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EVIDENCE FOR CONSTITUTIVE PROTEIN SYNTHESIS IN HIPPOCAMPAL LTP STABILIZATION

A.-K. ABBAS *

Institute of Neuroscience and Physiology, University of Gothenburg, P.O. Box 433, SE-40530 Gothenburg, Sweden

Abstract—The notion that blockade of constitutive protein synthesis underlies the effect of protein synthesis inhibitors (PSIs) on long-term potentiation (LTP) stabilization was examined using the rat hippocampal CA3–CA1 synapse. Using a biochemical assay we found protein synthesis rate largely recovered 1 h after wash-out of cycloheximide (CHX). Nonetheless, a 4-h CHX application followed by wash-out 1 h prior to LTP resulted in a significant decrement of LTP stabilization. Wash-out initiated just prior to LTP, thus extending protein synthesis inhibition well into the post-LTP period, resulted in no further effect on LTP. However, short pre- and continuous post-tetanization application of PSIs failed to influence LTP persistence for up to 7 h. Addition of hydrogen peroxide (H_2O_2) 5–25 min following LTP induction resulted in parallel depression of potentiated and non-potentiated inputs, leaving LTP seemingly unaltered. However, in the presence of cyxloheximide the H_2O_2 application resulted in a significant reduction of LTP. In conclusion: LTP stabilization was impaired by pre-LTP application of protein synthesis inhibition but not by post-LTP application unless the slices were exposed to oxidative stress. We submit that these results favor the notion that constitutive rather than triggered protein synthesis is important for LTP stabilization.

Key words: anisomycin, cycloheximide, neuronal plasticity, oxidative stress, protein synthesis, synapse.

INTRODUCTION

Long-term potentiation (LTP) is a cellular model for some forms of learning and memory (Martin et al., 2000; Lynch, 2004). In common with memory LTP has been differentiated temporally into an early vs. late phase, the latter believed to require the synthesis of novel proteins for its expression. In fact, a large number of studies have shown that application of protein synthesis inhibitors (PSIs) during a critical time window (often ±30 min) around the LTP induction event makes an LTP, that would otherwise be long-lasting, to vanish within few hours, i.e., prevents late-phase LTP. Thus, an LTP-inducing tetanization can result in late-phase LTP only if this tetanization triggers synthesis of proteins stabilizing LTP. However, results from other studies have questioned this idea. For example, this effect of PSIs was not observed when also protein degradation was blocked indicating that constitutively expressed proteins may suffice for LTP stabilization (Fonseca et al., 2006). We and others have also recently failed to observe any effect of protein synthesis inhibition on LTP during its first 7–8 h (Abbas et al., 2009; Villers et al., 2012). Moreover, the stability of LTP following a given induction protocol may depend critically on experimental conditions, such as slice preparation and recovery conditions (for references, see Villers et al., 2012).

That the state of the slice may affect LTP raises the question to what extent procedures affecting the protein content and/or the state of the proteins in the slice may affect LTP stabilization. Thus, can protein depletion per se result in an impaired ability of the synapses to undergo the structural/functional rearrangements necessary for stable LTP? In the present study we have introduced a long (4 h) pre-application of PSI to examine whether this procedure may result in less stable LTP. In another set of experiments we applied a mixture of anisomycin and cycloheximide (CHX) for 30 min before LTP induction throughout the time-course of recorded LTP. Furthermore, we also transiently applied hydrogen peroxide (H_2O_2) to the slice in order to induce rapid protein modification/degradation. Our findings suggest that proteins synthesized in response to the LTP-inducing stimulation are unlikely to be critical for the subsequent stabilization of LTP.

EXPERIMENTAL PROCEDURES

Animals

The experimental biomedicine (EBM) animal facility is fully accredited by the Swedish Council for Laboratory Animals. Fourteen- to 22-day-old Albino rats (strain Sprague–Dawley) of mixed sexes were obtained from Charles River (Scanbur AB, Sollentuna, Sweden) and were prior to the experiment kept group-housed. By using a multi-recording chamber system allowing four slices to be studied in parallel, the number of animals used was kept at a minimum.

*Tel: +46-768-235900; fax: +46-31-7863840.
E-mail address: abdul-karim.abbas@neuro.gu.se

Abbreviations: ACSF, artificial cerebrospinal fluid; AP-5, D-(−)-2-amino-5-phosphonopentanoic acid; CHX, cycloheximide; DMSO, dimethysulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; fEPSP, field excitatory postsynaptic potentials; $FeSO_4$, ferrous sulfate; H_2O_2, hydrogen peroxide; LTP, long-term potentiation; PSIs, protein synthesis inhibitors; TBS, theta-burst stimulation; TCA, trichloroacetic acid.

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In vitro electrophysiology and drugs preparation

The animals were killed in accordance with the guidelines of the local ethics committee of University of Gothenburg. They were deeply anesthetized with isoflurane (Baxter Medical AB, Oslo), decapitated and the brain was removed. Transverse hippocampal slices (400-μm-thick) were cut using a McIlwain-type tissue chopper and submerged in a holding chamber containing room-tempered artificial cerebrospinal fluid (ACSF) composed of (in mM): 119 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 26 $NaHCO_3$, 1.0 NaH_2PO_4, and 10 D-glucose, equilibrated with 95% O_2 and 5% CO_2. After pre-incubation for at least 90 min, slices were transferred as needed to one or several recording chambers where they were kept submerged and covered with nylon net to prevent their movement. The recording chamber consisted of a circular well of low volume (1.5–2 ml), and the solution was recycled (1.5–2.0 ml/min) using a peristaltic pump (Ismatec, Labinett Lab AB, Sweden). The perfusion solution was the same as in the holding chamber except for 2.5 mM $CaCl_2$ and 1.3 mM $MgCl_2$. Experiments were performed at 31 °C.

Extracellular field potentials were recorded with a glass micropipette filled with 1 M NaCl (resistance 3–5 MΩ) positioned in the middle of the CA1 stratum radiatum. Microelectrodes were pulled from microfiber (o.d. 1.5 mm, i.d. 0.86 mm, Warner Instruments, LLC, Hamden, CT, USA) capillary tubing. To stimulate independent inputs to the same cell population, two monopolar tungsten stimulating electrodes (0.1 MΩ; World Precision Instrument, Inc., Sarasota, FL, USA) were positioned on either side of the recording microelectrode. Their positions were arranged so that the same amount of stimulating current evoked field potentials of similar magnitude. Stimuli were delivered to the commissural–Schaffer collateral afferents as 100-μs negative constant-current pulses (20–50 μA) using a programmable pulse generator. While one synaptic input (test pathway) was employed to induce and monitor LTP, the other one (control pathway) was used to verify the stability of basal synaptic transmission. Pulses were delivered alternately to the two pathways, usually at a rate of once every 40 or 60 s, with stimuli to the two pathways separated by 20 or 30 s, respectively. Baseline field excitatory postsynaptic potentials (fEPSPs) were recorded for 60–90 min to ensure stability of responses. LTP was induced generally by theta-burst stimulation (TBS) composed of three trains (inter-train interval 5 s), each consisting of 10 bursts of four pulses at 100 Hz, with an inter-burst interval of 200 ms (Larson et al., 1986). In one group of experiments, LTP was induced by three tetanus trains (100 Hz, 1 s, inter-train interval 5 s).

The PSIs anisomycin (2-[p-methoxybenzyl]-3,4, pyrrolidinediol-3-acetate) and CHX 4-{(2R)-2-[(1S,3S,5S)-3,5-dimethyl-2-ococyclohexyl]-2-hydroxyethyl} piperidine-2,6-dione, H_2O_2 (3%), ferrous sulfate ($FeSO_4$) and dimethysulfoxide (DMSO) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and D-(−)-2-amino-5-phosphonopentanoic acid (AP5) were obtained from Tocris Bioscience (UK) or Ascent Scientific Ltd (UK). [^{3}H]leucine was obtained from Amersham, Buckinghamshire, UK. Milli-Q deionized water (Millipore, Bedford, MA, USA) was used in all preparations of buffers and solutions. Other chemicals used were all of highest grade commercially available. For biochemical assay, anisomycin, or CHX, was added to ACSF to different final concentrations derived from different stocks prepared in distilled water at the time of each experiment. For electrophysiological experiments, CHX and DPCPX were first dissolved in DMSO to a concentration of 100 and 3 mM, respectively, and stored at −18 °C until diluted in physiologic buffer to final concentration of 100 μM and 50 nM, respectively. The largest final concentration of DMSO was 0.1%, which had no effect on basal synaptic transmission as verified by lack of effect on the baseline fEPSP when added as vehicle. Stocks of AP5, anisomycin and $FeSO_4$ were prepared in distilled water and diluted at final concentrations of 50 μM, 25 mM and 100 μM, respectively. The rational for $FeSO_4$ addition was to enhance the generation of hydroxyl radical from H_2O_2 via a Fenton-like reaction.

Biochemical assays

Effect of PSIs on protein synthesis was measured by [^{3}H]leucine incorporation (Lipton and Heimbach, 1978) in whole slices. Hippocampal slices from 13- to 24-day-old rats were maintained under similar conditions as in the electrophysiological work but without stimulation. Slices were put on a multiwall plastic dish (Corning Incorporated, Corning, NY, USA) and assigned to a PSI group or a control group in an interleaved manner to minimize inter-slice variability with respect to weight and metabolism. To test the effect of PSIs on rate of protein synthesis in dose-dependent manner, two slices per group were pre-incubated for 10 min with the drug (or vehicle, if necessary) before addition of [^{3}H]leucine; final activity 0.5–1 μCi/ml. Uptake and incorporation of leucine into proteins were allowed to proceed for 50 min either with or without PSI. To test PSI reversibility, a corresponding total duration of pre-incubation with the drug (60 min) was followed by different pre-incubation intervals (30, 60 or 90 min) in drug-free solution before allowing slices to be incubated in a [^{3}H]leucine-containing solution for additional 50 min. Radioactive amino acid incorporation was then terminated by the addition of ice-cold saline, and the slices were subsequently lysed in ∼1 ml of 5 mM NaOH. After protein purification, incorporation of leucine into trichloroacetic acid (TCA)-precipitable proteins was measured in a scintillation counter (LKB Wallace, 1219 Rackbeta, Finland). Percentage inhibition of leucine incorporation produced by drug treatment was calculated by comparing counts in treated slices with those of control ones.

To investigate whether protein content was influenced by the addition of H_2O_2, two groups of slices were incubated with tritiated leucine for 50 min at 31 °C following a pre-incubation period equivalent to that for the electrophysiological experiments. The slices were subsequently washed by continuous perfusion with