Nitric oxide-induced calcium release: Activation of type 1 ryanodine receptor by endogenous nitric oxide

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Abstract

Ryanodine receptors (RyRs), located in the sarcoplasmic/endoplasmic reticulum (SR/ER) membrane, are required for intracellular Ca^{2+} release that is involved in a wide range of cellular functions. In addition to Ca^{2+}-induced Ca^{2+} release in cardiac cells and voltage-induced Ca^{2+} release in skeletal muscle cells, we recently identified another mode of intracellular Ca^{2+} mobilization mediated by RyR, i.e., nitric oxide-induced Ca^{2+} release (NICR), in cerebellar Purkinje cells. NICR is evoked by neuronal activity, is dependent on S-nitrosylation of type 1 RyR (RyR1), and is involved in the induction of long-term potentiation (LTP) of cerebellar synapses. In this addendum, we examined whether peroxynitrite, which is produced by the reaction of nitric oxide with superoxide, may also have an effect on the Ca^{2+} release via RyR1 and the cerebellar LTP. We found that scavengers of peroxynitrite have no significant effect either on the Ca^{2+} release via RyR1 or on the cerebellar LTP. We also found that an application of a high concentration of peroxynitrite does not reproduce neuronal activity-dependent Ca^{2+} release in Purkinje cells. These results support that NICR is induced by endogenous nitric oxide produced by neuronal activity through S-nitrosylation of RyR1.

There are an increasing number of studies showing that protein S-nitrosylation, the addition of a nitric oxide (NO) group to cysteine thiols, provides important roles in a wide range of signaling pathways.[1,2]   Because the ryanodine receptor (RyR), a Ca^{2+} release channel expressed in the SR/ER, has about 100 cysteine residues per subunit and almost half of these thiols are kept in a reduced state under the resting condition,[3] RyR is thought to be one of the substrate proteins for S-nitrosylation.   In accordance with this notion, the open probability of RyR1 measured in lipid bilayers is increased by NO donors,[4-7] and this increase is accompanied by an increase in S-nitrosylation of the Ca^{2+} release channel at cysteine 3635.[4,7-9]   In addition, NO-induced activation of RyR1 in lipid bilayers and in SR vesicles is influenced by pO2 levels: RyR1 is activated by submicromolar levels of NO in the tissue pO2 level (~10 mmHg) but not in the ambient O2 level (~150 mmHg).[4]   In these previous studies, the redox regulations of RyRs were extensively studied using *in vitro* experimental systems, especially in lipid bilayers and SR samples isolated from skeletal muscles.[1,10,11]   Although S-nitrosylation of RyR1 is implicated in the enhancement of Ca^{2+} leak from the skeletal muscle SR in pathological conditions,[12,13] physiological significance of S-nitrosylation-mediated modulation of RyR1 activity by endogenous NO remained elusive.

We previously found that burst stimulation (BS) of parallel fibers (PFs) generates a localized increase in NO concentration in Purkinje cell (PC) dendrites, as well as NO-dependent long-term potentiation (LTP) at the PF-PC synapse in the cerebellum.[14]   Because this LTP is also sensitive to the intracellular Ca^{2+} concentration, we examined a crosstalk between NO and Ca^{2+} signaling in PCs.   Bath application of 1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7), a NO donor, induced an increase in the intracellular Ca^{2+} concentration in PCs.   The NO-induced Ca^{2+} increase was independent of Ca^{2+} influx from the extracellular medium, but was dependent on the activity of RyR1 and the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), suggesting that the Ca^{2+} increase is mediated by Ca^{2+} release from the intracellular store through RyR1.   In addition, our study using RyR1-expressing HEK293 cells indicated that S-nitrosylation of RyR1 at cysteine 3635 is both necessary and sufficient for the NO-induced Ca^{2+} increase, which is hereafter referred to as "NO-induced Ca^{2+} release (NICR)".[15]   We then showed that BS to PFs induces intracellular Ca^{2+} release in PC dendrites. When BS which induces NO-dependent-LTP was applied to PF, Ca^{2+} levels were transiently but clearly elevated in PC dendrites.   This BS-induced Ca^{2+} increase was indicated to be due to Ca^{2+} release mediated by RyR1,

4

because the Ca^{2+} increase was abolished by the application of thapsigargin and cyclopiazonic acid, SERCA inhibitors, and dantrolene, a RyR antagonist[16,17]. In addition, the Ca^{2+} release was inhibited by the application of N^G-Nitro-L-arginine methyl ester (L-NAME), a broad inhibitor of NO synthases (NOS), and was not observed in the cerebellum of mice deficient in neuronal type NOS (nNOS).[18] These results strongly suggest that BS to PFs induces NICR in the PC dendrites.

NO signaling has two types of downstream pathways: activation of soluble guanylyl cyclase (sGC) and S-nitrosylation of target proteins. Because BS-induced NICR was inhibited by intracellular application of ascorbic acid, a reducing reagent, to Purkinje cells, but not by 1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase, NICR was suggested to be dependent on S-nitrosylation of proteins including RyR1. Correspondingly, biochemical analysis demonstrated that NICR induced by the application of NOC7 is accompanied with S-nitrosylation of RyR1 in cerebellar slice preparations. Furthermore, all manipulations that we used to inhibit NICR also abolished PF-LTP. Taken together, our study indicated that NICR is a novel Ca^{2+} mobilizing mechanism in neuronal cells and is essential for the induction of PF-LTP.

However, neuronal activity in conjunction with certain forms of synaptic

plasticity may be associated with superoxide generation,[19] and NO

generation leads to peroxynitrite formation when superoxide is

simultaneously generated.[20]   Because peroxynitrite is known to regulate

cell signaling via molecular modifications, including protein nitration,[20] it

seems possible that NICR is affected by peroxynitrite produced from

endogenous NO in the presence of superoxide.   We, therefore, examined the

potential role of peroxynitrite in the BS-induced Ca^{2+} release and LTP.

Bath application of the peroxynitrite scavenger, uric acid,[21] and the

peroxynitrite decomposition catalyst,

5,10,15,20-Tetrakis(4-sulfonatophenyl)prophyrinato iron (III) (FeTPPS),[22]

had no effect on BS-induced Ca^{2+} increase (Fig. 1), although the

concentration of uric acid (100 µM) and FeTPPS (10 µM) were thought to be

high enough.[23,24]   We subsequently examined effects of uric acid and

FeTPPS on the induction of PF-LTP.   In accordance with the insensitivity

of NICR to these reagents, neither uric acid nor FeTPPS impaired the

induction of PF-LTP induced by BS (Fig. 2).   These results do not support

the involvement of peroxynitrite in NICR and PF-LTP induction, although

there remains a possibility that the scavengers did not completely

antagonizing the effect of peroxynitrite.   We therefore examined the effect

of an application of peroxynitrite on the intracellular Ca^{2+} concentration of

Purkinje cells in cerebellar slice preparations. Because our previous NO imaging experiments during BS-induced NO generation from parallel fibers indicates that the peak NO concentration in the center of response is about 5 μM,[14] we applied 10 μM peroxynitrite to the cerebellar slice preparations. However, even at this very high concentration, peroxynitrite induced only very little changes in the Ca^{2+} concentration (Fig. 1D). The slow increase in the Ca^{2+} concentration became absent in the presence of uric acid or FeTPPS. However, it was resistant to an intracellular application of 10 mM ascorbic acid, which completely abolishes BS-induced NICR.[15] Therefore, the peroxynitrite-dependent slow response seems to have a mechanism different from that of NICR and might be accounted for by an irreversible effect of peroxynitrite on the endoplasmic reticulum that has been reported previously.[5] These results suggest that the role of peroxynitrite, if any, in BS-induced Ca^{2+} release is very little.

In conclusion, in combination with the observations in our previous study,[15] it is suggested that NICR is dependent on the NO-dependent S-nitrosylation of RyR1 rather than on indirect actions of peroxynitrite or cyclic GMP (Fig. 3). Biochemical analysis of RyR1 purified from the cerebellar cortex after the LTP induction may further confirm specific involvement of endogenous NO in NICR and PF-LTP.

7

Materials and Methods

Preparation of cerebellar slices, Ca^{2+} imaging and electrophysiological analysis were carried out essentially as described previously.[15,25]   C57BL/6 mice (postnatal days 24–32) were sacrificed by cervical dislocation under anesthesia with diethyl ether. The cerebellum was excised, and parasagittal cerebellar slices (250 µm thick) were prepared from the vermis.   Whole-cell recordings were obtained from visually identified PCs at room temperature (23–25°C).

For intracellular Ca^{2+} imaging in PCs, a Ca^{2+}-sensitive dye, Oregon Green 488 BAPTA-1 (100 µM), was introduced into Purkinje cells through the patch pipette.   Five to nine sequential confocal images (excitation at 488 nm), obtained at 3–4 µm z-axis intervals, were acquired every 0.8 s using an upright microscope (BX51WI; Olympus) equipped with a confocal scanning unit and an argon laser (FV300, Olympus), and projected onto a plane to obtain dendrite images at 10 s intervals.

For focal stimulation of PFs, a stimulation pipette was filled with standard bathing solution and used to apply square pulses (0.1 ms in duration, 0–20 V in amplitude) to the molecular layer, specifically in the middle portion of the molecular layer approximately one-third of the

distance below the pial surface. The intensity of each stimulus was adjusted to evoke PF-EPSCs with an amplitude of 60–120 pA. The ionic current was recorded from PCs using a patch-clamp amplifier (EPC-9, HEKA, Lambrecht/Pfalz, Germany) at a holding potential of −90 or −80 mV, after compensating for liquid junction potential. The signals were filtered at 2 kHz and digitized at 20 kHz.　After obtaining a stable initial recording for at least 10 min, 60 burst stimulations (1 burst stimulation: 5 pulses at 50 Hz) were repeatedly applied at 1 Hz to induce LTP.　Series resistance and membrane resistance were monitored throughout the experiments, and the data were discarded when either of these resistances varied by more than 10%.　The data were also discarded when the slope of PF-EPSC amplitude averaged every minute during the initial recording for 10 min was larger than 2% or when the amplitude did not become stable within 20 min after the onset of whole-cell configuration.[14]

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Figure Legends

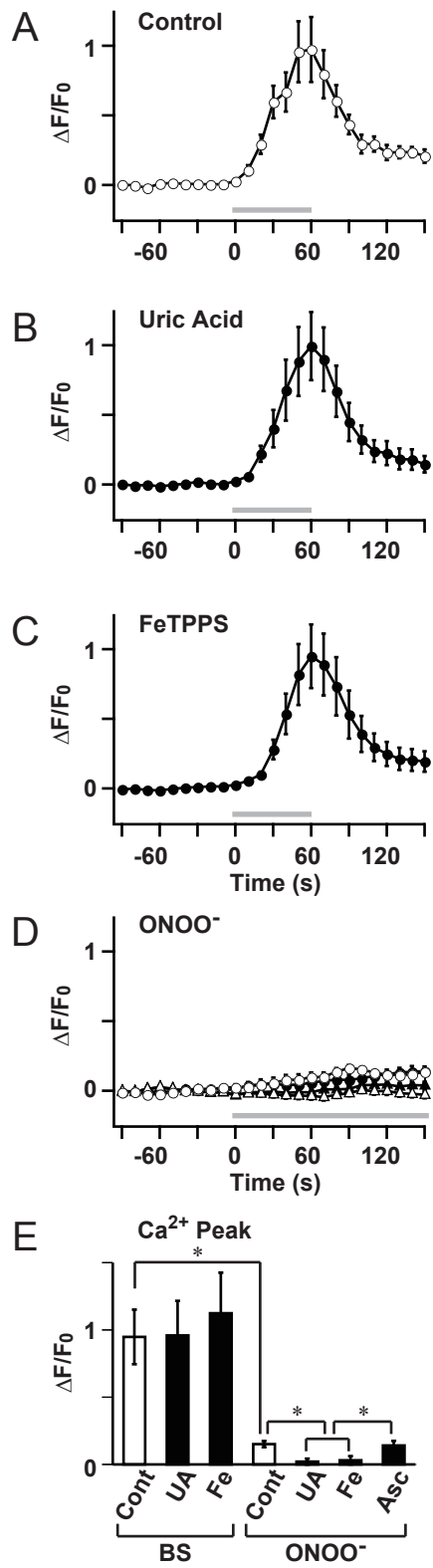**Figure 1.** NO-induced Ca^{2+} release (NICR) is not affected by peroxynitrite. (A-C) Ca^{2+} response, induced by a series of 60 BS at 1 Hz (BS; Gray bar) in the standard ACSF (A; data were obtained from 10 cells in 10 slices), was not inhibited by peroxynitrite scavenger, uric acid (B; 100 μM, n = 7 cells in 7 slices), or peroxynitrite decomposition catalyst, FeTTPS (C; 10 μM, n = 6 cells in 6 slices). Uric acid or FeTPPS was present in bathing solution from 30 min before the recording. (D) Ca^{2+} response to bath application of 10 μM peroxynitrite ($ONOO^-$) (open circle; n = 6 cells, 6 slices). It was abolished by 100 μM uric acid (open triangle; n = 7 cells, 7 slices) or 10 μM FeTPPS (closed triangle; n = 7 cells, 7 slices), but was insensitive to 10 mM ascorbic acid applied intracellularly through the patch pipette (closed circle; n = 8 cells; 8 slices). (E) Summary of data in A–D. The peak Ca^{2+} responses are shown. UA: uric acid; Fe: FeTPPS; Asc: ascorbic acid. Data are presented as Mean ± SEM. *p < 0.05, ANOVA / Tukey-Kramer test.

Figure 2. Cerebellar LTP is not affected by peroxynitrite. (A-C) Cerebellar LTP, induced by BS in the standard ACSF (A; n = 8 cells in 8
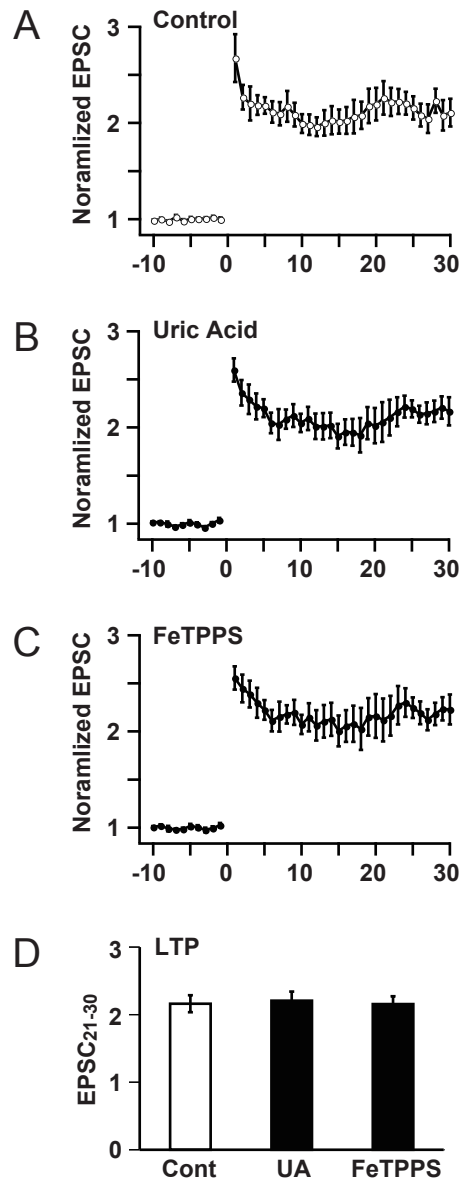
slices), was not inhibited by 100 μM uric acid (B; n = 5 cells in 5 slices) or

10 μM FeTPPS (C; n = 5 cells in 5 slices).　(D) Effects of uric acid (UA) and

FeTPPS on the magnitude of LTP (normalized EPSC averaged between

21-30 min after BS).　Data are presented as Mean ± SEM.

Figure 3.　A schematic diagram of signaling pathways for nitric

oxide-induced Ca^{2+} release (NICR) in cerebellar Purkinje cells.　Nitric

oxide (NO) is produced at the parallel-fiber terminal in response to

neuronal activity, and diffuses into the cerebellar Purkinje cell.　Then, type

1 ryanodine receptors (RyR1), located in the endoplasmic reticulum (ER)

membrane, are S-nitrosylated and activated, resulting in Ca^{2+} release from

the ER.　Cyclic GMP and peroxynitrite, which is produced by the reaction

of NO and superoxide, are unlikely to be involved in NICR.　nNOS:

neuronal NO synthase; IP_3R1: inositol 1,4,5-trisphosphate receptor;
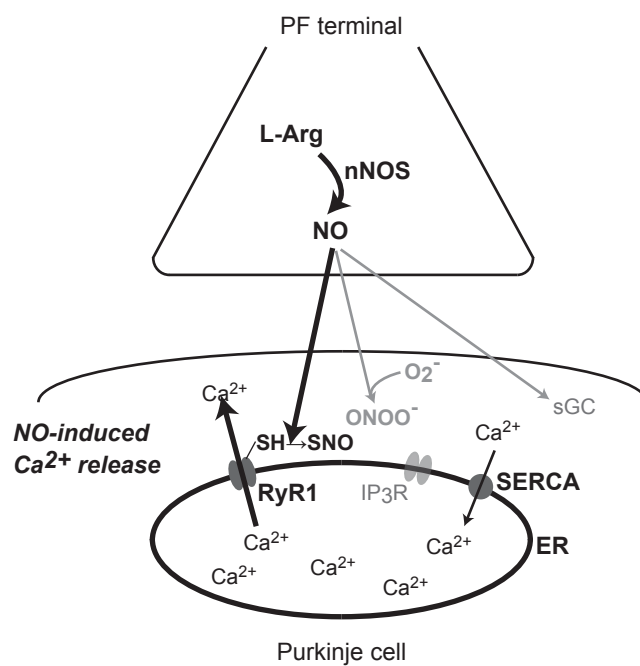
SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase.

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PF terminal

L-Arg
nNOS
NO

O_2^-

$ONOO^-$

sGC

Ca^{2+}

NO-induced
Ca^{2+} release

SH→SNO

Ca^{2+}

RyR1

IP3R

SERCA

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

ER

Purkinje cell

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