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Type-3 ryanodine receptor involved in Ca^{2+}-induced Ca^{2+} release and transmitter exocytosis at frog motor nerve terminals

Masakazu Kubota [a,1], Kazuhiko Narita [d], Takashi Murayama [c], Shinichi Suzuki [a], Satoko Soga [a], Jiro Usukura [b], Yasuo Ogawa [c], Kenji Kuba [a,e,*]

[a] *Department of Physiology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[b] *Department of Anatomy, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[c] *Department of Pharmacology, Juntendo University, School of Medicine, 2-2-1 Hongo, Bunkyoku, Tokyo 113-8421, Japan*
[d] *Department of Physiology, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-0192, Japan*
[e] *Laboratory of Anatomy and Physiology, Faculty of Nutrition, Nagoya University of Arts and Sciences, 57 Takenoyama, Iwasaki-cho, Nissin, Aichi 470-0196, Japan*

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Abstract

Ca^{2+}-induced Ca^{2+} release (CICR) occurs in frog motor nerve terminals after ryanodine receptors (RyRs) are primed for activation by conditioning large Ca^{2+} entry. We studied which type of RyR exists, whether CICR occurs without conditioning Ca^{2+} entry and how RyRs are primed. Immunohistochemistry revealed the existence of RyR3 in motor nerve terminals and axons and both RyR1 and RyR3 in muscle fibers. A blocker of RyR, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) slightly decreased rises in intracellular Ca^{2+} ($[Ca^{2+}]_i$) induced by a short tetanus (50 Hz, 1–2 s), but not after treatment with ryanodine. Repetitive tetani (50 Hz for 15 s every 20 s) produced repetitive rises in $[Ca^{2+}]_i$, whose amplitude overall waxed and waned. TMB-8 blocked the waxing and waning components. Ryanodine suppressed a slow increase in end-plate potentials (EPPs) induced by stimuli (33.3 Hz, 15 s) in a low Ca^{2+}, high Mg^{2+} solution. KN-62, a blocker of Ca^{2+}/calmoduline-activated protein kinase II (CaMKII), slightly reduced short tetanus-induced rises in $[Ca^{2+}]_i$, but markedly the slow waxing and waning rises produced by repetitive tetani in both normal and low Ca^{2+}, high Mg^{2+} solutions. Likewise, KN-62, but not KN-04, an inactive analog, suppressed slow increases in EPP amplitude and miniature EPP frequency during long tetanus. Thus, CICR normally occurs weakly via RyR3 activation by single impulse-induced Ca^{2+} entry in frog motor nerve terminals and greatly after the priming of RyR via CaMKII activation by conditioning Ca^{2+} entry, thus, facilitating transmitter exocytosis and its plasticity.

Keywords: Ca^{2+}-induced Ca^{2+} release; Type-3 ryanodine receptor; Presynaptic terminal; Transmitter exocytosis; Plasticity; Frog neuromuscular junction

1. Introduction

Much evidence has been accumulated for activation of CICR [1–4] via ryanodine receptors (RyRs) in neurons [4–11] (see reviews [12–14]). Its physiological roles in the regulation of the cell membrane excitability [15–20] and neuronal differentiation [21] have well been established. Evidence for the role of CICR in synaptic transmission, however, is limited to certain types of presynaptic terminals [22–29].

Frog neuromuscular junction had been the preparation that provided the basic concept of transmitter release mechanism at presynaptic nerve terminals. Ca^{2+} entry through voltage-gated Ca^{2+} channels is well known to activate a sequence of events for transmitter exocytosis [30]. Recent experiments in frog motor nerve terminals, however, have shown strong evidence for the role of CICR as an additional origin of Ca^{2+} in

* Corresponding author. Tel.: +81 561 75 2559; fax: +81 561 75 2559.
E-mail address: kubak@nuas.ac.jp (K. Kuba).
[1] Present address: Department of Sports Medicine, Graduate School of Medicine, Nagoya University, Nagoya 464-8601, Japan.

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the activation of transmitter exocytosis [22,23]. CICR in frog motor nerve terminals has unique properties that the full activation requires the conditioning large Ca^{2+} entry by repetitive nerve activity, which converts the state of ryanodine receptors from the 'sleeping' (incapable of being activated) to an activable state [22]. Henceforce, we call this mechanism of conversion the priming of RyR or CICR. A further study showed that this mode of activation of CICR contributes to the generation of short-forms of synaptic plasticity, augmentation and potentiation [23].

Among many unsolved problems as to the mechanism of CICR in frog motor nerve terminals, three specific questions were asked in this study. First, what is the type of RyR in frog motor nerve terminals? Two isoforms of RyR, type 1(RyR1) and type 3 (RyR3), which are also referred to as α-RyR and β-RyR, respectively, exist in almost equal amount in frog skeletal muscle [31,32]. mRNAs of both isoforms were also detected in frog brain [32]. However, it is not known which type of RyR exists in the frog motor nerve terminals. Second, does CICR occur in response to impulse-induced Ca^{2+} entry even without the conditioning Ca^{2+} entry to prime RyRs? Third, how RyRs are primed by repetitive Ca^{2+} entries into the terminals? The mechanism may involve a Ca^{2+}-dependent signaling process.

We studied these issues by applying histochemical, Ca^{2+} imaging and intracellular recording techniques to frog motor nerve terminals. Antibodies raised against frog RyR1 and RyR3 or both were used to solve the first issue, while blockers of RyR and Ca^{2+}/calmoduline-activated kinase II (CaMKII) were applied to clarify the second and third issues. The results indicate that CICR normally occurs via activation of RyR3 to some extent in response to single impulse-induced Ca^{2+} entry in frog motor nerve terminals, contributing to transmitter exocytosis to a considerable extent. They further suggest that CaMKII activation by a large Ca^{2+} entry is involved in the priming of the mechanism of CICR.

2. Materials and methods

2.1. Immunohistochemistry

Frogs (*Rana nigromaculata*) were decapitated and cutaneous pectoris muscles were isolated with the innervating nerve. The muscles were fixed with 4% paraformaldehyde in sodium phosphate-buffered solution (0.11 M, pH 7.4: PBS) at 4 °C for 2 h, washed with PBS, permeabilized with 0.5% Triton X-100 in PBS containing 4% bovine serum albumin (BSA) at room temperature for 2 h and rinsed with PBS. The muscles were exposed to an antibody against frog RyR1, RyR3 or both in PBS with 1% bovine serum albumin at room temperature at the IgG concentration of 1/100 dilution.

Antibodies used were polyclonal antibodies raised against particular sequences of peptides: for anti-RyR1 antibody, RTKKKRRGDRYSVQ, which corresponds to 3478–3491 of RyR1; for RyR3 antibody, KKRRRGQKVEKPE (4369–4381 of mammalian RyR3, which was the same as that of frog RyR3 except for K at 4377 instead of V) [33]; and for anti-pan RyR antibody, HPASKQRSEGEKVR (151–164 of RyR1 and 152–165 of RyR3) [34]. These antibodies were produced in rabbits and affinity-purified by procedures described previously [31]. Anti-RyR1 and anti-RyR3 antibodies react specifically with RyR1 and RyR3, respectively, and there was no cross-reaction between them. Anti-pan RyR antibody reacts with all kinds of RyR isoforms, whose entire sequences were reported. Mouse monoclonal anti-chicken ryanodine receptor antibody (34C, Biomol, Plymouth, PA) [35] that reacts with both frog RyR1 and RyR3 was also used. In some experiments, each of these peptides was added to the PBS containing the corresponding antibody.

The preparations were stained with Alexa 488-conjugated rabbit or mouse IgG (Molecular Probes) at the concentration of 15 μg/ml in PBS containing 1% bovine serum albumin at room temperature for 1 h. The muscles were mounted with an anti-fading agent (ProLong, Molecular Probes). Fluorescence images were taken by a confocal scan unit (MRC-600, Nippon BIO-RAD) attached to an inverted microscope (Nikon TMD-300, Water immersion 40× objective, NA 1.15). Stained muscles were scanned in a *X–Y* plane with blue laser (488 nm, 0.15–0.45 mW) at *Z* levels varied at a step of 0.2 or 0.3 μm. The diameter of the confocal aperture was set to be 1.9–3.1 mm for the images of the tissues stained with anti-RyR1, anti-RyR3 and anti-pan RyR antibody. The 3.1 mm diameter yielded the lateral and axial resolutions of 0.15 μm and 1.25 μm, respectively. For the images of the tissues stained with 34C antibody and those with anti-RyR1, anti-RyR3 or anti-pan RyR antibody in the presence of the corresponding epitope, the diameter was set to be maximum (9.3 mm).

2.2. Ca^{2+} imaging and intracellular recording

Experimental procedures are essentially similar to those described [23]. Frogs (*R. nigromaculata*) were anesthetized by cooling and cutaneous pectoris muscles were isolated with the innervating nerve. The composition of normal Ringer's solution (mM) was NaCl, 112; KCl, 2; $CaCl_2$, 1.8; glucose, 5.0; Hepes-Na, 5.0 (pH 7.4). K-salt of Oregon Green BAPTA-1 (OGB-1) was loaded into the terminals as described previously [22]. Fluorescence of OGB-1 was measured with a cooled CCD-camera (Argus/HiSca, C6790-81, Hamamatsu Photonics, Hamamatsu, Japan) through an image intensifier (Stardancer 2, Videoscope International, Sterling, VA) attached to an upright microscope (Zeiss Axioscope, objective 60× water/N.A. 0.95, Karl Zeiss Japan, Tokyo). The ratios of the images during and after nerve stimulation to that before stimulation were analyzed (Argus or Aquacosmos, Hamamatsu Photonics, Hamamatsu, Japan [23]). The ratios were converted to $[Ca^{2+}]_i$ values using the equation