Activity-dependent Expression of Inositol 1,4,5-Trisphosphate Receptor Type 1 in Hippocampal Neurons*

Received for publication, December 5, 2003, and in revised form, February 7, 2004
Published, JBC Papers in Press, March 11, 2004, DOI 10.1074/jbc.M313296200

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There are several lines of evidence showing that synaptic activity regulates the level of expression of inositol 1,4,5-trisphosphate receptor type 1 (IP$_3$R1) in neurons. In this study, we examined the effect of chronic activity blockade on the localization and level of IP$_3$R1 expression in cultured hippocampal neurons. We found that chronic blockade of NMDA receptors (NMDARs), one of the major Ca$^{2+}$-permeable ion channels, increased the number of neurons that express a high level of IP$_3$R1 without any apparent changes in its intracellular localization. Interestingly, this up-regulation was time-dependent; there was no clear change in IP$_3$R1 expression level up to day 5 of the NMDAR blockade, but expression increased at day 6, and the increased expression level persisted for at least a week. The up-regulation of IP$_3$R1 depended on transcription and protein synthesis and required cAMP-dependent protein kinase activity. Moreover, although most of the control neurons did not respond to the metabotropic glutamate receptor (mGluR) stimulation, the 2-amino-5-phosphonopentanoic acid-treated neurons with high IP$_3$R1 expression became sensitive to mGluR stimulation. Furthermore, we also found that hippocampal neurons transiently overexpressing green fluorescent protein-tagged IP$_3$R1 released Ca$^{2+}$ in response to mGluR and muscarinic acetylcholine receptor stimulation. These findings suggested that chronic NMDAR blockade increased the IP$_3$R1 expression and enhanced sensitivity to mGluR stimulation. The change in IP$_3$R1 expression level in response to alteration of synaptic activity may be an important determinant of the sensitivity of Ca$^{2+}$ stores to Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores (1–3), and three types have been identified thus far: IP$_3$R1, IP$_3$R2, and IP$_3$R3 (4, 5). One of the members of the IP$_3$R family, IP$_3$R1, is thought to be the predominant isoform in the central nervous system (6), and various approaches, including gene knockout and functional blockade by specific antibodies, have shown that IP$_3$R1 plays crucial roles in several neuronal functions, including synaptic plasticity (7–11), neurite extension (12), and nerve growth cone guidance (13). Thus, the precise regulation of Ca$^{2+}$ release through IP$_3$R1 is one of the important factors in these physiological functions in the brain.

IP$_3$R channel activity largely depends on direct modulation by numerous intracellular signals, including Ca$^{2+}$, ATP, calmodulin, FKBP12, calcineurin, BANK, IRAG, chromogranin, and Huntington-associated protein-1A, which acutely control the channel opening (3, 14–18). In addition, several recent studies suggest that, by changing the sensitivity of Ca$^{2+}$ stores to IP$_3$, the level of IP$_3$R expression may also be an important factor affecting intracellular Ca$^{2+}$ mobilization (19). For example, retinoic acid up-regulates IP$_3$R1 expression in HL-60 cells, a human leukocyte cell line, and HL-60 cells exposed to retinoic acid show a 2–3-fold increase in Ca$^{2+}$ mobilization in response to IP$_3$ stimulation (20). Overexpression of IP$_3$R1 in Xenopus oocytes results in an increased velocity of Ca$^{2+}$ wave propagation (21). Mouse L fibroblasts stably expressing approximately an 8.5-fold amount of IP$_3$R1 show 4-fold increase in the sensitivity to IP$_3$-induced Ca$^{2+}$ release compared with control cells (22). These cells also show a greater frequency of agonist-induced Ca$^{2+}$ oscillation and have a lower threshold of agonist concentrations for the transition from oscillatory to peak-and-plateau Ca$^{2+}$ patterns (23). Because the Ca$^{2+}$ release and Ca$^{2+}$ oscillation patterns profoundly affect the activity of downstream targets that play crucial roles in a myriad of cellular functions (24), it is important to determine how the IP$_3$R expression level is controlled in various types of cells.

In neurons, control of the IP$_3$R1 expression level is closely related to synaptic activity. In cerebellar granule cells, Ca$^{2+}$-influx through L-type Ca$^{2+}$ channels and N-methyl-D-aspartate receptors (NMDARs) increases the expression level of IP$_3$R1 (25). Similarly, activation of L-type Ca$^{2+}$ channels and NMDA receptors increases IP$_3$R1 expression through the calcineurin/NFATC4 (nuclear factor of activated T-cells) pathway in hippocampal neurons (26). In terms of more long-term effects of synaptic activity, chronic activation of muscarinic or metabotropic glutamate receptors results in down-regulation of the IP$_3$R1 expression level in neuroblastoma cells and cerebellar tansic acid; RT, reverse transcription; DIV, days in vitro; IBMX, 3-isobutyl-1-methylxanthine; CHX, cycloheximide; DHPG, (S)-3,5-dihydroxyphenylglycine.

This paper is available on line at http://www.jbc.org

* This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (to K. M. and T. I.) and a grant-in-aid for Young Scientists (B) (to C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate receptor; IP$_3$R, inositol 1,4,5-trisphosphate; mGluR, metabotropic glutamate receptor; PRA, cAMP-dependent protein kinase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; NMDAR, NMDA receptor; AP-2, activating protein-2; APV, 2-amino-5-phosphono-
Fig. 1. Chronic NMDA receptor blockade increases the number of hippocampal neurons with intense IP3R1 immunoreactivity without any apparent change in IP3R1 intracellular localization. A, hippocampal neurons from E18 mice were cultured and exposed to (APV) or unexposed to (Control) 100 μM APV for the 7 days from 14 to 21 DIV. At 21 DIV, cells were fixed and stained with anti-IP3R1 and anti-MAP2 antibodies. Arrowheads indicate neurons with intense IP3R1 immunostaining. Scale bar, 20 μm. B, no difference was observed for IP3R1 subcellular distribution between control and the APV-exposed hippocampal neurons of intense IP3R1 immunoreactivity. Lower panels show the IP3R1 immunoreactivity in the proximal dendrites of hippocampal neurons. Scale bars, 10 μm (upper panel) and 5 μm (lower panel). C, a histogram of the intensity of the IP3R1 immunoreactivity in chronically APV-exposed (black bar) or unexposed (Control; white bar) hippocampal neurons. Fluorescence intensity of the IP3R1 immunoreactivity at soma of neurons (MAP2-positive cells) was measured, and a representative histogram from one experiment was shown here. The experiments were repeated six times, and similar results were obtained. D, Western blot analysis of the lysates from APV-exposed (APV) or unexposed (Control) hippocampal neurons probed with anti-IP3R1 and anti-β-actin antibodies. Experiments were performed three times, and representative data are shown here.

granule cells, respectively (27, 28). However, although many studies have examined the effects of various stimuli on IP3R1 expression, little is known about the effect of chronic activity blockade on IP3R1 expression in neurons.

In this study, we examined the effect of chronic activity blockade on IP3R1 expression and its intracellular localization in cultured hippocampal neurons that have spontaneous synaptic activity (29). The results showed that chronic NMDAR blockade by 2-amino-5-phosphonopentanoic acid (APV) increases the IP3R1 expression level in hippocampal neurons. Transcription, protein synthesis, and cAMP-dependent protein kinase (PKA) activity were also found to be necessary for APV-induced IP3R1 expression. In addition, chronic NMDAR blockade increased the number of neurons that released Ca2+ in response to the mGluR stimulation, and sensitivity to mGluR stimulation was correlated with the IP3R1 expression levels. Furthermore, we showed that neurons transiently overexpressing GFP-tagged IP3R1 become sensitive to mGluR and muscarinic acetylcholine receptor stimulation. Thus, these results indicate that chronic NMDAR blockade increases IP3R1 expression, which in turn enhances sensitivity of Ca2+ stores to mGluR stimulation. Control of the IP3R1 expression level, which was regulated by the extracellular environment, may be one of the important mechanisms in maintaining intracellular Ca2+ homeostasis.

MATERIALS AND METHODS

Cell Culture—Hippocampal neuronal cultures were prepared from 17–18-day embryonic ICR mice as described previously (30). Briefly, hippocampi were dissociated by trypsin treatment and trituration, and were plated on poly-L-lysine-coated glass coverslips in 15-mm culture dishes at a density of 5.0–6.0 × 104 cells/cm2 in 1.0 ml of medium. After plating, cells were incubated at 37 °C, 5% CO2/air, and 400 μl of medium was replaced once a week. The composition of the medium is Neurobasal™ medium (Invitrogen) containing 2.0% B-27 supplement (Invitrogen), 0.5% insulin-transferrin-selenium A (Invitrogen), 1 mM 1-methylxanthine (IBMX) (25 M; Calbiochem), KT5720 (2.0 M; Calbiochem), L-glutamine (Nacalai Tesque, Kyoto, Japan), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. When medium was replaced, medium of the same composition except L-glutamine was used.

Pharmacological Agents—d-APV (100 μM; Tocris Cookson Ltd., Bristol, United Kingdom), cycloheximide (5.0 μM; Calbiochem), 3-isobutyl-1-methylxanthine (IBMX) (25 μM; Calbiochem, La Jolla, CA), 8-bromo-cAMP (10 μM; Calbiochem), KT5720 (2.0 μM; Calbiochem), and (S)-3,5-dihydroxypyrophospholine (DHPG) (100 μM; Tocris Cookson Ltd.).

Immunocytochemistry and Quantification—Neurons were fixed in 4% paraformaldehyde/PBS for 10 min and subsequently in 100% methanol (−20 °C) for 10 min, and then permeabilized in 0.2% Triton X-100/PBS for 10 min at room temperature. Coverslips were blocked with 5% skim milk/PBS and incubated with primary antibodies in 5% skim milk/PBS and incubated with primary antibodies for 1 h at room temperature. After washing, tissues were incubated with secondary antibodies for 1 h and then were washed again with PBS. Finally, the tissues were stained with Hoechst 33,342 (1 μg/ml). Coverslips were mounted on glass slides with Prolong Gold antifade reagent (Invitrogen) and sealed with nail polish. Fluorescence images were captured with a fluorescence microscope (Eclipse Ti; Nikon, Tokyo, Japan) attached to a digital camera (DP70; Olympus, Tokyo, Japan) and analyzed using Velocity version 2.2 (PerkinElmer, Waltham, MA).
Control of the IP$_3$R1 Expression by NMDAR Activity

RESULTS

Chronic NMDA Receptor Blockade Increased the IP$_3$R1 Expression Level in Hippocampal Neurons—We cultured mouse embryonic hippocampal neurons and examined their level of IP$_3$R1 expression and its intracellular distribution immunocytochemically with anti-IP$_3$R1 and anti-MAP2 antibodies. As shown in Fig. 1A (Control), individual neurons showed various levels of IP$_3$R1 expression at 21 DIV in culture. In neurons that were intensely immunoreactive for IP$_3$R1 (e.g. arrowhead in Fig. 1A), IP$_3$R1 was predominantly localized in the cell body and proximal dendritic shafts in a diffuse punctate pattern, as reported previously (Fig. 1B, Control) (34, 35). However, there

nullipotent with the alkaline phosphatase-conjugated secondary antibodies. To detect signals, the polyvinylidene difluoride membrane was incubated with AttoPhos substrate (Amersham Biosciences) and scanned on FluorImager (Amersham Biosciences). Band intensities were analyzed using ImageQuant software (version 4.1, Amersham Biosciences).

Isolation of RNA and Reverse Transcription (RT)-PCR—Total RNA was prepared from hippocampal cell cultures by using TRIZol reagent according to instructions from the manufacturer (Invitrogen). First-strand cDNA was prepared from the total RNA, using reverse transcriptase Superscript II (Invitrogen) and oligonucleotide (dT) primers. PCR was performed using specific primers for IP$_3$R1 isoform (forward, 5′-CGTGGATGGTCACAGACCAG-3′; reverse, 5′-CTTTCAGAACCCTCTTGATTC-3′) (32). The primers used for β-actin were as follows: forward, 5′-GGAATTCACTGGATGGAC-3′; reverse, 5′-ACGGATCCACACGATCTGGCC-3′. After an initial cycle of 2 min at 94 °C, the reaction was cycled 30 times for 30 s at 94°C, 30 s at 50°C, and 70 s at 72°C. We confirmed that the PCR fragments were exponentially amplified at least from 25 to 35 cycles (data not shown). For analysis, the PCR products were separated on 3% polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide. We performed densitometry by scanning the gel on FluorImager and analyzed the band intensities using ImageQuant software. The IP$_3$R1 mRNA levels were normalized relative to those of β-actin. The PCR products were separated by electrophoresis in 2% agarose gel, extracted, and purified with the GeneClean kit (Bio 101, Vista, CA). Nucleotide sequencing was performed using a DNA sequencer (ABI Prism 377; PerkinElmer Applied Biosystems, Foster City, CA).

Intracellular Ca$^{2+}$ Imaging—The hippocampal neurons were grown on cover glass engrafted with grids (CELLocate, Eppendorf, Hamburg, Germany) coated with 50 μg/ml poly-L-lysine (Nacalai Tesque) and treated with or without 100 μM APV for 7 days from 14 to 21 days in vitro (DIV). The neurons were loaded with 5 μM fura-2-AM (Dojindo, Kumamoto, Japan) for 30 min at room temperature in recording solution containing (in mM): 115 NaCl, 5.4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 20 Hepes, 10 glucose, 1 μM tetrodotoxin, pH 7.4, washed once, and then kept in the recording solution in the dark at room temperature for 30 min. Fura-2 fluorescence images were analyzed using an inverted microscope (IX50, Olympus, Tokyo, Japan) and a video image analysis system (Argus-50/CA, Hamamatsu Photonics, Hamamatsu, Japan) with excitation filters at 340 ± 10 and 380 ± 10 nm, dichroic beam splitter at 400 nm, and a band pass emission filter at 520 and 550 nm. After taking basal fura-2 fluorescence images every 10 s for 2.5 min, the cells were stimulated with 50 μM kainic acid for 15 s to fill Ca$^{2+}$ stores. After another 4.5 min, the cells were stimulated with 100 μM DHPG for 3 min. During these periods, fura-2 fluorescence images were taken every 5 s. After Ca$^{2+}$ imaging, the cells were fixed with 4% PFA for 10 min at room temperature, permeabilized with 0.2% Triton/PBS for 5 min, and subjected to immunostaining for IP$_3$R1 and MAP-2 as described above. Scoring of IP$_3$R1 staining intensity was done by two independent observers who were blind to the result of the DHPG sensitivity. The cells showing no IP$_3$R1 staining intensity were counted as IP$_3$R1-negative cells and indicated as (−); the cells showing saturated levels of staining (maximally stained cells) were considered as having strong staining intensity and indicated as (+); the cells labeled with intermediate intensity between these two categories were considered to be moderately stained and expressed as (+).
NMDAR blockade increased the IP3R1 expression level in hippocampal neurons to 100 μM APV from 15 to 21 DIV and 5.0 μM CHX from 20 to 21 DIV. Taken together, these results indicated that the chronic blockade of NMDAR activity, increased the number of hippocampal neurons with high IP3R1 immunoreactivity in their cell bodies (arrowheads in Fig. 1A, APV), but did not induce any apparent change in the intracellular localization of IP3R1; IP3R1 clusters remained diffused within the dendritic shafts and soma in a punctate pattern, and there was no apparent change in the size of IP3R1 clusters after APV exposure (Fig. 1B). Because IP3R1 immunoreactivity in the proximal dendritic shafts was similar in both the APV-treated neurons and control neurons, translocation of IP3R1 from dendrites to the cell body was unlikely to be the mechanism for the increase in IP3R1 intensity observed in the cell body (Fig. 1B, lower panels). Fig. 1C shows a histogram of the intensity of IP3R1 immunoreactivity in neuronal cell bodies (MAP2-positive cells), confirming the increased number of neurons with high IP3R1 immunoreactivity (arbitrary units, 9–20) in the APV-exposed neurons (black bar) compared with the control neurons (white bar).

To further confirm the increase in IP3R1 expression level, we analyzed the amount of IP3R1 expression by Western blot analysis. As shown in Fig. 1D, the IP3R1 expression level in the chronically APV-exposed neurons was clearly increased compared with the control cells, but there was no difference in expression level of β-actin and calreticulin between the groups. Quantitative analysis revealed that the IP3R1 protein level in APV-exposed neurons was 1.64 ± 0.34-fold higher than in the control cells (Control; n = 65 wells, APV; n = 24 wells, p < 0.01). Taken together, these results indicated that the chronic NMDAR blockade increased the IP3R1 expression level in hippocampal neurons.

Time Course of the APV-induced Increase in IP3R1 Expression Level in Hippocampal Neurons—To examine the time course of the APV-induced increase in IP3R1 expression, as shown in Fig. 2A, we exposed hippocampal neurons to 100 μM APV for various times (1, 3, 5, 6, 7, and 14 days), and determined the IP3R1 expression level by Western blot analysis at 21 DIV. At days 1–5 of APV-treatment, there was no apparent change in IP3R1 expression level, except for slight decrease after 1 day of APV exposure at day 1 (Fig. 2B). Because NMDAR activation is known to increase the IP3R1 expression through L-type Ca2+ channel activation (26), the slight decrease after 1 day of APV exposure was probably because of the suppression of basal NMDAR activity. After 6 days of APV exposure, however, the IP3R1 expression level clearly increased (Fig. 2, B and C), and the increase was observed until day 14. Thus, these results indicated that long term APV exposure, i.e. for more than 6 days, increases the IP3R1 expression level in hippocampal neurons.

Transcription and Protein Synthesis Were Required for the APV-induced Increase in IP3R1 Expression—To assess the contribution of protein synthesis to the APV-induced increase in IP3R1 expression level, a protein synthesis inhibitor, cycloheximide (CHX), was added to the APV-exposed cultures from 20 to 21 DIV, when the IP3R1 expression level had greatly increased (Fig. 3A). As shown in Fig. 3B, CHX significantly inhibited the APV-induced increase in IP3R1 expression and there was no significant difference in IP3R1 expression level between the APV+CHX-exposed and CHX-exposed neurons, indicating that IP3R1 degradation occurred to a similar extent irrespective of the presence of APV (Fig. 3, B and C). Next we examined the contribution of transcription of IP3R1 to the APV-induced increase in IP3R1 expression by measuring IP3R1 mRNA by RT-PCR. Total RNA was prepared from cultured hippocampal cells exposed to and unexposed to APV for 7 days, and semiquantitative RT-PCR analysis was performed using specific primers for IP3R1. As shown in Fig. 3D, the amount of IP3R1 mRNA in the APV-exposed neurons increased compared with the controls, whereas the β-actin mRNA level and the SERCA 2b mRNA level (data not shown) did not change significantly. Normalization of the amount of IP3R1 mRNA to that of β-actin revealed that the amount of IP3R1 mRNA had increased by 177 ± 34% in the APV-exposed neurons (n = 3, p < 0.02). These results demonstrated that long lasting NMDAR blockade increases the IP3R1 expression level through a mechanism involving transcription and protein synthesis rather than decreased IP3R1 degradation.
Involvement of PKA Activity in the APV-induced IP3R1 Expression—Chronic NMDAR blockade has been shown to activate cAMP-dependent protein kinase (36). To assess the involvement of PKA activity in the APV-induced IP3R1 expression, we added a specific PKA inhibitor, KT5720, to the APV-exposed cells for the 24 h from 20 to 21 DIV (Fig. 4A). The result was complete abolition of the increase in IP3R1 expression by APV (Fig. 4, C and D). Moreover, activation of PKA by a mixture of IBMX (an inhibitor of phosphodiesterase), and 8-bromo-cAMP, a membrane-permeable analogue of cAMP, for the 48 h from 19 to 21 DIV was sufficient for the increase in IP3R1 expression level (Fig. 4D). The histograms of IP3R1 immunoreactivity in neuronal soma (MAP2-positive; Fig. 4D) clearly show a decreased population of cells with relatively high IP3R1 immunoreactivity (arbitrary units, 9–12) and an increased population of cells with relatively low IP3R1 immunoreactivity (arbitrary units, 1–3) among the APV+KT5720-exposed neurons compared with the APV-exposed neurons. These results demonstrated that the APV-induced increase in IP3R1 expression required PKA activity.

APV-exposed Hippocampal Neurons with High IP3R1 Expression Are More Sensitive to mGluR Stimulation—To explore the functional effect of the increased IP3R1 expression on IP3-induced calcium release, we imaged Ca2+ signals evoked by application of 100 μM DHPG, an agonist of group I metabotropic glutamate receptors, in chronically APV-exposed and unexposed hippocampal neurons. To distinguish neurons from other types of cells and quantify the IP3R1 expression level, we transplanted the mGluR1-expressing cells into the APV-exposed neurons. These findings suggested that the increased IP3R1 expression level in APV-exposed neurons is an important factor in the increased number of DHPG-responsive neurons among the APV-exposed neurons.

Hippocampal Neurons Expressing GFP-tagged IP3R1 Released Ca2+ in Response to G-protein-coupled Receptor Stimulation—To further confirm the relationship between the IP3R1 expression level and the sensitivity of Ca2+ store to mGluR stimulation in hippocampal neurons, we transiently expressed GFP-tagged IP3R1 (38) and examined Ca2+ release in response to mGluR stimulation. As shown in Fig. 6, the cells exogenously expressing GFP-tagged IP3R1 showed transient Ca2+ release upon DHPG application (7 of 8 neurons), whereas no cells expressing GFP alone responded to the agonist stimulation (n = 4 neurons). The transient Ca2+ release in GFP-tagged IP3R1-expressing cells was also observed by stimulation of muscarinic acetylcholine receptor, another type of G-protein-coupled receptor (Fig. 6), suggesting that this increased sensitivity of Ca2+ store is not a specific up-regulation of mGluRs but rather a general phenomenon for G-protein-coupled receptor signaling. Taken together, these results indicated that the expression level of IP3R1 is a crucial determinant for the Ca2+ release upon G-protein-coupled receptor stimulation in hippocampal neurons.
DISCUSSION

The results of this study showed that chronic blockade of NMDAR activity by APV for more than 6 days leads to an increase in IP₃R1 expression level, and that this up-regulation of IP₃R1 requires RNA transcription, protein synthesis, and PKA activity. They also showed that APV-exposed neurons acquire the ability to mobilize intracellular Ca²⁺ in response to mGluR stimulation and that this Ca²⁺-mobilizing property is correlated with the IP₃R1 expression level in the APV-exposed neurons. Thus, our findings indicated that long lasting NMDAR blockade increases IP₃R1 expression and enhances the sensitivity of hippocampal neurons to mGluR stimulation. Consistently, we demonstrated that overexpression of IP₃R1 in hippocampal neurons was sufficient for Ca²⁺ release upon G-protein-coupled receptor stimulation. As far as we know, this is the first direct evidence that the expression level of IP₃R1 in hippocampal neurons determines the sensitivity of Ca²⁺ stores upon G-protein-coupled receptor stimulation.

Numerous factors, including Ca²⁺, calmodulin, ATP, and phosphorylation, have been reported to directly modulate IP₃R channel activity (3, 19), and, in addition to these direct modifications, regulation of the expression level and subunit combination of IP₃Rs can also affect Ca²⁺ mobilization from Ca²⁺ stores (19). The former can immediately and directly change channel activity, although the effects on intracellular Ca²⁺ mobilization may be transient, whereas the latter takes a longer time to influence intracellular Ca²⁺ mobilization, but can affect it for a longer time. Such prolonged regulation of IP₃R-mediated signaling would be important for cells to respond to protracted events, including development and lengthy changes in inhibitory or excitatory environment in various pathological states. The results of the present study revealed a new form of long term regulation of IP₃R1 expression levels. Up-regulation of IP₃R1 expression might respond to a demand for a Ca²⁺ source to maintain the intracellular Ca²⁺ level after blockade of another Ca²⁺ source through the NMDAR channel. It should be mentioned that there are multiple lines of evidence indicating that chronic stimulation of plasma membrane receptors linked to IP₃ production leads to a decrease in IP₃R protein level via calpain, caspase, and ubiquitin-proteasome pathways, which would attenuate excessive Ca²⁺ release from Ca²⁺ stores (19). Control of the IP₃R1 expression level on the endoplasmic reticulum in response to changes in the extracellular environment would be an important mechanism in maintaining intracellular Ca²⁺ homeostasis.

DHPG-insensitive cells were still observed among the strongly IP₃R1-positive neurons, suggesting the existence of other determinants of mGluR-mediated Ca²⁺ release in those neurons. Differences in Ca²⁺ signaling upon mGluR stimulation were also observed in acute hippocampal slices; mGluR-mediated Ca²⁺ release was observed in CA1 (39, 40) and CA3 (41, 42), but not in the granule cells of the dentate gyrus² or

² T. Nakamura, personal observation.

**Fig. 5.** Chronic APV exposure increases the number of hippocampal neurons that show IP₃-induced calcium release in response to mGluR stimulation. (Ca²⁺), changes in response to mGluR stimulation (100 μM DHPG) was examined in fura-2-loaded hippocampal cultured neurons. The preparation used for Ca²⁺ imaging was subsequently subjected to immunostaining with anti-IP₃R1 and anti-MAP2 antibodies to analyze the cell type and the expression level of IP₃R1 in recorded neurons. Upper panel, control cultured neurons. No neurons (MAP2-positive) of 25 neurons in this optical field (0%) responded to DHPG stimulation. Lower panel, APV-exposed neurons. Six of 38 neurons in this optical field (16%) responded to DHPG stimulation. Not all IP₃R1 strongly positive (+ +) cells responded to mGluR stimulation (asterisks). The intensity of IP₃R1 immunoreactivity was judged as described under “Materials and Methods.”
interneurons (see discussion in Ref. 43). Because IP3R1 is predominantly expressed in CA1, with substantially less expression in CA3 and only moderate levels in the granule cells of the dentate gyrus (34, 35), other factors, including the expression level and the cell surface expression level of mGluRs (44, 45), and mGluR-IP3R coupling status through an anchoring protein, Homer (46), may also be responsible for these differences in mGluR-IP3R signaling in different types of neurons.

NMDAR activation has been reported to increase the IP3R1 expression level in hippocampal neurons (26). This apparent contradiction of our results is probably caused by the difference in the duration of NMDAR blockade, because we observed a slight decrease in the IP3R1 expression level after 1 day of APV exposure (Fig. 2). Thus, NMDAR activation up-regulating IP3R1 expression in the short term seems consistent with their findings (26). After longer NMDAR blockade, however, the decreased IP3R1 expression level recovered to the basal level by day 3, and then increased after 6 days of APV exposure (Fig. 2). Thus, our results indicated that, although the IP3R1 expression level tends to decrease in response to NMDAR blockade for a short time, long lasting NMDAR blockade increases the IP3R1 expression level. This, together with the report that NMDAR activation increases IP3R1 expression through a transcription factor, cAMP response element-binding protein (47). Our finding that the IP3R1 up-regulation by prolonged NMDAR blockade involved PKA activation suggests a new signal cross-talk between PKA and phosphatidylinositol pathways over a span of days. Because IP3R1 plays an important role in synaptic plasticity (7–11), PKA probably regulates long term synaptic plasticity by phosphorylating cAMP response element-binding protein, as well as by controlling IP3R1 expression at the transcription level. One of the candidates for the link between PKA and IP3R1 expression is activating protein-2 (AP-2), a transcription factor. AP-2 is highly expressed in the hippocampus and cerebellar Purkinje cells, where IP3R1 is predominantly expressed (48). In addition, AP-2 has been shown to directly regulate the IP3R1 promoter (49), and it is directly phosphorylated and activated by PKA (50). Further studies on the correlation between AP-2 phosphorylation levels and IP3R1 expression levels in APV-exposed hippocampal neurons would help to understand the mechanism of the up-regulation of IP3R1 by prolonged NMDAR inhibition.

In summary, we have shown that chronic NMDAR blockade increases IP3R1 expression and that the neurons highly expressing IP3R1 protein are more sensitive to mGluR stimulation. Moreover, we have shown that the neurons overexpressing GFP-tagged IP3R1 became to show Ca2+ release upon G-protein-coupled receptor stimulation. Our findings, thus, suggest that the IP3R1 expression level is an important determinant of the sensitivity of Ca2+ stores to mGluR activation in hippocampal neurons. Further studies, including in vivo experiments, are necessary to understand the mechanism of the APV-induced IP3R1 expression.

![Hippocampal neurons expressing GFP-tagged IP3R1 showed Ca2+ release upon G-protein-coupled receptor stimulation.](image)

Hippocampal neurons were transiently transfected with GFP-tagged IP3R1 (GFP-IP3R1) or GFP (control) expression vectors. After 36 h, the cells were loaded with fura-2 and the change of [Ca2+]i upon KCl (50 mM), DHPG (100 μM), and acetylcholine (30 μM) stimulations were examined. Whereas no GFP-transfected neurons (n = 4 neurons) showed Ca2+ release upon stimulation by those agonists (7 of 8 neurons, lower panel). Asterisk indicates a glial cell, which was identified by sustained Ca2+ transients (data not shown).
iments, may elucidate the physiological significance of the APV-induced IP$_3$R1 expression in hippocampal neurons.

Acknowledgments—We thank Dr. A. Mizutani for fruitful discussions, Dr. M. Hattori for critical reading of the manuscript, and T. Nakayama and Y. Tateishi for GFP-IP$_3$R1 expression vector.

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J. Biol. Chem. 2004, 279:23691-23698.
doi: 10.1074/jbc.M313296200 originally published online March 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313296200

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