca^{2+} release may play a key role in hippocampal synaptic plasticity. Furthermore, conditions that promote oxidative/nitrosative stress may result in excessive stimulation of RyR-mediated Ca^{2+} release that if unchecked can provoke pathological responses and neuronal death.

4. CONCLUSIONS

Through either depolarization-induced Ca^{2+} release or via CICR, RyR channels have a pivotal role in the generation or amplification of Ca^{2+} signals that are required for muscle contraction and neuronal plasticity. Endogenous activation of RyR channels by cellular ROS/RNS may represent a physiological mechanism of cross talk between Ca^{2+} and redox signalling pathways. Thus, cells may use redox-modulated RyR-mediated Ca^{2+} release as an additional mechanism to either amplify or inhibit Ca^{2+} signals as needed for a specific response. In neurons, generation of ROS/RNS such as hydrogen peroxide or NO, which may act as diffusible signal molecules in synaptic plasticity, may modify cellular processes that depend on RyR-mediated Ca^{2+} release from the ER, including LTP and LTD, and presumably learning and memory. Oxidative stress and alterations in Ca^{2+} homeostasis may contribute to neuronal apoptosis and excitotoxicity, which may underlie the pathogenesis of several neurodegenerative disorders

(Mattson 2000; Mattson & Chan 2003). In particular, RyR channels may be involved in the pathophysiology of neurodegeneration in Alzheimer's disease (Kelliher *et al.* 1999; Chan *et al.* 2000; Mungarro-Menchaca *et al.* 2002). As discussed here, oxidative stress is likely to enhance RyR-mediated CICR in neurons. Thus, redox modification of RyR channels may have physiological and pathological consequences for neuronal function.

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