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Dephosphorylation and internalization of cell adhesion molecule L1 induced by theta burst stimulation in rat hippocampus

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Abstract

The neural cell adhesion molecule L1 may participate in initiating and maintaining synaptic changes during learning in the hippocampus. One prominent form of synaptic change in the hippocampus is long-term potentiation (LTP) that occurs following specific patterns of synaptic activity. We present evidence that Y1176 of the YRSL motif within L1 cytoplasmic domain is dephosphorylated in LTP-induced hippocampus. The dephosphorylated L1 is associated with AP-2 and AP180 that are required for clathrin-mediated internalization of L1. These data suggest that clathrin-mediated recycling of L1 at presynaptic sites is enhanced by certain kinds of neural activity, and that maintenance of LTP-induced synaptic changes is regulated by L1 recycling.

Introduction

Activity-dependent changes in the patterning of pre- and postsynaptic arborizations occur during neural plasticity as a result of learning and memory (Hawkins et al., 1993). However, the mechanisms by which electrical activity influences the expression and distribution of cell adhesion molecules (CAMs), as well as the signal transduction machinery affecting subsequent changes in cell–cell interactions remain poorly understood. Changes in neuronal activity can lead to changes in neuronal function and morphology. CAMs, such as L1, are known to influence cell–cell interactions and can regulate the kinds of cell motility that could underlie neuronal plasticity. So it is worthwhile to study how regulation of L1 expression and function can lead to alterations of neuronal plasticity, including the formation of neuronal processes and synapses. To this end, we examined the relationship between neuronal activity and L1 molecular interactions during long-term potentiation (LTP).

The regulatory mechanisms underlying expression of L1 are beginning to be elucidated. Expression of L1 is up-regulated by treatment with high KCl and NMDA (Scherer et al., 1992) and down-regulated by electrical stimulation at low frequency (Itoh et al., 1995), suggesting that neuronal excitability could regulate the expression of L1. If L1 was influenced by specific impulse activity, molecular interactions controlling neuronal plasticity could be regulated by nervous system function. L1 has been implicated in LTP (Luthl et al., 1994) although L1-knock out mice do not have abnormal LTP (Bliss et al., 2000; Law et al., 2003), but L1-deficient mice have been shown to have a reduction in inhibitory post-synaptic currents (Saghatelian et al., 2004). Consequently, the mechanism by which L1 regulates synaptic

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plasticity is still not clear. Recently, Shiosaka et al. reported that L1 could be cleaved by neuropsin in early stages of LTP (Matsumoto-Miyai et al., 2003). Previously, Bailey et al. (1992) have shown that apCAM, a neural cell adhesion molecule in *Aplysia*, is endocytosed during activity induced plasticity, raising the possibility that L1 expression could be regulated during synaptic changes via endocytosis.

Over the past 7 years, a substantial literature has evolved showing the L1 trafficking to axons and internalization from the plasma membrane involves both clathrin and a tyrosine based-sorting motif in the L1 cytoplasmic domain, YRSLE (reviewed in Kamiguchi, 2003). Additionally, the internalization of L1 is regulated by phosphorylation of L1 at Y1176, the tyrosine that initiates the tyrosine based sorting motif (Kamiguchi and Lemmon, 1998; Kamiguchi et al., 1998). When Y1176 is phosphorylated, probably by a src family kinase, L1 cannot be internalized (Schaefer et al., 2002). Fortunately, a monoclonal antibody (mAb), 74-5H7, has been characterized that only binds to L1 when Y1176 is dephosphorylated and this antibody binds to L1 in clathrin coated pits and vesicles but only poorly to L1 at the cell surface (Kamiguchi et al., 1998). So mAb74-5H7 can be used to monitor the internalization state of L1. In this report, we present evidence that the L1 cytoplasmic domain (CD) is dephosphorylated by LTP and that this dephosphorylation is associated with clathrin-mediated L1 internalization.

Results and discussion

LTP induction on MED probe

To investigate the spatial distribution of LTP in the hippocampus, we used a system designed for delivering conditioning stimulation to a slice and observing the amplitude of fEPSPs at nearby locations. Fig. 1 shows typical LTP elicited by the stimulation of the stratum radiatum of area CA1. In this experiment, the stimulating electrode (#27) was chosen in stratum radiatum to stimulate Schaffer collateral/commissural fibers projecting to area CA1. Control fEPSPs were recorded once per min, for 10 min before the conditioning theta burst stimulation (TBS, described in methods, Larson et al., 1986). After the TBS, fEPSPs were recorded at 20 s intervals using a paired-pulse stimulation with an inter-pulse interval of 50 ms for 60 min. Fig. 1A showed the waveforms of fEPSP at an adjacent site (#28) of the stimulation site (#27) at 5 min before and 30 min after the TBS. LTP occurred in CA1 and lasted for at least 30 min (Fig. 1B). The hippocampal slice on the electrode array and two-dimensional current source density analysis for the evoked response is presented in Fig. 1C, showing the site where TBS was delivered to Schaffer collateral/commissural fibers. Evoked current sinks were observed on the stratum radiatum (3rd and 4th row of electrode array) where Schaffer collateral/commissural fibers are localized. In contrast, evoked current sources were observed on the stratum oriens (2nd row of electrode array). The responses at the stratum lacunosum moleculare were very small but were still sources, as expected. Both current sinks and sources were potentiated by TBS. Thus, these evoked currents and potentiations were mainly observed in stratum radiatum and oriens of CA1 but not in the dentate gyrus (DG) (Fig. 1C) and CA3 (data not shown).

LTP leads to dephosphorylation of L1 at Y1176

To assess L1 changes before and after LTP in the hippocampus, L1 was extracted from hippocampus by immunoprecipitation and analyzed by immunoblotting (IB). We have previously characterized the mAb 74-5H7 and shown that it recognizes the tyrosine-based sorting motif, YRSLE, in the L1CD when the tyrosine is dephosphorylated (Schaefer et al., 2002). We have also shown that mAb 74-5H7 binds to L1 in clathrin-coated pits and vesicles but not L1 in the plasma membrane of growth cones (Kamiguchi et al., 1998). Therefore, this phosphorylation state-specific antibody to L1 can be used as an indicator of L1 internalization.

Total L1 levels did not significantly change as a result of LTP induction (Fig. 2A) when compared to control slices that did not receive TBS. However, IB with the mAb 74-5H7 demonstrate a 60% increase in immunoreactivity in LTP-induced hippocampi at 30 min after theta burst stimulation compared to control hippocampi that received repetitive stimulation (10 Hz) but no theta burst stimulation. This indicates a significant dephosphorylation of L1 occurred at tyrosine 1176 as a result of theta burst stimulation.

Dephosphorylated L1 was associated with clathrin-associated proteins in LTP-induced CA1 region

Areas CA1, CA3 and the DG were microdissected from control and LTP-induced hippocampal slices (Fig. 3A). Levels of L1 expression were not significantly changed by LTP induction in each of these regions (Fig. 3B). However, levels of protein recognized by mAb 74-5H7 in L1 immunoprecipitates from area CA1 at 30 min after TBS increased approximately 3-fold in comparison with that of other regions (Fig. 3F). To examine whether L1 associates with clathrin-associated proteins (AP2, AP180) in LTP-induced hippocampi, L1 immunoprecipitates from hippocampal region extracts were probed with antibodies against two of the subunits of AP2, and against AP180. Anti- α -adaptin antibody, which is known to react with 105 and 110 kDa forms of α -adaptin in brain preparations (Ahle and Ungewickell, 1990), labeled two bands of the corresponding sizes in L1 immunoprecipitates (Fig. 3D). These data indicate that the AP2 complex associates with L1 in three regions of hippocampus. AP180, which is a clathrin assembly protein in coated vesicles (Ahle and Ungewickell, 1986), co-immunoprecipitated with L1 from three regions of hippocampus (Fig. 3E), further confirming that L1 forms a complex with clathrin-associated proteins in the hippocampus. The clathrin-associated proteins, AP2 and AP180, in L1 immunoprecipitates from CA1 at 30 min after TBS increased approximately 1.5 and 2 fold, respectively, in comparison with that of CA3 and DG regions, as shown in Figs. 3D, E and F. Negative control showed that neither AP2 nor AP-180 co-immunoprecipitated with NCAM from CA1 region, although antibodies to NCAM readily detected NCAM (data not shown). These data indicate that dephosphorylation of L1 at Y1176 is enhanced by LTP, and then, the L1CD is bound with clathrin-associated proteins.

A variety of assays have shown that the YRSL sequence in the L1CD specifically binds the μ 2 chain of AP2 (Kamiguchi et al., 1998). These results suggest a mechanism by which L1 can be internalized from the cell surface at presynapses upon LTP induction. In theory, these changes would tend to increase structural plasticity by reducing adhesion at synapses. Application of antibody against L1 or recombinant fragments of L1 have been shown to inhibit LTP induced by TBS in CA1 hippocampal neurons in a dose-dependent fashion (Luthi et al., 1994). LTP can be initiated in the presence of these inhibitors, but at high concentrations of L1 antibody, synaptic strength returns to baseline within 30 min of stimulation. However, antibody against L1 is not effective in inhibiting LTP when added 10 min after LTP has been induced. This suggests that L1 participates in the stabilization phase of LTP, but interestingly, the long-term maintenance of LTP cannot be perturbed by antibodies applied minutes after LTP has been expressed. Studies of LTP and learning suggest a possible role of L1 in two phases of synaptic plasticity. In the early phase of LTP, L1 may be down-regulated by activity-dependent proteolysis by neuropsin (Matsumoto-Miyai et al., 2003). Shortly thereafter, L1 surface levels are further reduced by dephosphorylation and association with clathrin-associated proteins. This would lead to L1 internalization. In combination, these two different mechanisms would lead to a decrease in adhesion and permit synapse rearrangement or sprouting in a fashion similar to that proposed for serotonin-mediated synaptic plasticity via apCAM internalization at sensory synapses in *Aplysia* or regulation of Fas II internalization at the neuromuscular junction in *Drosophila* leading to changes in synaptic size (Mathew et al., 2003; Schuster et al., 1996). Regulation of adhesion molecule expression at and near

synapses by internalization, exocytosis and proteolysis is likely to be key factor in the short-term alteration of synaptic structure (Packard et al., 2003).

Experimental methods

Acute rat hippocampal slice preparation

One-month-old Sprague–Dawley rats were sacrificed by decapitation after anesthesia with sodium pentobarbital. Brains were immediately put in an ice-cold oxygenated preparation solution of the following composition: 124 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂ and 2 mM MgCl₂ for 2 min. Brain slices were cut at a 300-μm thickness using a vibrating tissue slicer (Dosaka EM; Model DTK-1500), and the hippocampal formation was dissected rapidly. The dissected hippocampal slices were incubated at room temperature for 1 h in preparation solution.

In hippocampal CA1 region using MED system

The hippocampal slices were gently placed on the center of the 0.1% polyethyleneimine-coated Multi-Electrode Dish (MED) with a multi-channel extracellular recording system (Alpha MED sciences Co., Ltd., Tokyo, Japan) (Oka et al., 1999). The device has an array of 64 planar microelectrodes, 50 μm², arranged in an 8 × 8 pattern with interelectrode spacing of 150 μm (MEDP515AP; Alpha MED sciences Co., Ltd.). Electrophysiological experiments were done in the CO₂ incubator while perfusing with artificial cerebro-spinal fluid (ACSF) of the following composition: 124 mM NaCl, 26 mM NaHCO₃, 10 mM glucose, 3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂ and 1 mM MgCl₂ at 32°C. ACSF was oxygenated with 95% O₂ and 5% CO₂ mixture gas before perfusion in the MED probe. Evoked field potentials at all 64 sites were simultaneously hardware filtered through a 0.1–10 kHz band pass filter and recorded with the MED64 system (Alpha MED sciences Co., Ltd.) at a 20-kHz sampling rate. Stimulations for recordings were performed using biphasic constant current pulses (5–50 μA, 0.1 ms) through one of the 64 electrodes. A stimulation electrode was selected in the stratum radiatum to stimulate Schaffer collateral/commissural fibers projecting to area CA1 (Fig. 1C). Field-excited potentials caused by stimulation were recorded from 64 points around CA1, CA3 and dentate gyrus (DG) regions. The recorded field potentials were confirmed to be field excitatory postsynaptic potentials (fEPSPs) by paired-pulse facilitation. Control fEPSPs were recorded for 10 min before the conditioning stimulation. LTP was induced by theta burst conditioning stimulation (TBS) (Larson et al., 1986), a ten burst train of four-pulses (100 Hz) with 200 ms inter-train intervals. After the theta burst stimulation, fEPSPs were recorded in the CA1, CA3 and DG regions every 20 s for more than 30 min. LTP occurred and lasted for at least 30 min.

Current source density analysis

Procedures for current source density (CSD) analysis of two-dimensional multielectrode data are described by Shimono et al. (2000, 2002a,b). Since the response at the stimulation electrode could not be recorded directly, it was estimated by averaging the left and right neighboring electrodes. The result was convoluted with a 3 × 3 Laplacian kernel (0 1 0, 1 −4 1, 0 1 0) to produce a discrete approximation of the second spatial derivative. The medium was considered ohmic with a homogeneous conductance.

Immunoprecipitation (IP) and immunoblot (IB) analyses

The three regions – CA1, CA3 and DG – were individually dissected from the LTP-induced hippocampal slices by micro-surgical knives (Fig. 3A). These hippocampal regions were each homogenized in 20 mM Tris (pH 7.4), 1 mM EGTA, 1% NP-40, 0.5% deoxycholate, 1 mM sodium orthovanadate, and 10 mM *p*-nitrophenyl phosphate containing 0.32 M sucrose, 200

mM Pefabloc SC, 0.1 mM leupeptin, and 100 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml egg white trypsin inhibitor, 1.0 mM benzamidine and 10 µg/ml pepstatin. The homogenates were centrifuged for 20 min at $10,000 \times g$ at 4°C to remove insoluble material. The solubilized crude membrane fractions were then incubated for 1 h at 4°C with rabbit anti-rat L1 antibody (Brittis et al., 1995). TrueBlotk™ anti-rabbit Ig IP beads (eBiosci., CA, USA) were added and incubated for 1 h at 4°C. The beads were washed six times with the homogenization buffer. Immuno-precipitates were mixed with SDS sample buffer and boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on 8–16% polyacrylamide gels (Novex, CA, USA). Proteins fractionated by SDS-PAGE were transferred to Immobilon-P (Millipore, MA, USA) and processed for western blot analyses with rabbit anti-rat L1 antibody (Brittis et al., 1995), mAb 74-5H7 (Lemmon et al., 1989), mAb AP2 and AP180 (Sigma) followed by HRP conjugated anti-rabbit and mouse Ig TrueBlotk™ (1:1,000, eBiosci., CA, USA) using Super-Signal WestPico chemiluminescent substrate (Pierce, IL, USA). The band densities on the western blots were semi-quantified using NIH Image J software.

Statistics

Significance was assessed by one-way analysis of variance (ANOVA) followed by the multiple means comparison test or the unpaired Student's *t* test.

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Fig. 1.

Long-term potentiation (LTP) in hippocampal slice on a MED probe. (A) The field postsynaptic potentials recorded 5 min before and 30 min after TBS are shown. Bar: 0.2 mV, 10 ms. (B) Summary of induction of LTP monitored from acute rat hippocampal slice. The EPSP amplitudes were normalized by the mean amplitude observed during the control period ($t = -10$ to 0 min). At arrow, TBS was delivered. (C) Two-dimensional current source density analysis of LTP on the top of micrograph of hippocampal slice on a MED probe. This representative photograph shows the placement of the hippocampal slice on the MED electrode array. The 8×8 array covered area CA1 (1st and 2nd row located in stratum oriens, 3rd and 4th row located in stratum radiatum, 5th row located in stratum lacunosum-moleculare, and 6th row located in boundary between CA1 and DG). The numbers (1, 8, 27, 57, 64) on the device indicate the position of electrodes. Typical postsynaptic currents elicited by a single stimulation pulse to the stimulation electrode (#27, closed red squares) on the Schaffer/commissural fiber in stratum radiatum of CA1 and measured from other electrodes (black squares) are shown. The traces showed computed current source density. Yellow traces show the control signal immediately preceding TBS and red traces show the signal 30 min post-TBS. The most significant potentiation can be seen near the stimulus electrode and clear current sinks and potentiations can be detected only along the Schaffer fibers in stratum radiatum (3rd and 4th row of electrode array, red arrows). The interelectrode distance of the MED probe is 150 μm . Bar: 10 ms.

Fig. 2.

Immunoprecipitation of L1 from hippocampal slices before and after LTP induction showing increases in dephosphorylation of Y1176. (A) L1 was immunoprecipitated from hippocampal (1) control slices and (2) 30 min after LTP with anti-L1 antibody. L1 in the immune pellets was detected by immunoblotting with anti-L1 antibody. The 200 kDa band is full-length L1. The 80-kDa band is a fragment containing the L1 cytoplasmic domain. (B) The same membrane filter was stripped and reprobed with mAb 74-5H7. (C) The levels of L1 proteins (200 kDa band) were determined semi-quantitatively by imaging densitometry comparing the intensity of each L1 specific band. The L1 and 74-5H7 values 30 min after LTP were divided by the values for L1 and 74-5H7 from control slices, respectively, and the numbers shown (means $\pm$ SEM) are represented as the percentage of control. Statistical significance was assessed by Student's *t* test, the change in 74-5H7 immunoreactivity (IR) was significant at $P < 0.01$. The experiments were performed four times with similar results.

Fig. 3.

Expression of L1, L1 dephosphorylated at Y1176, AP2 and AP180 in immunoprecipitation of L1 complex from microdissected CA1, CA3 and DG regions of the hippocampal slices after LTP induction. (A) Diagram depicting the microdissected areas of the hippocampal slice. (B) Immuno-blots with antibodies to total L1 (C) dephosphorylated L1 (74-5H7), (D) AP2 and (E) AP180. (F) The levels of L1, AP2 and AP180 proteins were determined semi-quantitatively by imaging densitometry comparing the intensities of each specific band. The L1, 74-5H7, AP2 and AP180 values were divided by the values for total L1, and the numbers shown (means $\pm$ SEM) are represented as the percentage of total L1. Statistical significance was assessed by one-way ANOVA. The increase in 74-5H7-IR, AP2-IR and AP180-IR between CA1 vs. CA3 and DG was significant at the $P < 0.01$. The experiments were performed three times with similar results. 1: CA1, 2: CA3, 3: DG.