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During the first year of this research project, it was demonstrated that distinct types of voltage-gated calcium channels are required for the exocytosis of glutamate and dynorphin peptides. We were also to confirm that the release of glutamate from hippocampal mossy fiber terminals is regulated by a presynaptic receptor that is sensitive to L(+)aminophosphonobutyric acid. In the second year of this research project we tested several specific hypotheses concerning presynaptic receptors and the autoregulation of the hippocampal mossy fiber synapse. Specifically, it was demonstrated that the transmitter(s) released from the mossy fiber terminals may mediate positive or negative feedback control of the mossy fiber synaptic input, under appropriate conditions, by activating presynaptic autoreceptors. Presynaptic facilitory kainate receptors are hypothesized to enhance mossy fiver transmitter release through a mechanism that involves the activation of a guanine nucleotide-binding regulatory protein (Gs) that stimulates adenylyl cyclase and increases the activity of voltage-gated calcium channels. This presynaptic facilitation may contribute to hippocampal neurodegeneration produced by the plant-derived toxins kainate and domoate. The goal of our research during the third year has been to determine whether presynaptic inhibitory kappa opioid

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receptors exert an antagonistic influence on mossy fiber transmitter release that may function ot limit the overexcitation of hippocampal neurons. Moreover, we have investigated the role of protein kinase C in the regulation of glutamate and dynorphin release from mossy fiber terminals. Overall, substantial progress has been made from the in vitro analysis of isolated hippocampal mossy fiber synaptosomes.

PRESYNAPTIC MODULATION OF THE HIPPOCAMPAL MOSSY FIBER SYNAPSE

AFOSR 89-0531

ANNUAL TECHNICAL REPORT

1. Summary

The overall goal of this research project is to systematically investigate a number of the possible ways through which presynaptic modulation might influence the effectiveness of local synaptic interactions at the mammalian hippocampal mossy fiber synapse. A hippocampal subcellular fraction that is highly enriched in large mossy fiber nerve endings was developed for this purpose. The morphological and metabolic properties of this synaptosomal preparation have previously been described, and both glutamate and prodynorphin-derived peptides have been shown to be released from these specialized nerve endings in response to membrane depolarization by calcium-dependent mechanisms. During the first year of this research project, it was demonstrated that distinct types of voltage-gated calcium channels are required for the exocytosis of glutamate and dynorphin peptides. We were also able to confirm that the release of glutamate from hippocampal mossy fiber terminals is regulated by a presynaptic receptor that is sensitive to L(+)aminophosphonobutyric acid. In the second year of this research project we tested several specific hypotheses concerning presynaptic receptors and the autoregulation of the hippocampal mossy fiber synapse. Specifically, it was demonstrated that the transmitter(s) released from the mossy fiber terminals may mediate positive or negative feedback control of the mossy fiber synaptic input, under appropriate conditions, by activating presynaptic autoreceptors. Presynaptic facilitory kainate receptors are hypothesized to enhance mossy fiber transmitter release through a mechanism that involves the activation of a guanine nucleotide-binding regulatory protein (G_s) that stimulates adenylyl cyclase and increases the activity of voltage-gated calcium channels. This presynaptic facilitation may contribute to hippocampal neurodegeneration produced by the plant-derived toxins kainate and domoate. The goal of our research during the third year has been to determine whether presynaptic inhibitory kappa opioid receptors exert an antagonistic influence on mossy fiber transmitter release that may function to limit the overexcitation of hippocampal neurons. Moreover, we have investigated the role of protein kinase C in the regulation of glutamate and dynorphin release from mossy fiber terminals. Overall, substantial progress has been made from the in vitro analysis of isolated hippocampal mossy fiber synaptosomes. Recent work in this laboratory has resulted in the development of an alternative model systems for use in this project; a primary culture of cortical neurons in which transient transfections can be obtained using plasmid constructions expressing specific PKC isoforms. Through the combined use of these complimentary preparations, both direct and indirect approaches will be employed for the investigation of the relationship between PKC activation, substrate phosphorylation, cytoskeletal reorganization and glutamate exocytosis.

It has been proposed that a controlled and restricted reorganization of the subsynaptic cytoskeleton plays a critical role in plasticity and the maintenance of cell polarity, an inherent feature of cells in the nervous system. However, neuronal processes would atrophy when this same process of cytoskeletal disaggregation (solation) persists beyond what is required to enhance synaptic strength. Our research has focused on specific

molecular components that are sorted and assembled into segregated microdomains surrounding voltage-regulated Ca^{2+} channels on either side of the hippocampal mossy fiber synapse. We postulate that the functional integrity of these microdomains is determined by Ca^{2+} channel activity. In collaboration with Dr. Huntington Potter (Harvard Medical School), we are conducting an experimental investigation of the mechanisms that target specific subsets of mRNA to the dendritic spines of the MF synapses.

2. Research Objectives

The research objectives for the funding period 15 September 1991-14 September 1992 were as follows:

a) To determine the proportion of endogenous glutamate which is released from synaptosomes which are of mossy fiber origin in the P_3 subcellular fraction.

b) Test the hypothesis that dynorphin, through activation of a presynaptic kappa subtype of opioid receptor, suppresses the release of both glutamate and dynorphin from isolated hippocampal mossy fiber terminals.

c) Test the hypothesis that acetylcholine suppresses the evoked release of glutamate from hippocampal mossy fiber synaptosomes through the activation of a muscarinic subtype of cholinergic receptor.

d) Test the hypothesis that the stimulation of protein kinase C-dependent phosphotransferase activity differentially influences the co-release of dynorphin and glutamate from hippocampal mossy fiber nerve endings.

e) Test the hypothesis that a subset of mRNA are specifically sorted and targetted to the dendritic spines which make intimate synaptic contact with the mossy fiber nerve endings.

3. Status of Research

3.1 To determine the proportion of endogenous glutamate which is released from synaptosomes which are of mossy fiber origin in the P_3 subcellular fraction.

Hippocampal mossy fiber (MF) nerve endings may be isolated in a subcellular fraction (P_3) that releases both prodynorphin-derived peptides and glutamate (Glu), in a calcium-dependent manner, when depolarized. Because dentate granule cells give rise to the MF pathway and are the only known source of hippocampal dynorphin (Dyn), it may be concluded that Dyn exocytosis originates exclusively from the MF synaptosomes present in the P_3 fraction. However, it remains to be demonstrated that the exocytosis of Glu originates from synaptosomes of MF origin. Here, we have addressed this issue by determining the degree to which a selective lesion of the MF system *in vivo* concomitantly reduces the exocytosis of Dyn and Glu from the P_3 subcellular fraction. Unilateral injections of colchicine into the dentate gyrus resulted in a substantial and selective degeneration of the granule cell-MF pathway in the rat hippocampal formation. However, measurements of the packing density for Timm-positive MF terminals indicated that the loss of MF boutons was not equal at all septotemporal levels. The overall integrated density

of the Timm-stained band, which corresponds to the position of the MF terminal field, was estimated to be reduced by 75%. Although destruction of MF boutons was incomplete, the colchicine-induced lesion reduced the K^+-evoked release of Dyn and Glu from the P_3 subcellular fraction by 95% and 51%, respectively. These results convincingly demonstrate that the majority of Glu released from this synaptosomal preparation is of MF origin and are consistent with the hypothesis that Dyn peptides are co-released with this excitatory amino acid from hippocampal MF terminals.

The results obtained in this study were used to devise a set of minimal criteria for determining whether a given parameter directly influences exocytosis from hippocampal MF nerve endings. For example, it may be concluded that MF terminals are an anatomic substrate for any parameter which alters Dyn B-LI release from the P_3 fraction. Various experimental conditions have previously been shown to satisfy this MF criteria (Gannon et al., 1989; Gannon and Terrian, 1991a; Gannon and Terrian, 1991b; Gannon and Terrian, 1992; Terrian et al., 1989b; Terrian et al., 1991c). However, a more conservative set of MF criteria must be employed when interpreting Glu release data obtained using this MF-enriched synaptosomal preparation. First, the K^+-evoked release of Glu from the P_3 fraction can not be reduced by more than 50% without inhibiting Glu exocytosis from MF nerve endings, since at least 50% of the Glu released is of MF origin. Therefore, it may be concluded that Glu exocytosis from MF nerve endings was directly influenced when the K^+-evoked release of Glu was decreased by more than 50%. This criterion has been shown to be satisfied by a variety of different κ opioid receptor agonists (Gannon and Terrian, 1991b; Gannon and Terrian, 1992), indicating that co-released prodynorphin-derived peptides, putative endogenous κ ligands, may play a role in the autoregulation of Glu exocytosis from MF terminals. Electrophysiological studies have recently provided additional evidence to support this hypothesis (Weisskopf et al., 1992). A second method we routinely employ for interpretation is to compare our Glu and Dyn B-LI release data. By examining the effect that a parameter has on the ratio of these two response data sets it is possible to estimate the likelihood that a causal relationship exists between the parameter and Glu exocytosis of MF origin. As this ratio approaches unity there is a corresponding increase in the confidence that such a relationship exists. For example, the protein kinase C activator, 4β-phorbol 12,13-dibutyrate, has been shown to produce proportional increases in the K^+-evoked release of both Glu and Dyn B-LI over a range of increasing concentrations (Terrian et al., 1991c). The most difficult result to interpret using the P_3 fraction is one in which the release of Glu but not Dyn B-LI is increased. It may be possible to facilitate Glu release through an enhancement of localized calcium entry without altering the release of neuropeptides (Verhage et al., 1991). However, the assignment of such a selective enhancement of Glu exocytosis to the MF synaptosomes must remain tentative until additional evidence can be obtained to support this conclusion. For example, it may be useful to compare the effects of such a treatment on the release of Glu from a P_3 fraction and crude mitochondrial fraction (P_2) prepared using the same hippocampal tissue. A preferential effect of the treatment on Glu release from MF synaptosomes would be indicated if there was a greater percent increase in Glu release from the P_3 than the P_2 fraction. Because MF synaptosomes are a major source of the Glu released from the P_3 fraction, the increase in Glu release would be understated if the stimulus preferentially enhanced Glu release from non-MF synaptosomes in this subcellular fraction. In this case, there would be a greater percent increase in the Glu released from the P_2 than the P_3 fraction. Finally, an equivalent enhancement of Glu release from the P_3 and P_2 fractions would require that the treatment be twice as effective in facilitating the release of Glu from non-MF synaptosomes in the P_3 than the P_2 fraction, since at least 50% of the Glu released from the later originates from MF synaptosomes. Until a more precise estimate can be obtained, it should not be assumed that any more than 50% of the Glu is

actually released from MF synaptosomes. Given this precaution, the P_3 fraction should continue to provide a useful model for investigating the presynaptic modulation of both Dyn and Glu release from hippocampal MF nerve endings.

3.2 Test the hypothesis that dynorphin, through activation of a presynaptic kappa subtype of opioid receptor, suppresses the release of both glutamate and dynorphin from isolated hippocampal mossy fiber terminals.

Opioid agonists specific for the μ, δ and κ opioid receptor subtypes were tested for their ability to modulate potassium-evoked release of L-glutamate and dynorphin B-like immunoreactivity from guinea pig hippocampal mossy fiber synaptosomes. The κ opioid agonists U-62,066E and (-)ethylketocyclazocine, but not the μ agonist [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO) nor the δ agonist [D-Pen^{2,5}]enkephalin (DPDE), inhibited the potassium-evoked release of L-glutamate and dynorphin B-like immunoreactivity. U-62,066E, but not DAGO or DPDE, also inhibited the potassium-evoked rise in mossy fiber synaptosomal cytosolic Ca^{2+} levels, indicating a possible mechanism for κ agonist inhibition of transmitter release. DAGO and DPDE were found to be without any effect on cytosolic Ca^{2+} levels or transmitter release in this preparation. The U-62,066E inhibition of the potassium-evoked rise in synaptosomal cytosolic Ca^{2+} levels was partially attenuated by the opioid antagonist quadazocine and insensitive to the δ-opioid specific antagonist ICI 174,864 and the μ opioid-preferring antagonists naloxone and naltrexone. Quadazocine also reversed U-62,066E inhibition of the potassium-evoked release of L-glutamate, but not dynorphin B-like immunoreactivity. These results suggest that κ opioid agonists inhibit transmitter release from mossy fiber terminals through both κ opioid and non-κ opioid receptor mediated mechanisms.

3.3 Test the hypothesis that acetylcholine suppresses the evoked release of glutamate from hippocampal mossy fiber synaptosomes through the activation of a muscarinic subtype of cholinergic receptor.

The presynaptic effects of muscarine on hippocampal mossy fiber (MF) synaptic transmission were examined using a subcellular fraction enriched in isolated MF synaptosomes. Muscarine significantly enhanced the K^+-evoked release of endogenous glutamate (Glu) but not dynorphin B-like immunoreactivity (Dyn B-LI) from superfused guinea pig hippocampal MF synaptosomes. The presynaptic facilitation of Glu release by muscarine was dose-dependent and was antagonized by the prior application of atropine. The effects of a variety of alternative cholinergic agonists on the depolarization-induced rise in cytosolic free calcium ($[Ca^{2+}]_i$) were also tested using the Ca^{2+} indicator dye, fura 2. Neither muscarinic nor nicotinic agonists produced a change in the extent to which membrane depolarization augmented the availability of $[Ca^{2+}]_i$ in MF synaptosomes. These results are consistent with the hypothesis that extrinsic septal cholinergic inputs to the hippocampal CA3 region may generate synchronized firing in pyramidal neurons through a mechanism which involves the presynaptic facilitation of the excitatory MF synaptic input.

3.4 Test the hypothesis that the stimulation of protein kinase C-dependent phosphotransferase activity differentially influences the co-release of dynorphin and glutamate from hippocampal mossy fiber nerve endings.

This has continued to be a very active field of investigation in this laboratory during the present funding period. In our most recent study we have investigated the delayed and persistent effects of 4β-phorbol 12,13-dibutyrate (PDBu) on the K^+-evoked release of endogenous glutamate and dynorphin B-like immunoreactivity (Dyn B-LI) from a subcellular fraction (P_3) that is enriched in hippocampal mossy fiber (MF) synaptosomes. It is demonstrated that the α, β, γ, ε, and ζ isoforms of protein kinase C (PKC) are present in the P_3 fraction obtained using the guinea pig hippocampus as starting tissue. The K^+-evoked release of glutamate was found to be selectively ehanced when MF-enriched synaptosomes were preincubated with PDBu for 15 min and extensively washed with a PDBu-free medium. The persistent enhancement of glutamate release observed under this condition was not reversed by the protein kinase inhibitor staurosporine and was desensitized to the potentiating effects of an acute re-exposure to PDBu. The overall content and activity of PKC was not substantially altered during the initial 15 min of treatment with PDBu (10 μM). More prolonged pretreatments with PDBu altered the substrate specificity of PKC and decreased the content of all PKC isoforms, but did not reverse the facilitation of glutamate release that followed preincubation in the presence of PDBu. It is concluded that the persistent activation of PKC enhances K^+-evoked glutamate release from hippocampal MF-enriched synaptosomes and that, once established, this presynaptic facilitation is sustained by a process that is no longer directly dependent on continued PKC phosphotransferase activity.

3.5 Test the hypothesis that a subset of mRNA are specifically sorted and targetted to the dendritic spines which make intimate synaptic contact with the mossy fiber nerve endings.

Previous studies have demonstrated that the branched spines of the MF-CA3 hippocampal synapse contain a particularly large number of polyribosomes (Chicurel and Harris 1989, Soc. Neurosci., abstract). We analyzed a preparation of synaptosomes isolated from this region and have found it to contain a restricted RNA population - certain mRNAs, presumably derived from the dendritic spines and the fine astrocytic processes surrounding the pre- and postsynaptic elements of the synapse are enriched in the synaptosomal preparation as compared to the total hippocampus; other mRNAs are less prevalent or altogether absent. In addition, neural BC-1, a small dendritic RNA thought to be involved in dendritic pre- or post-translational regulatory processes, is a major RNA component of the dendritic spine. These results support the hypothesis that local translational regulation of gene expression may be important in establishing and modulating synaptic function.

4. Publications

4.1 Full papers and review articles

1. Gannon, RL, TERRIAN, DM: Kappa opioid agonists inhibit transmitter release from guinea pig hippocampal mossy fiber synaptosomes. Neurochem. Res., 1992; 17:741-747.

2. Simpson, J., Gannon, RL, McGinty, JF, TERRIAN, DM: Kainic acid depresses the ex vivo release of dynorphin B and glutamate from rat hippocampal mossy fiber synaptosomes. Neurosci. Lett., 1992; 137: 149-153.

3. Loewen, JJ, Peters, RI, TERRIAN, DM: Adenosine modulation of dynorphin B release by hippocampal synaptosomes. Brain Res., 1992; 577: 318-320.

4. TERRIAN, DM, Ways, DK, Gannon, RL, Zetts, DA: Transduction of a protein kinase C-generated signal into the long-lasting potentiation of glutamate release. Hippocampus, In Press.

5. Chicurel, ME, TERRIAN, DM, Potter, H: mRNA at the synapse: analysis of a synaptosomal preparation enriched in hippocampal dendritic spines. J. Neurosci., In Press.

6. Conner-Kerr, TA, Simmons, D, Peterson, GM, TERRIAN, DM: Evidence for the co-release of dynorphin and glutamate from rat hippocampal mossy fiber terminals. J. Neurochem., Submitted.

7. Conner-Kerr, TA, TERRIAN, DM: Presynaptic inhibition of glutamate release from hippocampal mossy fiber synaptosomes: a potential mechanism for the anticonvulsant effects of U-54494A. Brain Res. Bull., Submitted.

4.2 Refereed Abstracts:

1. Simpson, JN, Gannon, RL, McGinty, JF, and TERRIAN, DM: Kainic acid causes a dissociation between the steady-state concentration and the KCl-evoked release of dynorphin B and glutamate from rat hippocampal mossy fiber synaptosomes. Trans. Soc. Neurosci. 1991; 17(1):412.

2. Chicurel, ME, TERRIAN, DM, Harris, KM, and Potter, H: mRNA at the synapse: analysis of a preparation enriched in hippocampal dendritic spine mRNA. Trans. Soc. Neurosci. 1991; 17(1):379.

3. Conner-Kerr, TA, Simmons, DR, Peterson, GM, Zetts, DA, and TERRIAN, DM: Evidence for co-release of dynorphin and glutamate from rat hippocampal mossy fiber terminals. Trans. Am. Soc. Neurochem. 1992; 23:222.

4. TERRIAN, DM, Ways, DK, Dorman, RV, and Zetts, DA: Presynaptic facilitation of glutamate exocytosis may be sustained by activation of a protein kinase cascade. Trans. Soc. Neurosci. 1992; 18:751.

5. Chicurel, ME, DeFranco, C, TERRIAN, DM, and Potter, H: Localization of RNA at synapses: identification of a synaptosomal GAP-43 RNA-binding protein. Trans. Soc. Neurosci. 1992; 18:787.

5. Professional Personnel Associated With the Research Project

David M. Terrian, Ph.D. - Principal Investigator
Robert L. Gannon, Ph.D. - Co-Investigator
Debbie A. Zetts, B.S. - Research Technician III
Meena H. Patel, B.S. - Research Technician II
Teresa A. Conner-Kerr - Graduate Student
Thomas H. Privette - Graduate Student
Jeffrey N. Simpson - Graduate Student
Duncan R. Simmons - Medical Student

6. Interactions

11/12/91 Invited Lecture, East Carolina Chapter of the Society for Neuroscience, Autoregulation and differential release of amino acids and neuropeptides from isolated nerve terminals.

11/17/91 Invited Lecture, United States Environmental Protection Agency, Neurotoxicology Division, Research Triangle Park, NC. Differential involvement of protein kinase C in the evoked release of glutamate and prodynorphin-derived peptides.

6/21/92 Invited Lecture, East Carolina University School of Medicine, Department of Biochemistry. Transduction of a protein kinase C-generated signal into the long-lasting facilitation of glutamate release: potential role in the maintenance of synaptic enhancement.

9/12/92 Invited Lecture, East Carolina University School of Medicine, Department of Physiology. Presynaptic facilitation of glutamate exocytosis.

7. New Discoveries, Inventions, or Patent Applications

None.

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Kappa Opioid Agonists Inhibit Transmitter Release from Guinea Pig Hippocampal Mossy Fiber Synaptosomes

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(Accepted December 11, 1991)

Opioid agonists specific for the μ, δ, and κ opioid receptor subtypes were tested for their ability to modulate potassium-evoked release of L-glutamate and dynorphin B-like immunoreactivity from guinea pig hippocampal mossy fiber synaptosomes. The κ opioid agonists U-62,066E and (-) ethylketocyclazocine, but not the μ agonist [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin (DAGO) nor the δ agonist [D-Pen^{2,5}]enkephalin (DPDE), inhibited the potassium-evoked release of L-glutamate and dynorphin B-like immunoreactivity. U-62,066E, but not DAGO or DPDE, also inhibited the potassium-evoked rise in mossy fiber synaptosomal cytosolic Ca^{2+} levels, indicating a possible mechanism for κ agonist inhibition of transmitter release. DAGO and DPDE were found to be without any effect on cytosolic Ca^{2+} levels or transmitter release in this preparation. The U-62,066E inhibition of the potassium-evoked rise in synaptosomal cytosolic Ca^{2+} levels was partially attenuated by the opioid antagonist quadazocine and insensitive to the δ-opioid specific antagonist ICI 174,864 and the μ opioid-preferring antagonists naloxone and naltrexone. Quadazocine also reversed U-62,066E inhibition of the potassium-evoked release of L-glutamate, but not dynorphin B-like immunoreactivity. These results suggest that κ opioid agonists inhibit transmitter release from mossy fiber terminals through both κ opioid and non-κ opioid receptor mediated mechanisms.

KEY WORDS: Hippocampus; mossy fiber; opioids; synaptosome; glutamate; dynorphin.

INTRODUCTION

The hippocampal mossy fiber (MF) terminal field of the CA3 region forms a suprapyramidal band, the stratum lucidum, that has been shown to possess a moderate to strong immunoreactivity to dynorphin and enkephalin peptides (1–5). Isolated hippocampal MF nerve endings (synaptosomes) release both types of opioid peptides in a calcium-dependent manner when depolarized (6–8; Terrian and Gannon, unpublished data) and autoradiographic studies have provided evidence for both μ and κ opioid receptor sites in the stratum lucidum of guinea pig hippocampus (4). These findings have raised the possibility that prodynorphin- and proenkephalin-derived peptides may be released from MF expansions and interact with multiple subtypes of opioid receptors in the immediate vicinity of their terminus (9,10) to influence the activity of hippocampal CA3 pyramidal cells. Indeed, pharmacological investigations have demonstrated that μ, κ, and δ opioid receptor agonists and antagonists exert a modulatory influence on the MF-CA3 pyramidal cell synapse (11–14). However, the proposed endogenous ligands for the κ opioid receptor subtype, the prodynorphin-derived peptides (15), produce only weak or mixed effects on the excitability of CA3 pyramidal cells in the hippocampus (16). Therefore, the location and pharmacological properties of the receptors that presumably mediate these actions of endogenous opioids in the hippocampal MF synapse have remained unclear.

It has recently been reported that the κ opioid agonist U-50,488H(17) inhibits the release of L-glutamate (Glu) and dynorphin B-like immunoreactivity (Dyn B-LI) from depolarized guinea pig hippocampal MF syn-

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aptosomes (18). The depressant effect of U-50,488H was the first direct biochemical evidence for an inhibitory κ opioid autoreceptor on MF terminals. However, relatively high (> 30 μM) concentrations of U-50,488H and its congeners are required to significantly depress either the electrophysiological activity of the MF-CA3 synapse in brain slices (13,19) or the release of transmitters from isolated MF synaptosomes (18). One possible explanation for the high concentrations of both U-50,488H and U-69,593 that are required for inhibition of MF-CA3 synaptic transmission may be that a non-opioid, local anaesthetic action directly on CA3 neurons may be responsible for the observed synaptic depression, rather than an opioid receptor mediated effect (19). However, the synaptic depression that is produced by micromolar concentrations of U-50,488H in the MF-CA3 synapse does not mimic the effects of the local anaesthetic procaine on CA3 cells (13). In reviewing this evidence, we have been unable to exclude the possibility that the synaptic depressant activity of κ opioids may be mediated primarily by a presynaptic action on MF terminals, rather than a direct interaction with the CA3 pyramidal cells. In fact, a distinct class of opioid binding site, the λ site, has tenatively been proposed to function as an opioid autoreceptor on the hippocampal MFs (20). The λ site has a ligand specificity that distinguishes it from classical opioid receptor subtypes (21). The dense λ binding that is present in the stratum lucidum (22) is substantially depleted following a colchicine-induced lesion of the dentate granule cells and their MF axons (20), and λ binding rapidly converts to a state of low affinity in in vitro preparations (21). The later finding may possibly account for the high agonist concentrations that are required to inhibit the release of neurotransmitters from isolated MF synaptosomes (18).

In the present study, we have tested agonists specific for μ, δ, and κ opioid receptor subtypes, as well as subtype-specific and nonspecific opioid antagonists for their effects on cytosolic Ca^{2+} levels in, and transmitter release from, MF synaptosomes. The results of this analysis confirm that the depressant activity of opioids on MF terminal function is specific to agonists of the κ opioid subtype, even at high concentrations. In addition, we provide evidence that the inhibitory effects of κ agonists at MF synapses cannot be attributed solely to a local anaesthetic mechanism.

EXPERIMENTAL PROCEDURE

Synaptosome Preparation and Superfusion Techniques. Male Hartley guinea pigs (200 – 250 g) were decapitated, the hippocampi removed, and MF synaptosomes prepared by manual homogenization and differential centrifugation as previously described (8). MF synaptosomes were initially incubated in Ca^{2+}-free buffer (see below) for 5 min at 30°C. $CaCl_2$ was then added (final concentration of 0.9 mM) and the incubation continued for an additional 15 minutes. Approximately 6 – 7 mg of synaptosomal protein (precise amounts were retrospectively determined by the method of Peterson (23)) were layered into superfusion columns constructed from 10 cc polypropylene syringe tubes, and superfused (0.5 ml/min) at room temperature. The procedure used to prepare superfusion columns has been explained in detail elsewhere (6). Superfusion buffers, pH 7.4, consisted of (in mM): $CaCl_2$ 0.9, NaCl 122.0, KCl 3.1, $MgSO_4$ 1.2, KH_2PO_4, 0.4 $NaHCO_3$ 5.0, glucose 10.0, Na-N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) 20.0, and 1 mg/ml bovine serum albumin. High potassium (30 mM KCl) buffers were prepared by equimolar substitution of KCl for NaCl. MF synaptosomes were superfused for 14 min with the low (3.1mM) KCl buffer to obtain steady basal release levels of Glu and Dyn B-LI. Synaptosomes were then exposed to low KCl medium containing 50 μM D-aspartate for 5 min (14 – 19 min of superfusion) in order to reduce cytosolic Glu levels, and enhance the Ca^{2+}-dependence of the Glu release measured in the superfusates (6,24). After 32 min of continuous superfusion, the MF synaptosomes were depolarized with a 2 min pulse of high (30 mM) KCl buffer and then superfused for an additional 10 min (34 – 44 min of superfusion) with a low KCl buffer to allow the rate of release to return to a basal level. The amounts of Glu and Dyn B-LI released into the superfusate fractions (5 min/fraction) were determined using fluorometric (25) and radioimmunoassay (8) procedures, respectively. The basal release levels of Glu and Dyn B-LI collected from a separate unstimulated column (3.1 mM KCl-containing buffer superfused throughout) were subtracted from 30 mM KCl-stimulated release levels in order to calculate evoked release values.

Measurement of Cytosolic Ca^2. Synaptosomal cytosolic Ca^{2+} levels were measured using the fluorescence ratio method for the Ca^{2+} indicator dye fura-2 (26). Approximately 5 mg of MF synaptosomal protein was incubated for 20 min with Fura-2/acetyloxymethyl ester (10 μM) at 37°C in Ca^{2+}-free buffer. Synaptosomes were pelleted (10 sec at 11,000 *g*), washed, and incubated for an additional 15 min at 37°C in 0.9 mM $CaCl_2$-containing buffer. After an additional wash, approximately 100 μg of MF synaptosomal protein was added to stirred quartz cuvettes containing 2.0 ml of low KCl buffer thermostatted to 37°C. Precise MF synaptosomal protein levels were determined retrospectively (23) and varied by less than 10% in all experiments. A Perkin Elmer LS 50 spectrometer was used to obtain fluorescence ratio measurements at 1.6 sec intervals. R_{max} was determined using 0.01% sodium dodecyl sulfate and R_{min} with 10mM TRIS(hydroxymethyl)-aminomethane/ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (TRIS/EGTA). The emission wavelength was set at 509 nm and the Kd for fura-2 was 224 nM. Results were calculated using the Intracellular Biochemistry® software provided by Perkin Elmer. Where appropriate, test compounds were added to the cuvettes in 100-fold greater concentrations to minimize the volumes added.

Materials. Drugs were obtained from the following sources: fura-2/AM (Molecular Probes, Eugene, OR), naloxone HCl and naltrexone HCl (Research Biochemicals Inc, Natick, MA), ICI 174,864 (Cambridge Research Biochemicals, Valley Stream, NY), [D-Pen^{2,5}]enkephalin (DPDE), [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO), tetrodotoxin (TTX) and veratridine (Sigma Chemical Co., St. Louis, MO). U-62,066E was kindly provided by The Upjohn Co. (Kalamazoo, MI), and (-)ethylketocyclazocine (EKC) and WIN 44441-3 (quadazocine) by Dr. Susan Ward and Sterling Drug Inc. (Rensselaer, NY).

RESULTS

Pharmacology of Opioid Effects on Cytosolic Ca^{2+} Levels

Agonists. The μ, δ, and κ selective opioid agonists DAGO, DPDE and U-62,066E (27,28), respectively, were initially evaluated for effects on cytosolic Ca^{2+} levels in MF synaptosomes. Agonists, from 1 nM - 100 μM, were applied 30 sec prior to depolarization of MF synaptosomes by 30 mM KCl. Neither DAGO nor DPDE had any effect on KCl-evoked rises in cytosolic Ca^{2+} levels at any of the concentrations tested (Figure. 1). U-62,066 E, however, did significantly depress stimulated cytosolic Ca^{2+} levels at concentrations greater than 30 μM, with mean inhibitions of 37% and 60% for 100 μM and 300 μM U-62,066E, respectively (Figure 1; when expressed as percent of control, 30 μM U-62,066E also inhibited the KCl-evoked rise in cytosolic Ca^{2+} by 29 ± 4 %, $P < 0.01$, Student's *t* test). No facilitory effects on cytosolic Ca^{2+} levels were observed upon application of opioid agonists under any condition tested. In addition, none of the agonists at the highest concentration tested (100 μM), affected basal MF cytosolic Ca^{2+} levels. Basal Ca^{2+} levels prior to and 30 sec after agonist application were as follows (nM, mean ± SEM): DPDE, 301 ± 4 and 306 ± 5 (n = 5); DAGO, 252 ± 8 and 263 ± 7 (n = 9); U-62,066E, 260 ± 4 and 265 ± 5 (n = 32).

Antagonists. A series of selective and broad spectrum opioid antagonists were evaluated for their ability to reverse the U-62,066E inhibition of KCl-evoked increases in MF synaptosomal cytosolic Ca^{2+} levels. Antagonists were applied 30 sec prior to U-62,066E (30 μM) and 60 sec prior to depolarization of MF synaptosomes by 30 mM KCl. Neither the μ-preferring antagonists naltrexone and naloxone (10,29) nor the δ-selective antagonist ICI 174,864 at concentrations up to 10 μM significantly reversed the inhibitory effect of U-62,066E (data not shown). However, quadazocine (1 μM) which has a greater affinity for κ receptors than either naloxone or naltrexone (30,31) significantly reversed the inhibitory effect of 30 μM U-62,066E on the stimulus-evoked rise in cytosolic Ca^{2+} levels, which amounted to an inhibition of 71 ± 2% (n = 9), to an inhibition of 61 ± 3% in the presence of quadazocine (n = 9; $P < 0.01$, Students *t* test). None of the antagonists, when applied without U-62,066E present, affected the basal or KCl-evoked rises in cytosolic Ca^{2+} levels (data not shown).

Agonist Sensitivity to Tetrodotoxin. U-62,066E inhibition of depolarization-induced rises in cytosolic Ca^{2+} levels was further examined in the presence of TTX to assess if U-62,066E was affecting voltage-dependent sodium channels. TTX (0.1 μM) was applied 60 sec, and U-62,066E 30 sec, prior to depolarization of MF synaptosomes by 30 mM KCl. TTX had no effect on the 30 mM KCl-evoked rise in cytosolic Ca^{2+} levels or the inhibitory activity of U-62,066E (Figure 2). In the presence of TTX, 100 μM and 300 μM U-62,066E inhibited KCl-evoked rises in cytosolic Ca^{2+} levels by an average of 44% and 76% respectively. TTX viability was assured by its ability to block veratridine stimulated rises in MF cytosolic Ca^{2+} levels (Figure 2, inset).

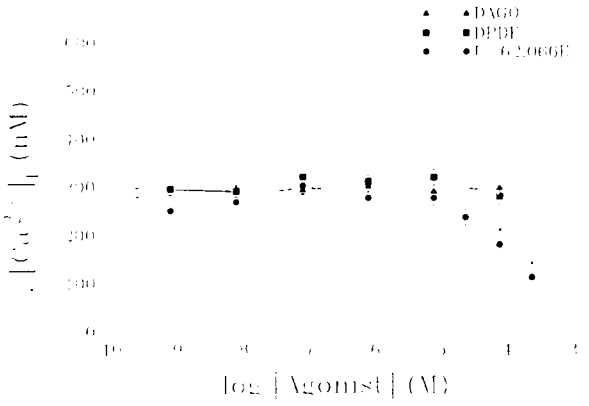

Fig. 1. Dose response of opioid agonists on the KCl-evoked rise in MF synaptosomal cytosolic Ca^{2+} levels. Data indicate the rise in cytosolic Ca^{2+} levels measured with fura-2 after depolarization of MF synaptosomes by 30 mM KCl. Agonists were present 30 seconds before depolarization. Open symbols are control levels (no agonist present). Basal Ca^{2+} levels for controls are: DAGO, 224 ± 7, n = 12; DPDE, 261 ± 3, n = 7; U-62,066E, 285 ± 17, n = 16. Data are expressed as the mean ± SEM (n = 5 – 15) from 9 experiments. * $P < 0.001$ from control, Student's *t* test.

Pharmacology of Opioid Effects on Transmitter Release

Agonists. U-62,066E also inhibited the release of Glu and Dyn B-LI from depolarized MF synaptosomes. U-62,066E was included in the superfusion medium 1 min prior to and during depolarization of MF synaptosomes by a 2 min pulse of 30 mM KCl-containing buffer. The application of U-62,066E (30 - 300 μM) significantly inhibited the KCl-evoked release of both Glu and Dyn B-LI in a dose-dependent manner (Figure 3). The kappa agonist (-)EKC also dose dependently inhibited the KCl-evoked release of Dyn B-LI, although (-) EKC displayed a weaker potency and efficacy than U-62,066E (Figure 3). (-)EKC could not be tested in the fluorometric assays for either Glu or Ca^{2+}, as we found this compound to have substantial emissions when excited at a 340 nm wavelength, which interfered with both assays.

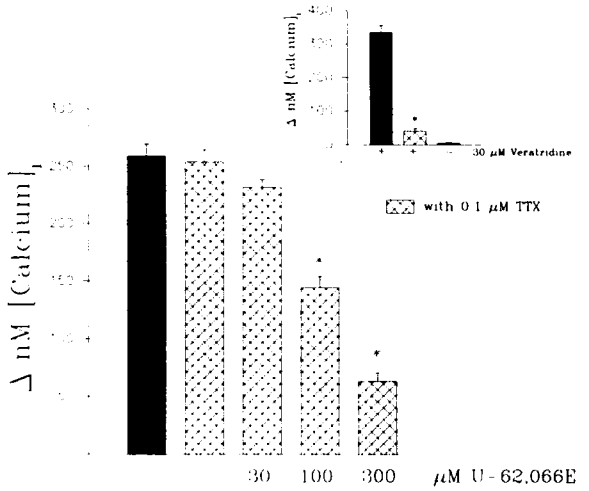

Fig. 2. Tetrodotoxin (TTX)-insensitive inhibition of KCl-evoked rise in MF synaptosomal cytosolic Ca^{2+} levels by U-62,066E. TTX was applied 60 sec prior to and U-62,066E 30 sec prior to depolarization of MF synaptosomes by 30 mM KCl. Basal Ca^{2+} level for control response was 243 ± 6 nM (mean ± S.E.M.). Inset: TTX inhibition of veratridine stimulated rise in synaptosomal cytosolic Ca^{2+} levels. TTX was applied 30 sec before veratridine. Basal Ca^{2+}level for veratridine control was 230 ± 5 nM (mean ± S.E.M.) Columns represent the average of 6 separate determinations from 2 experiments. * $P < 0.001$, Student's *t* test.

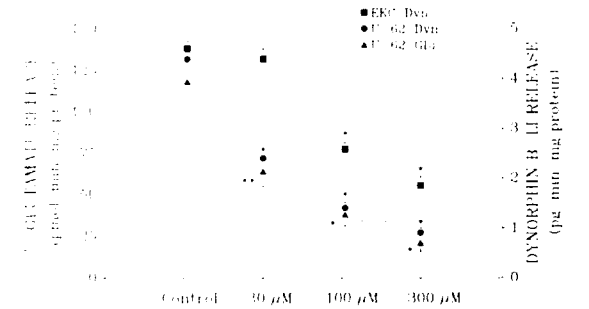

Fig. 3. U-62,066E and (-)EKC inhibition of Glu and Dyn B-LI release from hippocampal MF synaptosomes. Triangle: U-62,066E effects on Glu release. Circle: U-62,066E effects on Dyn B-LI release. Square: (-)EKC effects on Dyn B-LI release. Agonists were applied one minute prior to and during depolarization of MF synaptosomes by a two minute pulse of 30 mM KCl-containing buffer. Data are expressed as the mean ± SEM, (n = 5 − 9) from 8 experiments. * $P < 0.001$, ** $P < 0.005$ from control, Student's *t* test.

The opioid agonists DAGO and DPDE were also tested for effects on KCl-evoked Glu and Dyn B-LI release. In a separate series of experiments, high concentrations (100 μM) of DAGO, DPDE, and U-62,066E were applied to parallel superfusion columns 1 min prior to and during depolarization of MF synaptosomes by a 2 min pulse of high (30 mM) KCl-containing buffer. As previously shown in Figure 3, 100 μM U-62,066E inhibited the KCl-evoked release of both Glu and Dyn B-LI from MF synaptosomes by 67 ± 5% and 78 ± 3% (mean ± SEM), respectively (Figure 4). However, neither DAGO nor DPDE had any significant effect on Glu or Dyn B-LI release, even at these high concentrations.

Antagonists. The inhibitory effect of U-62,066E on Glu release could be completely reversed by the opioid antagonist quadazocine. Quadazocine (1 μM) was applied for 5 min prior to and during depolarization of MF synaptosomes (30 mM KCl for 2 min) and 30 μM U-62,066E was added with or without quadazocine for 1 min prior to and during depolarization. As shown in Table I, U-62,066E (30 μM) inhibited the KCl-evoked release of both Glu and Dyn B-LI in these experiments by an average of 44% and 35%, respectively. Quadazocine (1 μM) blocked the inhibitory effect of U-62,066E on KCl-evoked Glu release, but had no effect on the concomitant release of Dyn B-LI (Table I). When applied in the absence of U-62,066E in this same series of experiments, quadazocine (1 μM) had no significant effect on the KCl-evoked release of either Glu or Dyn B-LI; with an average release in the presence of quadazocine being 99 ± 9% and 94 ± 11% (mean ± SEM, n = 3) of control values, respectively.

DISCUSSION

The principal finding of this study is that κ, but not μ or δ, opioid agonists inhibit the KCl-evoked release of Glu and Dyn B-LI from guinea pig hippocampal MF

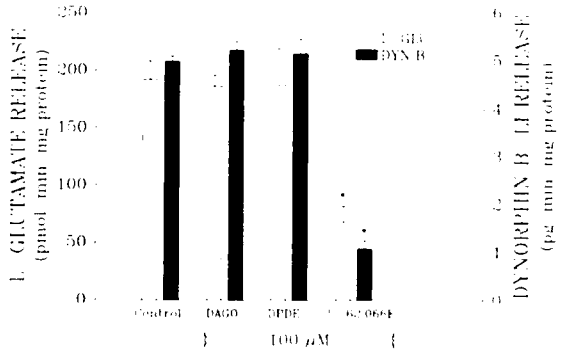

Fig. 4. Effects of opioid agonists on the KCl-evoked release of Glu and Dyn B-LI from MF synaptosomes. Agonists were applied one minute prior to and during depolarization of MF synaptosomes by a two minute pulse of 30 mM KCl-containing buffer. Data are expressed as the mean ± SEM, (n = 3 − 8) from three experiments. * $P < 0.001$, from control, Student's *t* test.

Table I. Quadazocine Reversal of the U-62,006E Inhibition of L-Glu but Not Dyn B-LI Release from MF Synaptosomes

	L-Glu (pmol/min/mg protein)	Dyn B-LI (pg/min/mg protein)
Control	97 ± 13 (n = 7)	4.9 ± 0.3 (n = 9)
U-62,066E (30 μM)	54 ± 8* (n = 6)	3.2 ± 0.3*** (n = 6)
U-62,066E (30 μM) & Quadazocine (1 μM)	85 ± 6**§ (n = 8)	3.2 ± 0.2*** (n = 9)

U-62,066E, was superfused 1 minute, and quadazocine 5 minutes, prior to and during depolarization of MF synaptosomes by a two minute pulse of 30 mM KCl-containing buffer. Samples were collected and assayed for L-Glu and Dyn B-LI as described in Experimental Procedure. Data are expressed as the mean ± SEM for the number of determinations (n) that is indicated in parentheses and obtained using 3 different synaptosomal preparations. *P < 0.05 from control, **P < 0.01 from U-62,066E alone, ***P < 0.005 from control, § no significant difference from control, Student's *t*-test.

synaptosomes. The presynaptic inhibition produced by the κ opioid agonists U-62,066E and U-50,488H (18) is associated with a significant reduction in the extent to which KCl-evoked depolarization elevates the level of cytosolic Ca^{2+} in MF synaptosomes. The inhibition of the KCl-evoked Glu release and rise in cytosolic Ca^{2+} by U-62,066E are both attenuated by quadazocine, suggesting a receptor-mediated effect of κ opioids.

The κ opioid U-62,066E and (-)EKC each produced a dose-dependent inhibition of the KCl-evoked release of Glu and Dyn B-LI from MF synaptosomes. In contrast, the μ and δ opioid agonists DAGO and DPDE, respectively, were without effect on the KCl-evoked rise in cytosolic Ca^{2+} levels or transmitter release. The δ specific antagonist ICI 174,864 and the μ-preferring antagonists naloxone and naltrexone did not affect U-62,066E inhibition of KCl-evoked rises in cytosolic Ca^{2+} levels. These results may be used to suggest that U-62,066E activity was not mediated to any significant degree by μ or δ opioid receptors. In a previous investigation (18) it was observed that U-50,488H also inhibited Glu and Dyn B-LI release from MF synaptosomes, as well as the KCl-evoked rise in cytosolic Ca^{2+}. The U-50,488H-induced depression of transmitter release and Ca^{2+} availability was partially reversed by the selective κ antagonist nor-binaltorphimine (30). In addition, the extent to which U-50,488H limited the availability of intrasynaptosomal Ca^{2+} was insensitive to naloxone and ICI 174,864 (18). The results of electrophysiological studies of the MF-CA3 synapse in guinea pig brain slice preparations would seem to be in agreement with the present results. In these studies, U-50,488H and U-69,593 were found to depress MF-CA3 synaptic responses but only at high micromolar concentrations (13,19). Supression of MF-CA3 synapse has also been observed with 100 μM concentrations of EKC and bremazocine, but not with morphine (100 μM) or [D-Ala^2,D-Leu^5]-enkephalin (1 μM)(13). Therefore, a consistent finding from these interdisciplinary studies has been that high concentrations of κ, but not μ or δ opioid agonists, inhibit MF synaptic activity.

The pharmacological properties of the κ agonist site that presumably mediates the presynaptic inhibition described in this report does not correlate well to any of the κ opioid receptor subtypes described in ^{3}H-ligand binding experiments. The guinea pig brain predominantly contains the high affinity κ_1 subtype of κ opioid receptor (32). Given the high concentrations of κ agonists required to significantly depress transmitter release, the presynaptic MF κ agonist site does not appear to be the high affinity κ_1 opioid binding site. Alternatively, a low affinity κ opioid site, reported to constitute approximately 15% of the total specific κ opioid binding in the guinea pig brain (32), may mediate the presynaptic inhibition observed in the present study. The inability of quadazocine to block the U-62,066E inhibition of Dyn B-LI release, as it did for Glu and cytosolic Ca^{2+} levels, may also indicate the involvement of κ opioid receptor subtypes. Subtypes of the κ opioid receptor that are insensitive to quadazocine have been suggested by the differential antagonism of U-50,488H and EKC activity on plasma corticosterone and thyroid stimulating hormone levels in rats (33). However, the parallel effects of U-62,066E, U-50,488H and (-)EKC, and the nor-binaltorphimine reversal of U-50,488H depressant activity on the KCl-evoked rise in cytosolic Ca^{2+} and the release of both Glu and Dyn B-LI (18), suggest that there is at least one common receptor involved in the inhibition of Glu and Dyn B-LI release from MF synaptosomes.

Non-opioid effects of κ agonists must also be seriously considered. It was recently suggested that U-50,488H and U-69,593 depression of CA3 neuronal activity could be attributed to a local "anaesthetic" action since sodium conductance was reduced by both compounds (19). However, the KCl-induced depolarization of the synaptosomal plasma membrane is independent of the sodium gradient across this membrane. For example, the addition of TTX to the incubation medium does not influence the extent to which intrasynaptosomal Ca^{2+} levels are raised following KCl-induced depolarization (Figure 2). Moreover, the present results show that U-62,066E depression of KCl-evoked rises in cytosolic Ca^{2+} levels is TTX-insensitive (Figure 2). Therefore, a reduction in sodium conductance is unlikely to account for our find-

ing that κ agonists depressed the KCl-evoked release of Glu and Dyn B-LI and that the depressant effects of opioid ligands at MF synapses are restricted to compounds identified as putative κ opioid agonists. The dissimilarity between the chemical structures of κ agonists such as U-50,488H EKC and bremazocine make it unlikely that they all would similarly affect MF synaptic transmission through a non-specific mechanism. The ability for nor-binaltorphimine and quadazocine to attenuate κ agonist depression of stimulated cytosolic Ca^{2+} levels and transmitter release from MF terminals indicates that a component of κ agonist activity is mediated through κ opioid receptors on MF terminals. However, there also appears to be a substantial non-κ opioid mediated component which may contribute to the depressant activity of the κ agonists.

U-62,066E inhibition of KCl-evoked cytosolic Ca^{2+} levels suggests that the κ agonist mediated inhibition of Glu and Dyn B-LI release may be mediated in part by limiting the availability of free cytosolic Ca^{2+} levels in MF terminals. It is not known from these studies if U-62,066E inhibits Ca^{2+} entry into, or Ca^{2+} mobilization within, MF terminals. However, a previous report has demonstrated that U-50,488H inhibits the entry of ^{45}Ca into rat cortical synaptosomes (34). Furthermore, the reduction in ^{45} Ca entry into rat cortical synaptosomes by U-50,488H (34) and the reduction of an inward calcium current by Dyn A (1–17) in mouse dorsal root ganglion neurons (35) have been attributed to a blockade of the N-type calcium channels in each preparation. We have previously reported that N-type Ca^{2+} channels are the most predominant presynaptic Ca^{2+} channel regulating the release of Dyn A(1–8)LI from MF synaptosomes (36) while more than one Ca^{2+} channel type appears to regulate Glu release (37). It is possible, therefore, that κ agonists inhibit Ca^{2+} entry into, and transmitter release from, MF synaptosomes through a blockade of at least N-type Ca^{2+} channels.

In summary, the depolarization-induced release of Glu and Dyn B-LI from guinea pig hippocampal MF synaptosomes is selectively depressed by κ, but not μ nor δ agonists. U-62,066E inhibition of KCl-evoked increases in cytosolic Ca^{2+} levels suggests a possible mechanism for κ agonist inhibition of transmitter release. The inhibitory efficacy of κ agonists on MF terminal exocytotic processes suggest that dynorphin peptides released from MF terminals may exert an inhibitory influence on MF synaptic activity through the activation of a presynaptic κ receptor. Collaterals of the hippocampal MF pathway undergo a reactive synaptogenesis in human temporal lobe epilepsy (38–40) that is associated with a redistribution of dynorphin immunoreactivity (38) and the formation of aberrant recurrent excitatory connections in the supragranular layer of the dentate gyrus (41,42). Such recurrent excitatory connections could possibly increase the frequency and magnitude of limbic seizure activity. However, experimentally induced limbic seizures have also been shown to enhance the transcription of genes encoding preprodynorphin and the content of these opioid peptides in the dentate granule cell MF pathway (43). This genomic response to intense synaptic activation may possibly provide a neuroprotective effect by limiting the release of endogenous glutamate from the MF presynaptic terminals. Pharmacological activation of MF κ autoreceptors would be expected to ameliorate any recurrent excitatory activity that may develop during MF synaptic reorganization in human temporal lobe epilepsy and, therefore, these receptors may represent an important target for the development of more efficacious anticonvulsant and antiepileptic therapies in the future.

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Kainic acid depresses the ex vivo release of dynorphin B and glutamate from rat hippocampal mossy fiber synaptosomes

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Key words: Kainic acid; Hippocampus; Dynorphin; Glutamate; Mossy fiber; Seizure; Neurotransmitter release

This experiment examined the effects of intracerebroventricularly (i.c.v.) administered kainic acid (KA) on the subsequent ex vivo release of L-glutamate (Glu) and dynorphin B-like immunoreactivity (Dyn B-LI) from isolated rat hippocampal mossy fiber (MF) synaptosomes at 4.5 h, 20 h or 48 h after administration of 0.5 $\mu g/\mu l$ KA. The Dyn B-LI content in the synaptosomal fraction initially decreased at 4.5 h and then rebounded and remained elevated above control levels at 20 h and 48 h. The K^+-evoked release of Dyn B-LI from the synaptosomes was markedly depressed at 4.5 h after KA and remained significantly below control levels at 20 h and 48 h. In contrast, KA caused no change in the K^+-evoked release of Glu at 4.5 h as compared to control levels, but did result in a significant decrease in Glu release at 20 h and 48 h. These data indicate a persistent effect of i.c.v. KA on neurotransmission at MF–CA3 synapses in rat hippocampus, resulting in a suppression of the release of Glu as well as the opioid peptide, Dyn B.

Kainic acid (KA), a structural analog of glutamate (Glu), has been used extensively to evoke experimental seizures and neuropathological sequelae that are similar to those observed in human temporal lobe epilepsy [2, 14]. KA-induced activation of dentate granule cells of the hippocampal formation produces transient increases in the expression of the messenger RNAs for several immediate-early genes [5, 6, 21], trophic factors [6] and the preprodynorphin [11] (Lason et al., submitted) and preproenkephalin [6, 10, 11] neuropeptides. Simultaneously, opioid peptide levels in the granule cell/mossy fiber (MF) axons decrease below control levels within 6 h following intracerebral KA administration and rebound above control levels within 48 h [9]. These findings suggest that during the ictal period that is rapidly induced by in vivo KA, the rate of neurosecretion from MF terminals may be enhanced to the extent that the vesicular pool of opioid peptides is depleted. The seizure-induced increase in opioid peptide mRNA may account for the finding that the steady-state levels of prodynorphin- and proenkephalin-derived peptides are subsequently elevated in dentate granule cells and their MF axons following seizure activity [6, 9]. However, it remains to be established if or how opioid peptide release is altered in the postictal period when the content of granule cell opioid peptides is markedly above normal.

An additional, and yet distinct, issue of considerable importance involves the question of whether limbic seizures have any persistent affect on glutamatergic neurotransmission in the hippocampal MF pathway. Numerous studies indicate that Glu mediates the excitatory MF synaptic input [4, 19, 20] and Glu release has been reported to be enhanced from hippocampal slices of regio inferior, which contains much of the MF terminal field, one month folowing entorhinal kindling [7]. However, it should not be assumed that enhanced neuronal activity influences peptidergic and glutamatergic transsynaptic communication in a similar fashion. Recent evidence suggests that the release of regulatory neuropeptides and fast-acting neurotransmitters can be independently stimulated [15] and replenished [3]. Indeed, given the substantial differences in their regulation [3], it seems reasonable to anticipate that the release of dynorphin-like immunoreactivity (Dyn-LI) and Glu may differ in their response to sustained epileptogenic activity.

A variety of prodynorphin-derived peptides have recently been shown to be released concomitantly with endogenous Glu from isolated rat hippocampal MF nerve endings (synaptosomes) [19]. Thus, it is now pos-

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sible to determine whether an alteration in the steady-state content of the prodynorphin-derived opioids and/or Glu is accompanied by parallel changes in their release from rat hippocampal MF terminals at different time points following in vivo administration of KA. In the present study, the release of Dyn B-LI and Glu from MF terminals was compared with the chronology of changes previously reported for preprodynorphin mRNA levels in the soma of dentate granule cells and Dyn-LI [9, 11] in the MF pathway after the i.c.v. administration of KA.

Male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing 200–290 g on a 12 h light/dark cycle with free access to food and water were used for all experiments. Two experiments were conducted for each time point investigated. Each experimental group consisted of 12 rats which received KA dissolved in phosphate-buffered saline (PBS) and 11 rats which received PBS alone. All rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and sterilized stainless steel guide cannulae (24 gauge) were implanted unilaterally 1.5 mm below the surface of the skull at the following coordinates (according to Paxinos and Watson [16]): AP −0.3 mm, ML −1.5 mm, DV −2.0. Four to six days after surgery, unanesthetized rats were given an i.c.v. injection with a stainless steel injection cannula (30 gauge, 3 mm longer than the guide cannulae) attached via polyethylene tubing to a Hamilton syringe. Rats were injected at a rate of 1 μl/min with either 0.5 μg KA (Sigma, St. Louis, MO) dissolved in 1 μl PBS or with 1 μl PBS alone. Both solutions were adjusted to pH 7.3 ± 0.2 prior to injection. Pontamine sky blue (0.1%) was included in both solutions in order to view the injection site prior to the removal of the hippocampi. Following KA injections, rats were observed for 3 h for typical KA-induced behavior [12]. Only KA-injected rats that displayed KA-induced behavioral effects and rats with accurate injection sites were included in the experiments. No more than one rat per experimental group failed to meet these criteria and these rats were not included in subsequent experiments.

At the proper time points, KA- and PBS-injected groups were decapitated and their hippocampi removed. The groups consisted of 10–11 KA-injected rats and 11–12 PBS-injected rats per experiment per time point. Large hippocampal MF synaptosomes were prepared separately for each group and protein concentrations were determined as described previously [18]. Mossy fiber synaptosomal suspensions (6 mg protein) were preincubated and superfused (0.5 ml/min) at room temperature with a buffer consisting of (in mM): $CaCl_2$ 0.9, NaCl 122.0, KCl 3.1, $MgSO_4$ 1.2, K_2HPO_4 0.4, $NaHCO_3$ 5.0, glucose 10.0 and Na-Tes 20.0, pH 7.4 and the superfusate was collected at 5 min intervals in polypropylene tubes [20].

In each experiment, MF synaptosomes prepared from KA-injected rats were layered into 5 superfusion columns. To measure the constitutive (basal) release of Dyn B-LI and Glu, one of these 5 columns was superfused with a standard buffer throughout the superfusion procedure. The remaining 4 columns were exposed to a 35 mM KCl-containing buffer (equimolar substitution of KCl for NaCl) for 2 min after the basal efflux of Dyn B-LI and Glu had become stable (t=17.5 min). The K^+-evoked release of Dyn B-LI and Glu was calculated as the difference between the basal release and the total release induced by exposure to 35 mM KCl. The same protocol was followed using synaptosomes isolated from PBS-injected rats to measure the evoked release of Dyn B-LI and Glu in 5 additional parallel superfusion columns. At each time point investigated, the experiment was repeated using separate MF synaptosomal preparations obtained from either KA-injected or PBS-injected rats. The data for each time point consisted of 3–4 determinations for each MF synaptosomal preparation, obtained using parallel superfusion columns, in two separate experiments. Therefore, the data for each time point are expressed as the mean ± S.E.M. of 7–8 total determinations unless otherwise specified. Student's t-test was used to determine significance.

The concentrations of Dyn B-LI and Glu in the superfusate fractions, the whole hippocampal homogenate, and MF (P_3) synaptosomal fractions were obtained using a radioimmunoassay procedure for Dyn B and a glutamic acid dehydrogenase-based fluorometric assay for Glu as described previously [20]. The selectivity of the Dyn B antiserum (Peninsula Labs, Belmont, CA) has previously been characterized [20]. This antibody does not recognize higher molecular weight precursor forms of Dyn.

An initial experiment was performed to determine the relative enrichment of Dyn B-LI in the MF synaptosomal preparation as compared to whole hippocampal homogenate and the percent of total synaptosomal Dyn B-LI released during superfusion experiments in control rats. There was a 64% relative enrichment of Dyn B-LI in the MF synaptosomal fraction as compared to whole hippocampal homogenates, calculated as the content of Dyn B-LI in the MF synaptosomal fraction (738 ± 21 pg Dyn B-LI/mg protein, n=12) divided by the content of Dyn B-LI in the homogenate (450 ± 9 pg Dyn B-LI/mg protein, n=12). On the average, the 6 mg of MF synaptosomal protein that was layered onto each column contained 5.66 ng of Dyn B-LI and the total amount of Dyn B-LI that was released during the entire superfusion protocol amounted to 159 pg. Therefore, it is estimated that

less than 3% of the MF synaptosomal content of Dyn B-LI was released in response to the stimulus conditions used in the present experiments. The percent recovery of Dyn B-LI during superfusion experiments (99.6%) was ascertained by dividing the total Dyn B-LI recovered during superfusion after adding a known amount of Dyn B standard (500 pg) to each column in the absence of tissue and assaying the total amount recovered during the course of the superfusion experiment (498 ± 45 pg Dyn B-LI recovered in the superfusate, n=12).

The i.c.v. injection of KA (0.5 $\mu g/\mu l$) resulted in a biphasic, time-dependent change in the content of Dyn B-LI in rat hippocampal MF synaptosomes. At 4.5 h after i.c.v. injection of KA, the content of Dyn B-LI in MF synaptosomes significantly decreased as compared to PBS-injected controls (705 ± 74 pg Dyn B-LI/mg protein compared to 1167 ± 139 pg Dyn B-LI/mg protein, P<0.05). Fig. 1 shows the data expressed as a percent of control. In contrast, at 20 h and 48 h post KA injection, the content of Dyn B-LI in MF synaptosomes was significantly elevated over PBS-injected controls (1592 ± 78 pg Dyn B-LI/mg protein compared to 1094 ± 65 pg Dyn B-LI/mg protein, P<0.001; and 1279 ± 70 pg Dyn B-LI/mg protein compared to 875 ± 66 pg Dyn B-LI/mg protein, P<0.001, respectively, Fig. 1). However, after i.c.v. injection of KA, the content of endogenous Glu in the MF synaptosomal preparation was slightly, but not significantly, depressed at all time points by an average of 20 ± 3% as compared to PBS-injected controls (43.2 ± 5.0 nmol Glu/mg protein compared to 54.1 ± 6.0 nmol Glu/mg protein, n=5, Fig. 1).

As Fig. 1 shows, the K^+-evoked release of Dyn B-LI from MF synaptosomes prepared 4.5 h after i.c.v. KA injection was significantly reduced as compared to the time matched PBS-injected controls (0.92 ± 0.18 pg Dyn B-LI/min/mg protein compared to 2.84 ± 0.12 pg Dyn B-LI/min/mg protein, P<0.001). At 20 h after i.c.v. KA, the K^+-evoked Dyn B-LI release rebounded somewhat but remained significantly below the PBS-injected control Dyn B-LI release values (2.51 ± 0.19 pg Dyn B-LI/min/mg protein compared to 3.81 ± 0.26 pg Dyn B/min/mg protein, P<0.05, Fig. 1). The K^+-evoked release of Dyn B-LI 48 h post i.c.v. KA remained significantly below control levels (2.06 ± 0.27 pg Dyn B-LI/min/mg protein compared to 3.66 ± 0.56 pg Dyn B-LI/min/mg protein, P<0.05, Fig. 1).

Fig. 1 shows that the K^+-evoked release of MF synaptosomal Glu was not significantly affected 4.5 h after i.c.v. injection of KA compared to the corresponding PBS-injected control levels (167 ± 11 pmol L-Glu/min/mg protein compared to 195 ± 9 pmol L-Glu/min/mg protein). However, at 20 h after i.c.v. KA injection, K^+-evoked MF synaptosomal Glu release was significantly

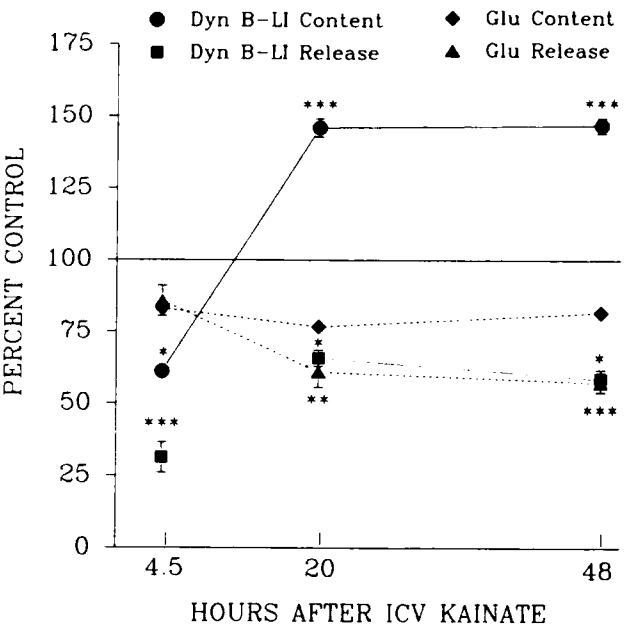

Fig. 1. Time course of changes in hippocampal mossy fiber synaptosomal content and K^+-evoked ex vivo release of Dyn B-LI and Glu after i.c.v. injection of kainic acid (0.5 μg/rat). Data are expressed as a percentage of PBS-injected controls. ($^*P$<0.05, $^{**}P$<0.005, $^{***}P$<0.001 compared to controls, non-normalized, Student's t-test.)

depressed compared to the respective PBS-injected control levels (149 ± 15 pmol L-Glu/min/mg protein compared to 246 ± 17 pmol L-Glu/min/mg protein, P<0.005, Fig. 1). In addition, similar to K^+-evoked Dyn B-LI release, the stimulated release of MF synaptosomal Glu remained at a constantly depressed level out to 48 h post i.c.v. KA as compared to corresponding control levels (197 ± 23 pmol L-Glu/min/mg protein compared to 341 ± 33 pmol L-Glu/min/mg protein, P<0.001).

This study demonstrates that although the genomic response of dentate granule cells to KA-induced seizures may be adequate to replenish the level of Dyn B-LI in hippocampal MF nerve endings, the K^+-evoked release of this opioid peptide remains significantly depressed for at least 48 h after KA injection. Intracerebroventricular administration of KA produced characteristic recurrent motor seizures in the rat that lasted 3–6 h and, at 4.5 h following KA, both the steady-state content and K^+-evoked release of Dyn B-LI were significantly decreased in the isolated hippocampal MF synaptosomes. Subsequently, however, the release of Dyn B-LI remained depressed despite the fact that the level of Dyn B-LI in the MF terminals had rebounded above control levels. At these later time points, glutamate release was found to be similarly depressed. These data suggest that KA-induced seizures may produce a prolonged disruption of the presynaptic mechanisms that mediate Dyn B-LI and Glu exocytosis from hippocampal MF synaptosomes

that cannot be attributed to a diminished steady-state content of either compound within the MF presynaptic terminals.

Intracerebral injections of KA have been reported to produce an initial decrease in hippocampal Dyn-LI, lasting from 3 to 12 h, followed by a subsequent elevation above saline-injected controls [9]. The alterations in the MF synaptosomal content of Dyn B-LI mimicked this temporal sequence of events. Therefore, the present results are consistent with the suggestion that a seizure-induced increase in the level of preprodynorphin mRNA in dentate granule cells [11] becomes translated into prodynorphin-derived peptides and that at least one of these opioid peptides is transported in increased amounts to the MF presynaptic terminals [9]. However, the replenishment of a transiently depleted pool of Dyn B-LI in the MF terminals did not reverse the depression of Dyn B-LI release that was evident within 4.5 h after KA administration. This finding is in agreement with the report that 3 h following the systemic injection of KA, the K^+-evoked release of another prodynorphin-derived peptide, α-neoendorphin, from rat hippocampal slices was also significantly reduced [10]. The concomitant release of endogenous Glu from hippocampal MF synaptosomes was also found to be significantly decreased at 20 h and 48 h following the intracerebral injection of KA. We conclude from these results that KA-induced seizures gradually lead to a persistent reduction in the ex vivo release of both Dyn B-LI and Glu from hippocampal MF nerve endings. However, previous investigations indicate that the depression of transmitter release by limbic seizures may not be permanent. Arias et al. [1] detected a significant reduction in the hippocampal release of [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) but not [^{14}C]Glu 3 days following the systemic injection of KA; Jefferys et al. [8] reported that tetanus toxin-induced seizures do not alter hippocampal Glu release 10–14 days after treatment; and Jarvie et al. [7] have reported that Glu release from slices is enhanced one month following entorhinal kindling of the rat hippocampus.

It has been estimated that less than 5% of the total Glu endogenous to isolated hippocampal MF synaptosomes is released under the stimulus conditions used in the present experiment [18]. Consequently, even substantial alterations in the 'metabolic' pool of Glu may not be accompanied by corresponding changes in the vesicular pool of Glu or in the amount of Glu released in response to depolarization. In addition, the present data indicate that less than 3% of the Dyn B-LI present in hippocampal MF synaptosomes is released in response to identical stimulation parameters. Therefore, it is suggested that the attenuation of MF neurotransmitter release is not related to a fall in the overall availability of either Dyn B-LI or Glu within the MF terminals. However, these results do not eliminate the possibility that the two separate pools of 'releasable' Dyn B-LI and Glu are selectively depleted and remain unreplenished during the 48 h following KA-induced seizures.

Exocytosis involves a highly regulated sequence of biochemical events that is initiated by calcium entry into the presynaptic terminal and is apparently sustained by a mechanism that is tightly coupled to the availability of high-energy phosphates. It has been reported that intracranial injections of KA result in a substantial decline in the level of intrastriatal ATP whereas ADP and AMP levels are both significantly increased [17]. This fall in high energy phosphate charge also appears to be accompanied by an increased level of lactate [17] and a decreased hexokinase activity [13]. Given these metabolic effects of intracranial KA injections, a more reasonable explanation for the present results may be that the prolonged seizures induced by KA gradually depleted available energy reserves within the MF nerve endings and disrupted energy metabolism, whereas a sufficient energy charge for enhanced opioid synthesis remained available in the soma of the dentate granule cells. Consequently, a gradual depression of Dyn B-LI and Glu release might be expected despite the increased steady-state content of Dyn B-LI.

In conclusion, an i.c.v. injection of KA caused a prolonged decrease in ex vivo release of Dyn B-LI and Glu from rat hippocampal MF synaptosomes. This prolonged decrease in neurotransmitter release may be due to a reduction in hippocampal high-energy phosphate reserves and/or glucose utilization. This is the first report of a decrease in release of Dyn B-LI from hippocampal MF synaptosomes following administration of KA. The relationship between altered synaptosomal energy metabolism and the concomitant release of opioid peptides and endogenous Glu following intracerebral injections of KA clearly warrants further investigation.

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