Functional Properties of Ryanodine Receptors in Hippocampal Neurons Change During Early Differentiation in Culture

MANANA SUKHAREVA, SUSAN V. SMITH, DRAGAN MARIC, AND JEFFERY L. BARKER
Laboratory of Neurophysiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

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Sukhareva, Manana, Susan V. Smith, Dragan Maric, and Jeffery L. Barker. Functional properties of ryanodine receptors in hippocampal neurons change during early differentiation in culture. J Neurophysiol 88: 1077–1087, 2002; 10.1152/jn.00557.2001. 6-(4,4-difluoro-5,7-dimethyl-1-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)hexanic acid ryanodine (BODIPY-ryanodine) binding and Ca2+ imaging were used to study the properties of ryanodine receptors (RyRs) and cytoplasmic Ca2+ (Ca2+cyt) changes in neurons cultured from the embryonic rat hippocampus during the earliest stages of differentiation. Baseline Ca2+ levels declined from 164 ± 5 (SD) nM at early stages to 70 ± 4 nM in differentiated neurons. Fluorescent BODIPY-ryanodine binding signals identified activated RyRs in somata, which were eliminated by removal of external Ca2+. Activated RyRs in somata, which were eliminated by removal of external Ca2+. Identified activated RyRs in somata, which were eliminated by removal of external Ca2+. Direct removal of extracellular calcium by removal of external Ca2+ or by blockage of Ca2+ entry through L-type but not N-type Ca2+ channels. The GABA synthesis inhibitor 3-mercaptopropionic acid completely abolished ryanodine binding. Caffeine or K+-depolarization inhibited the activity of RyRs at very early stages of differentiation but had stimulatory effects at later stages after a network of processes had formed. BayK-8644 stimulated RyRs throughout all regions of all differentiating cells. The results suggest that in differentiating embryonic hippocampal neurons the activity of RyRs is maintained via Ca2+ entering through L-type Ca2+ channels. The mode of activation of L-type voltage-gated Ca2+ channels with either membrane depolarization or specific pharmacological agents affects the coupled activity of RyRs differently as neurons differentiate processes and networks.

INTRODUCTION

Changes in cytoplasmic free Ca2+ concentration ([Ca2+]cyt) play an ubiquitous and crucial role in intracellular signaling in excitable and nonexcitable cells. In muscle, [Ca2+]cyt changes regulate contraction (Fraznini-Armstrong 1999). In neurons, changes in [Ca2+]cyt regulate excitability, neurotransmitter release, synaptic plasticity, gene expression, cell growth, and cell death (Berridge 1998; Kennedy 1989; Korkotian and Segal 1998; Llano et al. 2000; Marks 1997; Mothet et al. 1998; Narita et al. 2000; Niishiyama et al. 2000). Although Ca2+ release from intracellular stores is clearly involved in these processes, little is known about the mechanisms that govern Ca2+ release during development of the CNS and at peripheral sites between the CNS and target cells. Ca2+ release occurs from Ca2+-storing organelles including the sarcoplasmic reticulum (SR) in muscle and the endoplasmic reticulum (ER) in all other types of cells. Neurons have an ER that begins in the soma and extends into the dendrites and the axon (Berridge 1998). Within the soma and the dendritic region, some portions of the ER come into close contact with the plasma membrane to form subsurface cisternae (Berridge 1998). Immunohistochemical and autoradiographic studies have detected a widespread and heterogeneous distribution of ryanodine receptors (RyRs) throughout the CNS (Nakanishi et al. 1992; Ouyang et al. 1997; Padua et al. 1996; Sharp et al. 1993). During the last decade, many studies have revealed different components underlying Ca2+ homeostasis one of which involves Ca2+ stores, particularly in hippocampal neurons (Berridge 1998). Caffeine mobilizes Ca2+ from intracellular pools of hippocampal neurons that are distinct from IP3-gated stores (Koizumi et al. 1999). Activation of ryanodine-sensitive Ca2+ stores is involved in synaptic plasticity (Caillard et al. 2000; Narita et al. 2000). Deletion of the brain type RyR impairs hippocampal synaptic plasticity and spatial learning (Balaschun et al. 1999). Activation of RyRs modulates miniature GABA-mediated synaptic currents in hippocampal neurons (Llano et al. 2000; Savic and Sciancalepore 1998). Neurons exhibit at least two pharmacologically and molecularly defined mechanisms by which Ca2+ may be released from the ER, activation of RyR and IP3R (Meldolesi and Pozzan 1998; Verkhratsky and Petersen 1998). Interaction between these two mechanisms of Ca2+ release can influence the dynamic behavior of Ca2+ signals in neurons. Therefore it is important to understand the distribution and functional properties of intracellular Ca2+ release channels and the Ca2+ signals, which result from their activation.

Analysis of ryanodine binding to its receptor site provides unique information about its functional properties. Previous ryanodine binding studies have been performed with radioactively labeled [H3]ryanodine and microsomal brain preparations (McPherson et al. 1991; Zimanyi and Pessah 1991). However, the binding properties of RyR in physiologically intact neurons could be different due to the presence of other endogenous factors like cyclic-ADP-ribose (Hashii et al. 2000; Mothet et al. 1998; Reyes-Harde et al. 1999a; Sitges et al. 2000) and nitric oxide (Reyes-Harde et al. 1999b). Fluorescently labeled ryanodine in combination with confocal microscopy offers a new strategy to investigate the distribution of RyRs directly in live cells under different conditions. Moreover, because ryanodine binds preferentially to the open state of the Ca2+ release channel (Coronado et al. 1994), 6-(4,4-difluoro-5,7-dimethy1-1-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)hexanic acid ryanodine (BODIPY-ryanodine) binding and Ca2+ imaging were used to study the properties of ryanodine receptors (RyRs) and cytoplasmic Ca2+ (Ca2+cyt) changes in neurons cultured from the embryonic rat hippocampus during the earliest stages of differentiation. Baseline Ca2+ levels declined from 164 ± 5 (SD) nM at early stages to 70 ± 4 nM in differentiated neurons. Fluorescent BODIPY-ryanodine binding signals identified activated RyRs in somata, which were eliminated by removal of external Ca2+. Activated RyRs in somata, which were eliminated by removal of external Ca2+. Direct removal of extracellular calcium by removal of external Ca2+ or by blockage of Ca2+ entry through L-type but not N-type Ca2+ channels. The GABA synthesis inhibitor 3-mercaptopropionic acid completely abolished ryanodine binding. Caffeine or K+-depolarization inhibited the activity of RyRs at very early stages of differentiation but had stimulatory effects at later stages after a network of processes had formed. BayK-8644 stimulated RyRs throughout all regions of all differentiating cells. The results suggest that in differentiating embryonic hippocampal neurons the activity of RyRs is maintained via Ca2+ entering through L-type Ca2+ channels. The mode of activation of L-type voltage-gated Ca2+ channels with either membrane depolarization or specific pharmacological agents affects the coupled activity of RyRs differently as neurons differentiate processes and networks.

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5,7-dimethyl-1-bora-3a,4a-diaza-2-indacene-3-propionyl-amino)hexanoic acid ryanodine (BODIPY-ryanodine) could be used to probe the distribution of activated Ca\(^{2+}\) release channels in physiologically intact cells.

The aim of this investigation was to study the functional and pharmacological properties of RyRs and associated Ca\(^{2+}\) signaling resulting from their activation in hippocampal neurons during early differentiation in culture. Our observations suggest that in hippocampal neurons the activity of RyRs is maintained via coupling to L-type Ca\(^{2+}\) channels. Physiological and pharmacological properties of RyRs during neuronal differentiation depend on resting Ca\(^{2+}\) baseline levels. Some of these results have been published in abstract form (Sukhareva et al. 2001).

**Methods**

**Cell dissociation and culture**

Time pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY) were killed by CO\(_2\) inhalation followed by cervical dislocation in compliance with the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The protocol was approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee. The details of cell dissociation have been previously reported (Vautrin et al. 2000). Briefly, hippocampal regions of 19-day-old rat embryos (E19) were dissected and completely dissociated into single-cell suspensions using papain (20 U/ml, Worthington, Freehold, NJ) digestion and gentle trituration. Cells were cultured in 35-mm plates with a glass coverslip bottom 14 mm in diameter, which was precoated with 2 mg/ml high-molecular-weight poly-D-lysine (PDL). Cells were plated at a density of approximately 9.5 \(\times\) 10\(^3\) cells/cm\(^2\) and used for experiments 3 h [3 h in vitro (hiv)] after attachment to PDL-covered glass, at 1 day in vitro (div) and at 5 div. Cultures were kept in a CO\(_2\) (5%) incubator at 37\(^\circ\)C in Neurobasal medium (Gibco, Gaithersburg, MD), which contained the B27 supplement (Gibco) and astrocyte-conditioned medium (ACM). ACM was generated by incubation of a confluent carpet of astrocytes (type-I) in Neurobasal medium plus B27 supplement. It was then collected and stored at –80\(^\circ\)C before use (Liu et al. 2000). ACM was used to facilitate neuronal differentiation and network formation among hippocampal neurons (Liu et al. 1996, 1998).

**BODIPY-ryanodine binding**

**LABELING WITH BODIPY-RYANODINE.** Ryanodine binding was studied by using ryanodine conjugated to a fluorescent probe 6-(4, 4-difluoro-5,7-dimethyl-1-bora-3a,4a-diaza-2-indacene-3-propionyl)amino)hexanoic acid (BODIPY\(^\text{FL}-\)X, Molecular Probes, Eugene, OR). Cultured neurons were washed in physiological Krebs solution and incubated with 1 \(\mu\)M BODIPY\(^\text{FL}\)-X ryanodine (BODIPY-ryanodine), at room temperature for 10 min. Ten minutes was sufficient to stain the majority of cells under these conditions. After incubation, the cells were washed three times with Krebs saline to remove unbound fluorescently conjugated label. Fluorescence measurements were carried out within 1 h. Nonspecific levels of fluorescence staining were examined by incubating cells with both 1 \(\mu\)M BODIPY-ryanodine and 100 \(\mu\)M ryanodine (Sigma, St. Louis, MO). In some experiments, the cells were exposed to caffeine, KCN, or the L-type Ca\(^{2+}\) channel agonist, BayK-8644, for 10 min during the incubation with BODIPY-ryanodine. In other experiments, the cells were exposed to EGTA, ryanodine, nifedipine (L-type Ca\(^{2+}\) channel antagonist), or o-conotoxin (N-type Ca\(^{2+}\) channel antagonist) for at least 5 min before labeling and also during the labeling with BODIPY-ryanodine. The same plate of cells, recorded under control conditions could not be used for subsequent experiments because the intracellular receptor for BODIPY-ryanodine is not accessible for wash out. For this reason, it was technically impossible to show paired fields and therefore we did population studies. Every experiment has been independently repeated with three different cell preparations, using from three to five different plates for each preparation, taking images from up to 10 different fields. BODIPY-ryanodine labeling of neurons in the presence of drug was done in parallel with “control” cells in the absence of drug and also in the presence of 100 \(\mu\)M unlabeled ryanodine to verify nonspecific binding.

**DATA COLLECTION AND ANALYSIS OF BODIPY-RYANODINE BOUND CELLS.** Digital transmission light with differential interference contrast (DIC) modulation and fluorescence images of the labeled cells were obtained on an inverted Zeiss LSM410 or LSM510 confocal microscope (Carl Zeiss) using an achromat 1.2 aperture 63\(\times\) oil-immersion objective and 2\(\times\) digital magnification. Excitation and emission filter wavelengths for BODIPY\(^\text{FL}\) were 488 and 515 nm, respectively. Images acquired on the LSM were stored as PIC files (proprietary Zeiss format), translated to TIFF format, and converted to a gray scale for the analysis and image representation using Scion Image (Scion, Frederick, MD) software or Adobe Photoshop 5.0.2. The color table of a gray value was inverted so that high-intensity pixels appear black and low-intensity appear white. The number of fluorescently labeled cells and total number of cells visualized in DIC images in each plate was counted. To estimate the fluorescence intensity of the labeled cells, the area of interest around each fluorescent cell was digitally outlined and the mean density of gray value within the selection was measured in arbitrary units (a.u.). The mean density of background fluorescence intensity was measured for each acquired field and the average from all experiments was 16 ± 11 a.u. (\(n > 220\), where \(n\) is total number of experiments).

**Measurement of [Ca\(^{2+}\)]\(_i\) levels**

**LOADING WITH Ca\(^{2+}\)-SENSITIVE DYES.** Cultured hippocampal neurons were loaded with 2.5 \(\mu\)M fluo-3 AM or 2 \(\mu\)M fura-2-AM together with 2.5 \(\mu\)M Pluronic-F-127 acid [20% (wt/vol) solution in DMSO, all from Molecular Probes] in Krebs saline. Cells were incubated in the loading solutions for 30 min with fluo-3 or for 1 h with fura-2 at room temperature. Cells were washed three times with Krebs saline and incubated in the same saline for a further 30 min to allow complete de-esterification of intracellular AM esters.

**MEASUREMENTS OF [Ca\(^{2+}\)]\(_i\), USING THE Zeiss LSM.** Cells were loaded with fluo-3 AM as described as in the preceding text. Images were acquired using the frame-scan mode of a Zeiss LSM510 (Carl Zeiss) confocal microscope equipped with an achromat 1.3 aperture 40\(\times\) oil-immersion objective. The time of each scan was 1.5 s with a total of 40 scans acquired over 60 s. Excitation and emission filter wavelengths for fluo-3 were 488 and 515 nm, respectively. Because fluo-3 is used at a single excitation wavelength, changes in [Ca\(^{2+}\)]\(_i\) were calculated as fluorescence intensity (\(F\)) at a given time divided by fluorescence at rest (\(F_0\)). To determine changes in [Ca\(^{2+}\)]\(_i\) in particular regions, areas of interest were digitally outlined and \(F/F_0\) values for each region were calculated using LSM510 software (Carl Zeiss). The changes in fluorescence intensity (\(F\)) were considered significant when they attained an amplitude of at least twice the SD of the optically detected noise. The changes in the intensity values over time were plotted as \(F/F_0\) (arbitrary units) using Microcal Origin 6.0. Ca\(^{2+}\) changes were quantified by integrating the area under the curve for the 60-s scanning period.

**MEASUREMENT OF [Ca\(^{2+}\)]\(_i\) USING QUANTITATIVE VIDEO MICROSCOPY.** These images from somata were acquired using quantitative microscopy. Fura-2-loaded cells were recorded using the Zeiss Axiofocus RatioVision workstation (Atto Instruments, Rockville, MD) equipped with an Axiovert 135 inverted microscope (Carl Zeiss, Thornwood, NY) and an ICCD camera (Atto Instruments, Rockville, MD). The
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fura-2 dye was sequentially excited at 500-ms intervals with a 100-W mercury arc lamp filtered at 334 ± 5 and 380 ± 5 nm and the respective emissions acquired through a 510-nm dichroic mirror and 520-nm long-pass filter set (Chroma Technology, Brattleboro, VT). To collect the fura-2 fluorescence data, areas of interest were digitally outlined around each of up to 60 cells per recording field. The fluorescence intensities from each area were digitized with a Matrox image processing board and plotted as line graphs using Attograph for Windows analysis software (Atto Instruments). Fura-2 fluorescence signals were calibrated in terms of [Ca²⁺]ᵣₑₚ using previously established protocols (Maric et al. 2000).

Solutions and reagents

The composition of Krebs saline was (in mM) 136 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 8 glucose, pH 7.4, with osmolarity 290 mmol/kg. Unless otherwise stated, BayK-8644 (BayK), ryanodine, 3-mercaptopropionic acid (3-MPA), and other experimental reagents were supplied by Sigma.

Statistical tests

Data are shown as means ± SD. Statistical analyses were performed using a one-way ANOVA with a post hoc pair contrast test. The values P for significant differences are indicated in the text and figure legends.

RESULTS

Detection of spontaneous Ca²⁺ release sites

Normal neuronal development requires Ca²⁺ influx, which could lead to Ca²⁺ release from internal stores (Berridge 1998; Holliday et al. 1991; Maric et al. 2000; Mhyre et al. 2000). To investigate the contribution of RyRs to [Ca²⁺]ₑₚ homeostasis during neurogenesis, we studied the distribution of RyRs and their pharmacological properties in live hippocampal neurons during the earliest stages of differentiation in vitro. In the micromosaic preparations, ryanodine was found to bind preferentially to the activated RyR/Ca²⁺ release channel (Coronado et al. 1994). We have used this property of RyRs and BODIPY-ryanodine to identify activated Ca²⁺ release channels in live cells. Fluorescence signals resulting from BODIPY-ryanodine binding should reveal Ca²⁺ release channels in the open conformational state. For our experiments, we used short-term cultured hippocampal neurons grown in ACM (see METHODS) that are found to differentiate, extend processes, and have amino acid transmitter-induced current densities beginning as early as 2 h in culture (Liu et al. 1996, 1997, 1998, 2000). The images presented in the figures are representative of 10 fields taken from each of nine plates from three different cell culture preparations. As shown in Fig. 1A, functionally active RyRs were readily detected by BODIPY-ryanodine in acutely adherent embryonic hippocampal cells 3 h after plating. Two types of cells were present in the hippocampal cultures shortly after dissociation: a population of small, round cells (Fig. 1A, thick arrow) and a population of larger, elliptical cells (Fig. 1A, thin arrow). The latter often exhibited one or more short processes, while the former was devoid of them. Fluorescence signals were found throughout most of the cell bodies of both populations of cells (99 ± 2%, n = 554) with intensity values averaging 228 ± 11 a.u. (Figs. 1Aii and 4). Here and later n indicates the total number of labeled and unlabeled cells counted in all examined plates and fields. After 1 day in vitro, many cells had begun to extend processes, and many processes as well as somata (95 ± 8%, n = 320) were stained with BODIPY-ryanodine with an average intensity of 228 ± 22 a.u. (Figs. 3E and 4). At 5 div most of the cells exhibited pyramidal shaped cell bodies with one long thick process and a number of short processes (Fig. 1B). Fluorescent ryanodine

FIG. 1. Identification of spontaneously opened ryanodine receptors (RyRs) in embryonic hippocampal neurons in vitro using 6-((4,4-difluoro-5,7-di- methyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)hexanoic acid ryanodine (BODIPY-ryanodine). Hippocampal neurons cultured for 3 h in vitro (hiv) (A) and 5 days in vitro (div) (B–D) were labeled with 1 μM BODIPY-ryanodine for 10 min at room temperature. Digital images were obtained using laser scanning confocal microscopy with 568-nm excitation wavelength and DIC modulation (i) or 488-nm excitation and 515-nm emission wavelengths for BODIPY-ryanodine (ii). Fluorescence signals, indicating activated RyR, were found throughout cell bodies (Aii and Bii), in adjacent processes (Bii) and in isolated discrete regions (Ci). D: nonspecific (NS) BODIPY-ryanodine binding was evaluated in the presence 100 μM ryanodine. Under these conditions the fluorescent signal was decreased to the background fluorescence level (Di). Scale bar: 8 μm.
signals (230 ± 22 a.u.) were detected in the somata and adjacent processes in 90 ± 8% of the cells (n = 461) at 5 div (Figs. 1Bi and 4). The results from measured mean fluorescence density were not significantly different (P > 0.05) in all neurons. BODIPY-ryanodine labeling tended to be more prominent in larger diameter processes (Fig. 1Bi, arrow). Cells cultured at this density in medium supplemented with ACM invariably generated an extensive network of processes within 5 days (Fig. 1, Bi and Ci). In fine distal processes, active Ca2+-release channels were detected in isolated discrete regions, some of which involved points of contact between processes (Fig. 1Cii, arrows). The specificity of BODIPY-ryanodine was determined by treating neurons with a combination of 1 μM BODIPY-ryanodine and 100 μM unlabeled ryanodine. Fluorescent signals under these conditions were reduced to 46% of control (Figs. 1Dii and 4). The means of nonspecific (NS) BODIPY-ryanodine binding in the neurons at 3 hiv and 1 and 5 div were not significantly different (P > 0.05), but they were significantly different from control (P < 0.001).

BODIPY-ryanodine binding found in cell bodies revealed active Ca2+-release channels. We investigated whether the activity of the channel and thus ryanodine binding may have occur due to spontaneous changes in the Ca2+ levels. Ca2+ levels were recorded using a confocal LSM510 microscope, which allowed us to monitor up to 10 cells independently before bleaching and photodynamic damage occurred. Experiments were performed with Ca2+ in the external solution to allow reloading of internal stores between trials when necessary. Shortly after dissociation (3 hiv) spontaneous Ca2+ transients were detected only in 4% of soma (n = 372, s = soma) with the integrated areas under the Ca2+ traces averaging 4.0 ± 0.6 a.u. Very few neurons at 3 hiv exhibited short processes, and these did not manifest any detectable changes in Ca2+. The probability of detecting spontaneous Ca2+ changes among the cultured cells increased with neuronal differentiation, but the net values of cytoplasmic Ca2+ transients were not significantly different (P > 0.05). The values of integrated areas under the F/F0 curve at 1 div were 5 ± 1 a.u. in 7% of soma (n = 407) and 3 ± 0.4 a.u. in 2% of areas on processes (n = 87, p = processes; data not shown). The values of integrated areas at 5 div were 5 ± 1 a.u. in 13% of soma (n = 481) and 8 ± 1 a.u. in 20% of areas on processes (n = 203). Representative images and traces of spontaneous [Ca2+] changes in neuronal soma and processes at 5 div are shown in Fig. 2. In this experiment, the peak values of spontaneous Ca2+ transients in soma and processes were not different, and at a given time resolution, the events in soma and processes appeared inseparable in time. Further in our study we will focus on the RyRs located primarily in soma.

**Activation of RyRs by Ca2+ entry via L-type Ca2+ channels**

The major mammalian brain RyR isoform is thought to be the cardiac type (type-2) (Berridge 1998), which is activated by Ca2+ entry through L-type Ca2+ channels (Franzini-Armstrong 1999). We investigated the contribution of extracellular Ca2+ to BODIPY-ryanodine binding during neuronal differentiation. BODIPY-ryanodine fluorescence signals in control cells were compared with those obtained in cells, which were experimentally manipulated. Control cells were labeled with BODIPY-ryanodine in Krebs saline without agonists or antagonists. If RyRs in neurons are primarily activated by Ca2+ entry, elimination of extracellular Ca2+ should abolish ryanodine binding. Indeed, when staining was carried out in the presence of 5 mM EGTA BODIPY-ryanodine fluorescence intensity decreased to the nonspecific intensity level in all neurons (Figs. 3 and 4). The means of intensities in 84 ± 19% of neurons (n = 647) at 3 hiv (Figs. 3B and 4), 100% of neurons (n = 323) at 1 div (Figs. 3F and 4) and 84 ± 22% of neurons (n = 269) at 5 div (Figs. 3J and 4) were significantly different from the controls (P < 0.001) and were not significantly different from the means of NS binding (Fig. 4). Hence, Ca2+ entry was required to activate RyRs and bind BODIPY-ryanodine.
In muscle, L-type Ca\(^{2+}\)/H\(_{11001}\) channels are the major pathway of Ca\(^{2+}\)/H\(_{11001}\) entry that activates RyRs (Franzini-Armstrong 1999), while in neurons there are several components contributing to Ca\(^{2+}\) entry, which could activate RyRs (Avery and Johnston 1996). We used pharmacology to identify which types of Ca\(^{2+}\)/H\(_{11001}\) channel is involved in the activation of RyRs in neurons. Block of L-type Ca\(^{2+}\)/H\(_{11001}\) channels with nifedipine (10\(^{-6}\)M) significantly reduced \((P < 0.001)\) fluorescence signals in 100% of the cells \((n = 102)\) at 3 hiv (Figs. 3C and 4), 98 ± 3% of the cells \((n = 154)\) at 1 div (Figs. 3G and 4), and in 89% of the cells \((n = 294)\) at 5 div (Figs. 3K and 4). The antagonist of N-type Ca\(^{2+}\)/H\(_{11001}\) channels, \(\omega\)-conotoxin (1\(^{-6}\)M), had no effect on the fluorescence intensity signal in neurons. At 3 hiv 82 ± 19% of the cells \((n = 204)\) exhibited 96% of the fluorescence signals in control (Figs. 3D and 4). At 1 and 5 div 86 ± 16% \((n = 96)\) and 75 ± 18% \((n = 176)\) neurons, respectively, emitted 100% of the fluorescence signals in control (Figs. 3, H and L, and 4). The differences in the means in the presence of \(\omega\)-conotoxin were significant \((P < 0.001)\) from the nonspecific binding but insignificant from the control. This experimental fact indicates that RyR activation in hippocampal neurons during early differentiation in culture required Ca\(^{2+}\) entry, predominantly via L-type Ca\(^{2+}\) channels.

**Inhibitory effect of 3-MPA on ryanodine binding**

The spontaneous activity of voltage-gated Ca\(^{2+}\) channels can be modulated by membrane potential controlled predominantly by K\(^{+}\) channels and, as was shown for early neuronal development, by Cl\(^{−}\) (Fukura et al. 1999). Thus Ca\(^{2+}\) entry in hippocampal neurons could be triggered by membrane depolarization due to an activation of GABA\(_{A}\) receptor/Cl\(^{−}\) channels via autocrine or paracrine secretion of GABA (Maric et al. 2001; Yokogawa et al. 2001). We examined the role of endogenous GABA in RyRs function. BODIPY-ryanodine binding experiments were done in neurons where GABA synthesis was inhibited. Neurons at 5 div were treated with 100 \(\mu\)M 3-MPA, a GABA synthesis inhibitor (Katoh et al. 1994) for 30 min and 1 and 1.5 h. We found that 1-h treatment with 3-MPA was sufficient to reduce significantly \((P < 0.001)\) BODIPY-ryanodine binding in 98 ± 2% \((n = 42)\) to the level close to the fluorescence intensity of nonspecific binding (Fig. 5, Bii and C). This suggests that during early differentiation of hippocampal neurons in culture endogenous GABA-mediated depolarization could constitutively open L-type Ca\(^{2+}\) channels, stimulating Ca\(^{2+}\) entry and activating RyRs/Ca\(^{2+}\) release channels.
membrane depolarization that opens voltage-dependent Ca\textsuperscript{2+} channels activated RyRs only in cultures with a morphologically differentiated neuronal network. When Ca\textsuperscript{2+} entry was potentiated by BayK, a selective agonist of L-type Ca\textsuperscript{2+} channel (Sanguinetti et al. 1986), BODIPY-ryanodine binding was observed in 82 ± 25% (n = 571) of the cells at 3 hiv and (Fig. 6D), in 93 ± 4% of the cells (n = 139) at 1 div (Fig. 6H) and in 95 ± 8% of cells (n = 102) at 5 div (Fig. 6L). Fluorescence signal intensity increased significantly (P < 0.001) compared with that detected in control cells at 3 hiv and 5 div along with changes in spatial distribution (Fig. 6L), but there was no significant change for cells at 1 div (Fig. 7). The means in all cells were significantly different (P < 0.001) from NS binding (Fig. 7). At 5 div activation of L-type Ca\textsuperscript{2+} channels resulted in the activation of RyRs not only in somata but also in all processes of differentiating neurons.

The previous set of BODIPY-ryanodine binding experiments demonstrated the inhibitory effects of caffeine and K\textsuperscript{+}.

Effect of caffeine, KCl and BayK on ryanodine binding and Ca\textsuperscript{2+}.

Caffeine is a RyR agonist that releases Ca\textsuperscript{2+} from intracellular stores by increasing the affinity of the ryanodine receptor for Ca\textsuperscript{2+} (Coronado et al. 1994). We compared BODIPY-ryanodine binding in neurons in the absence (Fig. 6, A, E, and I) or in the presence of caffeine (Fig. 6, B, F, and J). The effect of 2 mM caffeine on BODIPY-ryanodine binding was developmentally regulated. At 3 hiv, 100% of the neurons (n = 179) exhibited 51% of the fluorescence signal in control after exposure to caffeine (Figs. 6B and 7). At 1 div, 72 ± 25% of the neurons (n = 132) displayed 60% of control fluorescence signals (Figs. 6F and 7). At 5 div, fluorescence intensity of BODIPY-ryanodine binding of 78 ± 2% of neurons (n = 131) in the presence of caffeine was 99% of the intensity recorded under the control conditions (Figs. 6J and 7). Thus stimulation of RyRs by low concentration caffeine resulted in a significant decrease (P < 0.001) in BODIPY-ryanodine binding during the earliest stages of differentiation (3 hiv and 1 div), but exhibited little or no effect after a morphologically visible network had formed (5 div; Fig. 7).

We used 20 mM KCl to depolarize neurons to see if moderate levels of membrane depolarization would affect ryanodine binding. The effect of 20 mM KCl on BODIPY-ryanodine binding in embryonic hippocampal neurons was also developmentally regulated. At 3 hiv and at 1 div 98 ± 4% (n = 223) and 87 ± 13% (n = 142) of neurons, respectively, showed significant reduction (P < 0.001) in the fluorescence signal intensities (Figs. 6, C and G, and 7). However, at 5 div, membrane depolarization increased the fluorescence intensity up to the threshold and the mean density of the fluorescence intensities were not significantly different from the control in 90 ± 15% of the cells (n = 134; Figs. 6K and 7). Noticeably the fluorescence signal had increased not only in the somata but also in the processes, especially thick ones (Fig. 6K). Hence
depolarization on ryanodine binding in neurons at early stages of development (3 hiv and 1 div). We investigated whether the cytoplasmic Ca\(^{2+}\) level in neurons at 3 hiv and 1 and 5 div was affected when caffeine and KCl were added to the bath. Because the effects of agonists on the BODIPY-ryanodine binding were observed predominantly in the soma and the thicker apical processes close to it, we were interested in this study only in the somatic Ca\(^{2+}\) responses and did not focus on the distal processes. Application of caffeine (2 mM final concentration) induced Ca\(^{2+}\) transients in 11% of neurons (n = 98) at 3 hiv, in 18% of neurons (n = 145) at 1 and in 54% of neurons (n = 79) at 5 div, illustrated in Fig. 8, A–C. Ca\(^{2+}\) transiently increased with caffeine addition and decayed back to control levels within 60 s. Figure 9 represents the average values of the integrated areas under the Ca\(^{2+}\) traces, reflecting both spontaneous events (control) and in response to different agents. The Ca\(^{2+}\) changes during spontaneous events in neurons cultured for 3 h and 1 and 5 days were not significantly different (P > 0.05, Fig. 9). The Ca\(^{2+}\) responses to caffeine were approximately seven times greater than spontaneous Ca\(^{2+}\) changes in neurons at 3 hiv and at 5 div but were not significantly different in neurons at 1 div (Fig. 9). Net Ca\(^{2+}\) responses to caffeine varied during differentiation. KCl addition to the bath immediately induced Ca\(^{2+}\) elevation in 88% of the neurons (n = 75) at 3 hiv, in 97% of neurons (n = 80) at
1 div and in 100% of the neurons \((n_s = 59)\) at 5 div, (Fig. 6, D–F). \(\text{Ca}^{2+}\) elevations at 5 div were observed not only in somata but also in thick neuronal processes (data not shown). In all three experiments with KCl, the \(\text{Ca}^{2+}\) levels increased as fast as occurred in response to caffeine, but the decay was much slower and did not return to the basal level within the 60-s recording time. Therefore the total \(\text{Ca}^{2+}\) response to KCl should be bigger than one recorded within 60 s. However, even within the 60-s period, \(\text{Ca}^{2+}\) responses were greater than those occurring during spontaneous \(\text{Ca}^{2+}\) events: 14 times at 3 hiv, 19 times at 1 div, and 23 times at 5 div (Fig. 7) and progressively increased during differentiation of neurons in vitro. Membrane depolarization caused \(\text{Ca}^{2+}\) entry via voltage-dependent \(\text{Ca}^{2+}\) channels, which then activated \(\text{Ca}^{2+}\) channels and induced \(\text{Ca}^{2+}\) release from internal stores. The L-type \(\text{Ca}^{2+}\) channel agonist BayK (5 \(\mu\)M), like caffeine and KCl, induced \(\text{Ca}^{2+}\) transients in variable numbers of cells: 44% of neurons \((n_s = 45)\) at 3 hiv, in 55% of neurons \(n_s = 69\) at 1 div and in 65% of neurons \(n_s = 31\) at 5 div (Fig. 8, G–I). The \(\text{Ca}^{2+}\) responses to BayK were not immediate like those recorded in caffeine or KCl. At 1 div low-amplitude transient fluctuations in the basal \(\text{Ca}^{2+}\) levels were observed in response to BayK (Fig. 8/I). The integrated \(\text{Ca}^{2+}\) responses to BayK were significantly greater \((P < 0.001)\) than those recorded under control conditions: 16 times greater at 3 hiv, 5 times greater at 1 div, and 22 times greater at 5 div (Fig. 9). Thus \(\text{Ca}^{2+}\) responses to BayK varied with differentiation similar to caffeine-induced transients.

The experiments with fluo-3 provide qualitative estimates of the intracellular \(\text{Ca}^{2+}\) changes in response to the application of agonists. We performed experiments with fura-2 to estimate the changes of \([\text{Ca}^{2+}]\) in response to caffeine, KCl, and BayK. We were not able to record intracellular \(\text{Ca}^{2+}\) transients due to fast responses to 2 mM caffeine and 5 \(\mu\)M BayK and limited time resolution of our video-imaging system. But we were able to estimate the levels of intracellular baseline levels of \([\text{Ca}^{2+}]\) in neurons on differentiation in vitro. In neurons at 3 hiv, the baseline level of intracellular \(\text{Ca}^{2+}\) in the soma was \(164 \pm 5\) nM \((n = 125)\), at 1 div \(95 \pm 4\) nM \((n = 143)\) and at 5 div \(70 \pm 4\) nM \((n = 68)\). The result indicates that the \(\text{Ca}^{2+}\) baseline level decreases progressively during differentiation of neurons in culture.

\(\text{FIG. 8.} \ \text{Ca}^{2+}\) responses induced by caffeine, KCl, and BayK in hippocampal neurons during differentiation in culture. The traces represent the time courses of cytoplasmic \(\text{Ca}^{2+}\) \((\text{Ca}^{2+})\) signals in neurons at 3 hiv \((A, D, and G)\), 1 div \((B, E, and H)\), and 5 div \((C, F, and I)\) under the different experimental conditions. The dashed line indicates baseline \(\text{Ca}^{2+}\) signals before drug application. Concentrated stock solutions of caffeine \((A, B, and C)\), KCl \((D, E, and F)\), and BayK \((G, H, and I)\) were added into the bath (\(\uparrow\)). Final concentrations were 2 mM caffeine, 20 mM KCl, and 1 \(\mu\)M BayK. Caffeine and BayK induce single or multiple transients \((A–C and G–I)\), while KCl generates a well-sustained \(\text{Ca}^{2+}\) elevation throughout neuronal differentiation \((D–F)\).
logical experiments showed that RyRs in neurons are coupled. RyRs are active in physiologically intact neurons. Pharmacological agents affect the coupled activity of RyRs differently as neurons differentiate processes and networks.

The presence of spontaneously active Ca\textsuperscript{2+} release channels was detected in cells at 3 hiv and 1 and 5 div. Spontaneous Ca\textsuperscript{2+} transients observed with fluo-3 paralleled the substantial level of ryanodine binding to RyRs in neurons throughout neuronal differentiation in vitro. The observation of BODIPY-ryanodine binding and cytoplasmic Ca\textsuperscript{2+} transients in somata and isolated spots in processes without overt external stimulation implies that Ca\textsuperscript{2+} can be spontaneously released from internal Ca\textsuperscript{2+} stores presumably due to spontaneously generated action potentials or perhaps to fluctuations in some endogenous factors. Spontaneous Ca\textsuperscript{2+} release was observed in spinal cord neurons in cultures and in slices (Gu and Spitzer 1997; Spitzer 1994) as well as in hippocampal pyramidal cells (Dailey and Smith 1994; Garaschuk et al. 1997; Llano et al. 2000). What factors may give rise to spontaneous RyR activity in neurons? We found that BODIPY-ryanodine binding was eliminated either by decreasing extracellular Ca\textsuperscript{2+} or by exposing neurons to the L-type Ca\textsuperscript{2+} channel antagonist, nifedipine, in the presence of extracellular Ca\textsuperscript{2+}. However, binding was insensitive to the N-type Ca\textsuperscript{2+} channel blocker, ω-conotoxin, which suggests that in embryonic hippocampal neurons during early differentiation in vitro, L-type Ca\textsuperscript{2+} channels mediate Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from internal stores. Similar coupling was previously reported for cerebellar granule cells (Chavis et al. 1996). The coupling of L-type Ca\textsuperscript{2+} channels and RyRs could maintain tonic or transient Ca\textsuperscript{2+} entry during differentiation and neurite outgrowth.

The contribution of tonic Ca\textsuperscript{2+} entry to the baseline Ca\textsuperscript{2+} levels and membrane potentials has been recently reported for pyramidal neurons from hippocampus (Garaschuk et al. 1997; Magee et al. 1996), cortical (Maric et al. 2001), and spinal cord neurons (Li et al. 1998). The inhibition of ryanodine binding following treatment with 3-AMP, which blocks glutamic acid decarboxylase-derived GABA synthesis from glutamate, reveals a critical contribution of GABA at the early stage of neuronal differentiation. Most likely GABA acts at GABA\textsubscript{A} receptor/Ca\textsuperscript{2+} channels to depolarize neurons and thus indirectly triggers constitutive Ca\textsuperscript{2+} entry. This GABA/GABA\textsubscript{A}/Ca\textsuperscript{2+} channel/L-type Ca\textsuperscript{2+} channel autocrine/paracrine circuit, which underlies process formation in differentiating embryonic cortical neurons (Maric et al. 2001), is also likely to be important in hippocampal neurite outgrowth and the formation of functional synapses.

Both caffeine and Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release from intracellular stores in several types of CNS and peripheral neurons (Garaschuk et al. 1997; Hernandez-Cruz et al. 1995; Kano et al. 1995; Koizumi et al. 1999; Llano et al. 1994; Seymour-Laurent and Barish 1995; Tsai and Barish 1995). In this study, Ca\textsuperscript{2+} transients were detected in response to 2 mM caffeine and the magnitude declined with the reduction in the baseline Ca\textsuperscript{2+} level in neurons at 3 hiv and at 1 div but not at 5 div. The increase in the magnitude of caffeine-induced Ca\textsuperscript{2+} transients was also found to be enhanced in proportion to the basal Ca\textsuperscript{2+} in the cytosol (Garaschuk et al. 1997; Hua et al. 1993; Krizaj et al. 1999; Sitones and Williams 1990). Consequently, in our BODIPY-ryanodine binding experiments, caffeine had an inhibitory effect on ryanodine binding in immature neurons (3 hiv), which exhibit high basal Ca\textsuperscript{2+} levels, then and gradually became stimulatory on more differentiated neurons, which exhibit low basal Ca\textsuperscript{2+} levels. This results suggest that caffeine-induced transients inhibited RyR activity in very immature neurons when basal Ca\textsuperscript{2+} level was high, but activated RyRs in differentiated neurons when the basal Ca\textsuperscript{2+} was markedly lower. The shift from a facilitatory to inhibitory effect of low concentrations caffeine when basal Ca\textsuperscript{2+} reached higher levels was reported for sympathetic neurons (Hernandez-Cruz et al. 1995). Caffeine is also able to stimulate Ca\textsuperscript{2+} removal from the cytosol (Friel and Tsien 1992), and this process is faster than any other Ca\textsuperscript{2+} ATPases and extrusion pumps (Cseresnyes et al. 1997). Thus it is possible to explain the inhibitory effects of caffeine on the ryanodine binding in neurons in culture during the early stages of differentiation by the same nonconventional Ca\textsuperscript{2+} transport mechanism.

Membrane depolarization by extracellular K\textsuperscript{+} was also used to stimulate RyR activity in our experiments. K\textsuperscript{+} depolarization applied to neurons at 3 hiv and 1 div inhibited RyRs/Ca\textsuperscript{2+} release channels, but at 5 div, it activated them and revealed activated RyRs not only in the soma but also in processes. Changes of Ca\textsuperscript{2+} induced by KCl were much greater than with caffeine, apparently because depolarization-induced Ca\textsuperscript{2+} transient contained two components, the entry of Ca\textsuperscript{2+} through...
voltage-dependent Ca\(^{2+}\) channels and subsequent Ca\(^{2+}\) release from intracellular stores. We also found that similar to caffeine the K\(^+\) depolarization effect on BODIPY-ryanodine binding in neurons was inhibitory during the early stages of differentiation and became facilitatory in more differentiated neurons, while K\(^+\) depolarization consistently elevated Ca\(_c\) through-out differentiation. The same mechanism as discussed in the preceding text for caffeine effects on RyRs may underlie the K\(^+\) -depolarization induced inhibition of RyRs.

BayK is a dihydropyridine L-type Ca\(^{2+}\) channel agonist (Hess et al. 1984) that can indirectly modulate the gating of RyRs in resting cells via dihydropyridine receptor-RyR interaction (McCall et al. 1996; Satoh et al. 1998). We found that BayK invariably induced cytoplasmic Ca\(^{2+}\) transients in hippocampal neurons at all stages of differentiation in vitro without external stimulation. This could be due to either an ambient level of L-type Ca\(^{2+}\) channel activity promoted by an autocrine GABAergic circuit (Maric et al. 2001) or the known ability of BayK to cause depolarization-independent Ca\(^{2+}\) release from the skeletal muscle SR (Oba et al. 1997). Ryanodine binding experiments showed little effect of BayK on BODIPY-ryanodine binding in soma but profound increase in processes of more differentiated neurons (5 div). This indicates that despite BayK’s stimulation of Ca\(^{2+}\) influx at all stages of neuronal differentiation RyR activity was enhanced only when a complex network of processes between neurons became evident. BayK effects on ryanodine binding can be via a direct physical and/or functional link between L-type Ca\(^{2+}\) channels and RyRs (Katoh et al. 2000).

In conclusion, we have utilized the BODIPY-ryanodine probe as a tool for identification of activated RyRs/Ca\(^{2+}\) release channels during neuronal differentiation in vitro. The results demonstrate the presence of RyRs functionally linked to L-type Ca\(^{2+}\) channels in neurons as they differentiate into networks. The activity of RyRs in the presence of depolarization, caffeine, and BayK depends on baseline Ca\(_c\) levels, which undergo substantial modification during neuronal differentiation in vitro. It is also possible that the observed changes in response to depolarization, caffeine, and BayK could be due to the developmental appearance of different types of RyRs during neuronal differentiation. Previous studies of mRNA levels coding for different types of RyRs in the hippocampal region during mouse brain development (Mori et al. 2000) have revealed type-1 RyR to be more prominent in early stages, while type-2 RyR and type-3 RyR appeared later. We also observed a spatial redistribution of RyR activity from soma to processes during differentiation that were revealed by the appearance of multiple independent hot spots in distal processes. These RyRs may play important roles in growth cone mobility and in the formation of synaptic contacts. Further understanding of these roles and associated mechanisms will be required to elucidate the types of RyRs and their physiological and pharmacological properties in these specialized regions.

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