Corticotropic-Releasing Factor Produces a Protein Synthesis–Dependent Long-Lasting Potentiation in Dentate Gyrus Neurons

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INTRODUCTION

We have previously demonstrated that the 41 amino acid polypeptide corticotropin-releasing factor (CRF) produces a long-lasting enhancement of synaptic efficacy in rat hippocampal neurons (Wang et al. 1998). This CRF-induced enhancement was shown to be dependent on the CRF receptor antagonist dose- and time-dependently diminishes the magnitude of LTP. In addition, CRF enhances synaptic transmission, and CRF and tetanus stimulation have an additive effect on hippocampal neuron excitation (Wang et al. 1998). Further, the N-methyl-D-aspartate (NMDA) receptor antagonist MK801, known to prevent tetanization-induced LTP (Collingridge and Bliss 1987), also partially blocks CRF-induced potentiation. Moreover, we have demonstrated that CRF-induced potentiation, like the case of LTP, is also mediated through the cyclase-adenosine-3,5-monophosphate (cAMP) signaling pathway because the cAMP inhibitor Rp-cAMP significantly blocks CRF-induced potentiation (Wang et al. 1998).

Physiologically, CRF is known to stimulate the secretion of adrenocorticotropic and β-endorphin from the pituitary (Rivier et al. 1981). CRF is also known to produce various behavioral activations (Dunn and Berridge 1990). For example, intraventricular injection of CRF enhances locomotion, rearing, and sniffing responses in rats (Sutton et al. 1982; Veldhuis and DeWied 1984). More related to the present study, both our and other laboratories have found that direct central injections of CRF improve acquisition and memory retention of rats in various learning tasks (Koob and Bloom 1985; Lee et al. 1993; Tershner and Helmstetter 1996). Further, CRF-induced memory facilitation is dependent on protein synthesis because protein synthesis inhibitors prevent the memory-enhancing effect of CRF (Lee et al. 1992). Because protein synthesis mediates CRF-induced memory facilitation and that CRF and LTP share some similar mechanisms in producing hippocampal neuron excitations, in the present study, we were aimed to investigate the following. 1) Is protein synthesis also involved in the cellular mechanism underlying CRF-induced long-lasting potentiation? 2) Does CRF-induced long-lasting potentiation share the same mechanism with tetanization-induced LTP? To study this, we examined whether maximum excitation of dentate gyrus neurons with tetanic stimulation occludes further potentiation of these neurons produced by CRF and vice versa. 3) Does tetanic stimulation activate CRF neurons in the dentate gyrus?

METHODS

Animals

Adult male Sprague-Dawley rats weighing 250–350 g bred in the Animal Facility of the Institute of Biomedical Sciences, Academia Sinica were used. Animals were housed three per cage in a room maintained at 23 ± 2°C and a 12-h light/dark cycle (light on at 6:30 A.M.) with food and water continuously available.

Drugs and drug application

Corticotropic-releasing factor, actinomycin-D and emetine were purchased from Sigma Company (St. Louis, MO). All other chemical reagents of the highest grade were purchased from Merck (Darmstadt, Germany) or Sigma. All drugs were dissolved in 0.9% saline. Thirty...
minutes after baseline recording, drugs were delivered to the dentate gyrus through Hamilton syringe at a rate of 20 nl/min. At the end of drug infusion, the Hamilton syringe was withdrawn, and the recording electrode was placed into the dentate gyrus. Tetanic stimulation was then given. The concentration of CRF, actinomycin-D, and emetine were given in the dose of 100 ng, 5 µg, and 5 µg in a volume of 100 nl, respectively. These concentrations were chosen because CRF at this concentration was shown to produce a consistent long-lasting potentiation in dentate gyrus neurons (Wang et al. 1998) and actinomycin-D and emetine were shown to prevent the memory-facilitating effect of CRF (Lee et al. 1992). For the drug interaction studies, actinomycin-D or emetine was given 90 min before CRF or tetanic stimulation.

**Stimulating and recording electrodes**

The stimulating electrodes were platinum concentric bipolar electrodes with a tip diameter of 25 µm. The stereotaxic coordinates were adjusted for variation in rat ages to maximize the monosynaptic responses of the excitatory postsynaptic potentials (pEPSP) produced by the granule cells in response to stimulation of the dorsomedial perforant path. The correctness of the coordinates was verified by the wave form that appeared on the oscilloscope on stimulation (McNaughton and Barnes 1977). The averaged stimulating coordinates were AP ~8.3 mm from bregma, ML +4.4 mm from the midline, and 2.7 mm below the skull surface. The recording electrodes were prepared from single-barrel borosilicate glass micropipettes 1.2 mm and 2.7 mm below the skull surface. The recording electrodes were positioned ~3.5 mm posterior to bregma and 2.0 mm from the midline. The dentate gyrus was identified by single-unit activity characteristic of granule cells, as well as by an electrode depth of 3.0–3.5 mm below the brain surface. After the stimulating and recording electrodes were positioned, 5% agar dissolved in 0.9% NaCl was applied over the exposed brain and skull to prevent surface drying and reduce movement artifacts.

**Electrophysiological recording**

Approximately 1 h before the surgery, animals were anesthetized with urethane (1.4 g/kg, 25% concentration ip). The rat was then placed on a stereotaxic instrument for surgery. At the beginning of the surgery, lidocaine was applied in small quantities to the incision area. The surface dura matter was incised. Throughout the surgery and experiments, body temperature was monitored and maintained at 35 ± 1°C with a feedback control system. Stimulation parameters for evoking population spikes in vivo were adopted from the protocol of Chiba et al. (1992). Briefly, stimulation consisted of 100 µA, a population spike of 7–8 mV in amplitude can always be seen with tetanization, and most of the time seen with CRF injection. Once determined, stimulus current remained constant throughout the experiment. After recording 30 min of baseline responses, four sets of tetanic stimulation were administered to induce stable and long-lasting potentiation. Each set contained 5 trains, 10 pulses per train at 400 Hz, delivered at a rate of 1 train per second for 5 s. The pulse widths in the trains were 50, 100, 150, and 200 µs, respectively. Tetanic stimulation was delivered at 10-min intervals for 40 min. LTP as well as CRF-induced potentiations were measured as the increase in slope during the first millisecond of pEPSP in millivolts. Percent increase in the pEPSP was calculated as follows: the slope in millivolts at each minute minus the mean baseline slope and then divided by the mean baseline slope. The granule cell pEPSPs were amplified by a conventional amplifier at a frequency band of 1.0 Hz to 10 kHz. All potentials were monitored on an oscilloscope. The pEPSP slope recorded at time point set at 1.52 ms was used for data analysis.

**Total RNA extraction**

Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Briefly, the hippocampal tissue from the recording site was dissected out at the end of recording using a brain slicer. The dentate gyrus was further dissected out using a punch with 1.5 mm diam (Ma et al. 1999). The dentate gyrus tissue was then homogenized with Ultraspex RNA isolation kit. The homogenate was kept on ice at 4°C for 5 min, added with 0.2 ml chloroform, and centrifuged at 14,500 g for 15 min. The aqueous phase was added with equal volume of isopropanol and kept on ice for 10 min. The reaction mixture was centrifuged at 14,500 g for 10 min. The pellet RNA was washed twice with 75% ethanol and precipitated by subsequent centrifugation at 11,500 g for 5 min. The pellet was briefly dried under vacuum and dissolved in diethyl-pyrocarbonate (DEPC)-treated water. To avoid DNA contamination, total RNA (20 µl) was further treated with DNase (0.1 U/µl) in the presence of RNase inhibitor (0.2 U/µl) for 30 min at 37°C in Taq buffer. After phenol/chloroform (3:1, vol/vol) extraction, the aqueous phase was recovered by centrifugation at 14,500 g for 15 min. It was then added with 1/10 volume of 2.5 M sodium acetate and 2 volume of 95% ethanol. The mixed solution was kept in a freezer (−20°C) for at least 2 h and centrifuged at 14,500 g for 15 min. The pellet was then washed with 75% ethanol and dried.

**Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of CRF mRNA level**

Because the total amount of RNA extracted from the dentate gyrus was <20 µg, and because of the minute amount of CRF present in the dentate gyrus, the quantitative RT-PCR method was adopted for the present study. Briefly, 0.05 µg of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMVRT) with oligo-dT (0.5 µg/µl) as primers in a 20-µl reaction buffer containing 25 mM Tris-HCl (pH 8.3), 25 mM KCl, 5 mM MgCl2, 5 mM dithiothreitol, 0.25 mM spermidine, 1 mM deoxyribonucleoside triphosphate (dNTPs), and 20 U RNase inhibitor for 1 h at 42°C. AMVRT was heat-inactivated by boiling for 5 min. The endogenously expressed rat hypoxanthine phosphoribosyl transferase (HPRT) mRNA was used as an internal control template, which was coamplified with the CRF mRNA. One set of PCR primers was used for CRF and HPRT, respectively. The oligonucleotide primer pairs for PCR were designed by using a computer program (Lowe et al. 1990). The sequences for primer pairs of CRF were 5’-(CAG AAC AGT GCG GCC GTA CTA CG-3’) and 3’-(GGGA AAA AGT TAG CCG CAG CCT CTT-3’), and that for HPRT were 5’-(CAG AAT TCC GTT TGT CTG AAG GGG GGC-3’) and 3’-(GGGA ACG CAG CAA CAG ACA TT T-3’), which flanked a region of 365 and 625 bp for CRF and HPRT, respectively. In PCR amplification, specific oligonucleotide primer pairs of CRF and HPRT (0.5 and 0.2 µl, respectively) were incubated with 2 µl of the RT product and 2 U of Taq polymerase in a 20-µl reaction mixture that included 1 × Taq buffer, 1.5 mM MgCl2, and 200 µM dNTPs. The parameters for PCR amplification were as follows: denaturation at 94°C for 2 min. The reactions were performed for 30 cycles and followed by 10-min incubation at 72°C for complete elongation. Ten microliters of each PCR product was investigated by 2% agarose gel electrophoresis.

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The gels were stained with ethidium bromide solution at a concentration of 1 μg/ml for 30 min and destained in distilled water for 15 min. For quantification, the gels were illuminated by ultraviolet lamp, and the intensity of each CRF cDNA band was normalized with the corresponding HPRT cDNA band and quantified with a densitometer (Eagle Eye II, Stratagene).

RESULTS

Effects of actinomycin-D on CRF-induced potentiation

To study whether RNA synthesis is involved in CRF-induced long-lasting potentiation, we have examined the effects of actinomycin-D, a transcription inhibitor, on CRF-induced potentiation. Results in Fig. 1A show that actinomycin-D at a dose of 5 μg/μl, which by itself did not markedly affect basal synaptic transmission in dentate gyrus neurons $[t_{(1,8)} = 0.61, P > 0.05]$, significantly prevented CRF-induced long-lasting potentiation $[t_{(1,8)} = 13.74, P < 0.001$; Fig. 1B] in a manner similar to actinomycin-D–induced impairment in tetanization-induced LTP in vivo $[t_{(1,8)} = 20.25, P < 0.001$; Fig. 1C]. Figure 1D illustrates the patterns of a single response evoked by CRF (1) and actinomycin-D + CRF (2), respectively.

Effects of emetine on CRF-induced potentiation

To investigate whether CRF-induced potentiation also depends on protein synthesis, we have examined the effects of emetine, a protein synthesis inhibitor, on CRF-induced potentiation.
tiation. Similar to the effects observed with actinomycin-D, emetine at 5 μg/μL, which did not markedly affect the baseline activity of dentate gyrus neurons \( t_{1,8} = 0.53, P > 0.05; \) Fig. 2A, markedly decreased the magnitude of CRF-induced long-lasting potentiation \( t_{1,8} = 10.93, P < 0.001; \) Fig. 2B in a way similar to emetine-induced inhibition of tetanization-induced LTP in vivo \( t_{1,8} = 18.25, P < 0.001; \) Fig. 2C. Figure 2D illustrates the patterns of a single response produced by CRF (1) and emetine + CRF (2), respectively.

**Effects of prior tetanization on CRF-induced potentiation**

We have previously demonstrated that tetanic stimulation and CRF, when both were not given at the maximum intensity and concentration, produced an additive effect on hippocampal neuron potentiation (Wang et al. 1998). If tetanic stimulation and CRF do share a common mechanism, then prior maximum excitation of dentate gyrus neurons should occlude further excitation of these neurons produced by the other stimulus. The present experiment examined this hypothesis. Results in Fig. 3A show that prior maximum excitation of dentate gyrus neurons with four trains of tetanic stimulation effectively prevented further excitation of these neurons produced by 100 ng of CRF \( t_{1,8} = 0.78, P > 0.05 \). Figure 3B illustrates the patterns of a single response produced by tetanic stimulation alone (1) and tetanic stimulation followed by CRF administration (2).
Effects of CRF pretreatment on tetanization-induced potentiation

To further test the above hypothesis, we have also examined the effects of tetanic stimulation and CRF on hippocampal neuron potentiation in an opposite way. Results in Fig. 4A indicate that if 100 ng CRF was given to produce a maximum excitation of dentate gyrus neurons (Wang et al. 1998), the following tetanic stimulation did not produce further consistent excitation on these neurons \( t(1,8) = 1.53, P > 0.05 \), but a transient increase in the amplitude of pEPSP was observed. Figure 4B illustrates the patterns of a single response produced by CRF alone (1) and CRF followed by tetanic stimulation (2).

**CRF mRNA expression on tetanic stimulation**

If CRF neurons are involved in the neural circuits underlying LTP, it is expected that CRF neurons are activated on tetanic stimulation. Results in Fig. 5 reveal that there was \( 30\% \), but a significant increase in CRF mRNA level in the dentate gyrus 1 h after tetanic stimulation \( t_{(4,18)} = 4.63, P < 0.05 \).
and tetanization do act additively and synergistically to activate production of hippocampal neurons suggest that CRF vise versa. Our results that the second stimulation could not have first maximally excited hippocampal neurons with mechanisms in producing hippocampal neuron potentiation, turned on on CRF administration require further investigation.

The specific genes that are expression and protein synthesis. The specific genes that are phosphorylation pathway and turn on the downstream genes for gene cAMP responsive element binding protein (CREB) phosphorylation, associated with memory facilitation in rats (Hung et al. 1995). It is possible that CRF may activate the cAMP and cAMP responsive element binding protein (CREB) phosphorylation pathway and turn on the downstream genes for gene expression and protein synthesis. The specific genes that are turned on for CRF administration require further investigation.

To address the issue that CRF and L-LTP may share similar mechanisms in producing hippocampal neuron potentiation, we have first maximally excited hippocampal neurons with four trains of tetanic stimulation followed by CRF injection or vice versa. Our results that the second stimulation could not produce further excitation of these neurons suggest that CRF and tetanization do act additively and synergistically to activate hippocampal neurons. But in the case of CRF followed by tetanization, a transient increase in the magnitude of LTP immediately after tetanic stimulation was still observed (Fig. 3A). This is probably due to the fact that the induction of LTP is mainly dependent on NMDA receptor activation (Collingridge and Bliss 1987; Morris et al. 1986), although NMDA receptor blockade only partially prevents CRF-induced potentiation (Wang et al. 1998). Therefore the transient increase in pEPSP following tetanization possibly reflects a transient activation of NMDA receptor and Ca\(^{2+}\) influx, rather than protein synthesis. This differential effect of NMDA receptor blockade on CRF and tetanization-induced potentiations also suggests that the mechanisms underlying the induction of these two potentiations are not completely the same.

Then how might CRF neurons involved in the neural circuits underlying LTP? To address this issue, we have analyzed CRF mRNA expression in the dentate gyrus on tetanic stimulation by quantitative RT-PCR analysis. Our results indicated that there was a significant increase in CRF mRNA level 1 h after tetanization. In an earlier study, we have similarly reported that hippocampal CRF mRNA level is increased in rats showing good retention performance on learning (Lee et al. 1996). These results together suggest that perforant path stimulation may directly or indirectly activate local CRF neurons in the hippocampus. This suggestion is also supported by the observation that in the paraventricular nucleus of the hypothalamus, ~70% of the CRF-positive neurons also express the NMDA receptor NR1R mRNA, and ~20–45% of these neurons also express other types of the glutamate receptors (Aubry et al. 1996). In a more recent study, we have demonstrated that intra-dentate CRF injection dose-dependently increases brain-derived neurotrophic factor (BDNF) mRNA level (Ma et al. 1999, while BDNF mRNA expression was also increased on LTP stimulation (Castren et al. 1993). However, the extent of CRF mRNA elevation was ~30%, indicating that the number of CRF neurons in the dentate gyrus is limited. Further, the present results indicated that actinomycin-D and emetine inhibited tetanization-induced LTP to a greater extent than CRF-induced long-lasting potentiation (Figs. 1, B and C, and 2, B and C). These results also suggest that, although CRF neurons are possibly involved in the neural circuits underlying LTP, the mechanisms underlying these two processes may not be completely the same. Tetanic stimulation may activate more neurons and involve more complicated neural networks. It would be ideal to examine CRF mRNA level after actinomycin-D and emetine treatments. Alternatively, CRF may activate the noradrenergic innervation to the hippocampus to produce potentiation. This suggestion is based on the findings that norepinephrine (NE) also produces long-lasting potentiations in dentate gyrus neurons in a protein synthesis-dependent manner (Stanton and Sarvey 1984) and that CRF facilitates hippocampal NE release (Lee et al. 1994) and activates noradrenergic neurons in the rat brain (Emoto et al. 1993). Further, NE receptor blockade prevents the memory-facilitating effect of CRF injected into the hippocampus (Lee et al. 1993). Otherwise, CRF may exist in GABAergic neurons in the hippocampus because \(\gamma\)-aminobutyric acid (GABA) neuron constitutes the major type of interneurons in the hippocampus (Freund and Buzsaki 1996) and CRF-immunoreactive neurons were found to be co-immunolabeled with glutamate decarboxylase (GAD) in this area (Yan et al. 1998). Further, amygdala kindling was found to increase the expression of CRF and CRF-binding protein in GABAergic neurons in the dentate hilus (Smith et al. 1997). Thus concurrent activation of CRF and GABA on tetanic stimulation phase of LTP. In both studies, ~5–10% of potentiation in pEPSP slope was still observed at the end of recording. This is probably because the dose chosen for the present study was moderate because higher concentration of both inhibitors was known to produce cell toxicity (Lindenboim et al. 1995; Wenzel and Cosma 1984). In another study, Huang and Kandel (1995) have similarly reported that dopamine D1/D5 receptor activation also produces a protein synthesis-dependent long-lasting potentiation of hippocampal neurons. Further, DA receptor activation-induced potentiation was also suggested to be mediated through the cAMP signaling pathway. Because CRF receptor activation is known to stimulate adenyl cyclase activity (Dave et al. 1985) and increase hippocampal cAMP level (Wang et al. 1998), CRF also increases protein phosphorylation associated with memory facilitation in rats (Hung et al. 1992). It is possible that CRF may activate the cAMP and cAMP responsive element binding protein (CREB) phosphorylation pathway and turn on the downstream genes for gene expression and protein synthesis. The specific genes that are turned on for CRF administration require further investigation.

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may therefore reduce the GABAergic inhibition that then leads to excitation. However, the exact synaptic relationship among CRF neuron, GABA neuron, glutamate neuron, and the neural circuits underlying LTP requires further investigation. On another topic, CRF has been shown to result in seizure activity in hippocampal CA3 layers in immature rats (Hol Zigel et al. 1998). Whether it also contributes to the potentiation observed in the present study is not known. Electroencephalogram (EEG) monitoring should help to delineate this point.

In conclusion, the present results demonstrate that CRF and tetanic stimulation share some similar mechanisms in producing long-lasting potentiation of dentate gyrus neurons, and protein synthesis appears to be one of the underlying mechanisms. Tetanic stimulation also seems to activate CRF neurons in the dentate gyrus because CRF mRNA level was increased on tetanization. In conjunction with our previous observation that RNA synthesis and protein synthesis inhibitors also effectively block the memory-facilitating effect of CRF (Lee et al. 1992), these results together suggest that CRF plays an important role in modulating synaptic efficacy of hippocampal neurons and behavioral plasticity in rats. Further, these processes critically depend on protein synthesis.

This work was supported by Research Grant NSC88-2314-B-001-012 from the National Science Council of Taiwan, Republic of China. Present address of H. L. Wang: Dept. of Medical Technology, Yuan-Pei Institute of Medical Technology, Hsin-Chu, Taiwan, Republic of China.

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Received 24 June 1999; accepted in final form 23 September 1999.

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