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BRYOSTATIN-1 PROMOTES LONG-TERM POTENTIATION VIA ACTIVATION OF PKCα AND PKCε IN THE HIPPOCAMPUS

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Abstract—Activation of protein kinase C (PKC) by bryostatin-1 affects various functions of the central nervous system. We explored whether bryostatin-1 influenced synaptic plasticity via a process involving PKC. Our purpose was to examine whether bryostatin-1 affected the induction of hippocampal long-term potentiation (LTP) in Schaffer-collateral fibers (CA1 fibers) of the hippocampus, and/or influenced the intracellular Ca^{2+} level of hippocampal neurons. We also determined the PKC isoforms involved in these processes. We found that bryostatin-1 strongly facilitated LTP induction, in a dose-dependent manner, upon single-theta burst stimulation (TBS). Further, intracellular Ca^{2+} levels also increased with increasing concentration of bryostatin-1. The facilitative effects of bryostatin-1 in terms of LTP induction and enhancement of intracellular Ca^{2+} levels were blocked by specific inhibitors of PKCα and PKCε, but not of PKCδ. Our results suggest that bryostatin-1 is involved in neuronal functioning and facilitates induction of LTP via activation of PKCα and/or PKCε.

Key words: bryostatin-1, long-term potentiation (LTP), synaptic plasticity, protein kinase C (PKC), hippocampus.

INTRODUCTION

Protein kinase C (PKC) is a member of a multigene family of protein kinases composed of phospholipid-dependent serine–threonine kinases. Based on homology and sensitivity to activators, the 12 isozymes of the family are divided into three subfamilies: the classical PKC subfamily (containing the cPKC isoforms α, β_I, β_{II}, and γ); the novel PKC subfamily (containing the nPKC isoforms δ, ε, η, and θ); and the atypical PKC subfamily (containing the aPKC isoforms ζ and λ/ι). PKC is involved in synaptic transmission, activation of ion flux, secretion, cell cycle control, proliferation, tumorigenesis, and apoptosis (Teyler and DiScenna, 1987). PKC enhances learning and memory (Sun and Alkon, 2005). Activation of PKC increases the numbers of spines and presynaptic vesicles in the hippocampal CA1 region (Hongpaisan and Alkon, 2007).

"Synaptic plasticity" is a term used to describe persistent activity-dependent changes in synaptic strength, and such plasticity is thought to play an important role in learning and memory (Ster et al., 2009). The principal forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP is a long-lasting activity-dependent enhancement of excitatory synaptic strength after delivery of a brief high-frequency train of electrical stimulation to the hippocampus (Kumar, 2011). LTP is mediated by several protein kinases and phosphatases. For example, the phosphorylation activity of calcium/calmodulin-dependent protein kinase II (CaMII) is enhanced following LTP induction (Fukunaga et al., 1993). Similarly, PKC activity is essential for induction of LTP in the hippocampus (Collingridge et al., 2004; Moriguchi et al., 2009).

A few studies have recently shown that activation of PKC by bryostatin-1 regulates neuritogenesis, axonal transport, and the synaptic plasticity essential for neuronal function (Gonzalez et al., 2002; Hama et al., 2004; Alkon et al., 2005b; Houeland et al., 2007; Sossin, 2007). Bryostatins are macrocyclic lactones produced by the marine organism *Bugulaneritina* (Pettit et al., 1986). Both bryostatins and phorbol esters are potent activators of PKC (Kraft et al., 1986; Zhang et al., 1996). *In vitro* trials have shown that bryostatins act synergistically with other anti-cancer drugs to prevent tumor growth via modulation of PKC activity (Kraft et al., 1986; Hofmann, 2004; Alkon et al., 2007). Studies on Alzheimer's disease (AD) using AD-transgenic mice have found that bryostatin significantly reduced mortality. Bryostatin also effectively reduced amyloid-β levels (Lahn et al., 2004).

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Abbreviations: ACSF, artificial cerebro-spinal fluid; AD, Alzheimer's disease; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; fEPSP, field excitatory postsynaptic potential; FPs, field potentials; LTP, long term potentiation; NMDAR, *N*-methyl-D-aspartate receptor; PKC, protein kinase C; PPF, paired-pulse facilitation; TBS, theta burst stimulation.

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However, the mechanism by which bryostatin-1 affects learning and memory has not been elucidated. Any role for PKC, activated by bryostatin-1, in induction of hippocampal LTP, has not been defined. The purpose of the present study was to examine whether the PKC activator bryostatin-1 affected induction of hippocampal LTP and/or influenced intracellular Ca^{2+} levels. We also identified the PKC isoforms involved in mediation of these effects.

EXPERIMENTAL PROCEDURES

Experimental animals

Male C57BL/6 mice (3–4 weeks of age when experiments commenced) were used. All animals were individually housed in a temperature-controlled room (22–25 °C) under a 12-h light/dark cycle; the lights went on at 07:00. Food and water were available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of Eulji University (Permit No. EUIACUC 11-12).

Preparation of hippocampal slices

The procedures for electrophysiological experiments were as described previously (Park et al., 2010). Briefly, mice were decapitated under deep enflurane anesthesia and brains were quickly removed and chilled in ice-cold dissection buffer containing sucrose (212.7 mM), KCl (2.6 mM), NaH_2PO_4 (1.23 mM), $NaHCO_3$ (26 mM), dextrose (10 mM), $MgCl_2$ (10 mM), and $CaCl_2$ (0.5 mM). Horizontal brain sections (400 μmin thickness) were prepared using a vibratome (Campden Instruments; Loughborough, UK); the slices were placed into dissection buffer that was continuously bubbled with 95% O_2/5% CO_2 (v/v). The slices were held at 35 °C for 1 h in a chamber filled with continuously oxygenated artificial cerebrospinal fluid (ACSF) of the following composition: NaCl (124 mM), KCl (5 mM), NaH_2PO_4 (1.25 mM), $NaHCO_3$ (26 mM), dextrose (10 mM), $MgCl_2$ (1.5 mM), and $CaCl_2$ (2.5 mM). The slices were next transferred to a submersion recording chamber, maintained at 30 °C, and perfused with oxygenated ACSF at a flow rate of 2 ml/min.

Electrophysiological recordings

A bipolar stimulating electrode was inserted into the stratum radiatum to activate the Schaffer collaterals of CA1 pyramidal cells. A glass micropipette filled with ACSF was inserted into the CA1 pyramidal layer to record field potentials (FPs). CA1 FPs were evoked by stimulating the Schaffer collaterals with electrical pulses 0.2 ms in duration delivered with the aid of concentric bipolar stimulating electrodes (FHC; Bowdoinham, ME, USA); the initial slopes of extracellular FPs were recorded in the CA1 stratum radiatum. Baseline responses were obtained upon application of 50% of the maximal stimulation, at 0.033 Hz. LTP was induced using a conventional stimulation paradigm; the theta-burst stimulation (TBS) protocol consisted of eight bursts, each of four 100-Hz pulses, administered at 200-ms intervals. The stimulus intensity during TBS was identical to that of the test pulse. All measurements are expressed as percentages of the average values calculated 20 min prior to LTP induction. Significant differences between groups were sought via evaluation of average LTP values 58–60 min after LTP induction. To measure paired-pulse facilitation (PPF), we used inter-stimulus intervals (ISIs) of 25 ms, 50 ms, 100 ms, 200 ms, 400 ms, 1000 ms, and 2000 ms. When input–output tests were conducted, the slopes of stabilized field excitatory postsynaptic potentials (fEPSPs) were recorded. Under stable conditions, each test commenced at zero intensity and the intensity was increased until the maximal synaptic response was obtained. Input–output responses represent changes in basal synaptic transmission mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Akopian and Walsh, 2002). Pharmacologically isolated NMDA receptor-mediated synaptic responses were measured using 0 mM $MgCl_2$ ACSF with 10 μM CNQX. APV (50 μM) was added at the end of each experiment to confirm the NMDA receptor-mediated responses.

Culture of primary neurons

Rat hippocampal neurons dissociated from the newborn pups (P1–P2) as described previously (Garrido et al., 2002; Codazzi et al., 2006). The hippocampus was dissected out from SD rat pups and carefully removed meninges. Cells were dissociated with papain for 30 min, and then discarded the media. Hippocampal neurons were plated onto a culture plates and maintained with neurobasal medium containing B27, and 1 mM L-glutamine at 37 °C, 95% O_2 and 5% CO_2 incubator. The medium must be half-changed every 3–4 days.

Determination of changes in $[Ca^{2+}]_i$

To monitor $[Ca^{2+}]_i$, neurons were incubated in culture medium containing fura-2/AM (1 μM) at room temperature (37 °C) for 10 min. To detect Ca^{2+}-dependent fluorescence signals, coverslips coated with fura-2-loaded cells were mounted in an experimental perfusion chamber and placed on the stage of an inverted epifluorescence microscope (Nikon TE300, Tokyo, Japan). A video microscopic image acquisition and analysis system was employed. Cells were alternatively excited, at 340/380 nm, with light from a xenon arc lamp passed through a filter wheel (LEPMAC 5000, Spectra Services Inc.; Ontario, NY, USA). Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (CoolSNAP HQ, Roper Scientific; Ottobrunn, Germany), and recorded with the aid of MetaFluor (Version 7.5, Molecular Devices; Downingtown, PA, USA). All fluorescence measurements were conducted in areas that we considered contained individual neurons. No apparent morphological changes, that could possibly introduce errors in Ca^{2+} measurements, were observed under our experimental conditions (data not shown). All stimulatory materials were dissolved in neurobasal medium and applied directly to cells in the perfusion chamber. All experiments were performed at room temperature (23–25 °C).

Drugs

We purchased the PKC activator bryostatin-1, the nonspecific PKC inhibitor Ro 31-8220, the selective PKCδ inhibitor rottlerin, the AMPA receptor antagonist CNQX and the *N*-methyl-D-aspartate receptor (NMDAR) antagonist DL-APV, from Sigma (St. Louis, MO, USA). The selective PKCα inhibitor Ro 32-0432 was from Calbiochem (San Diego, CA, USA) and the PKCε-inhibitor TAT-εV1-2 peptide from Anaspec (San Jose, CA, USA). All drugs were dissolved in DMSO, except for DL-APV, which was dissolved in normal saline.

Data analysis

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc.; San Diego, CA, USA) or SPSS 10.0 (SPSS Inc.; Chicago, Illinois). All values are given as means ± SEMs; the error bars in the figures also represent SEMs. Statistical significance was assessed using *t*-test or ANOVA, followed by Fisher's PLSD post hoc testing.