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Synaptic Activation of Presynaptic Kainate Receptors on Hippocampal Mossy Fiber Synapses

Dietmar Schmitz, Matthew Frerking, and Roger A. Nicoll*
Department of Cellular and Molecular Pharmacology and
Department of Physiology
University of California, San Francisco
San Francisco, California 94143

Summary

Kainate receptors (KARs) are a poorly understood family of ionotropic glutamate receptors. A role for these receptors in the presynaptic control of transmitter release has been proposed but remains controversial. Here, KAR agonists are shown to enhance fiber excitability, and a number of experiments show that this is a direct effect of KARs on the presynaptic fibers. In addition, KAR activation inhibits evoked transmitter release from mossy fiber synapses. Synaptic release of glutamate from either neighboring mossy fiber synapses or associational/commisural (A/C) synapses results in the activation of these presynaptic ionotropic KARs. These results, along with previous studies, indicate that KARs, through the endogenous release of glutamate, mediate excitatory postsynaptic potentials (EPSPs), alter presynaptic excitability, and modulate transmitter release.

Introduction

Neurotransmitters can act at two sites on neurons in the central nervous system: postsynaptically, changing the excitability of the neuron, or presynaptically, altering the amount of neurotransmitter released. In general, neurotransmitters that act presynaptically do so via metabotropic receptors. However, considerable recent evidence raises the possibility that ionotropic neurotransmitter receptors can be present on presynaptic terminals and that their activation can modulate the release of neurotransmitter (MacDermott et al., 1999). In particular, a number of recent publications have suggested that kainate receptors (KARs) may act on the presynaptic terminal (Lerma, 1997; reviewed in Frerking and Nicoll, 2000). However, these studies have not definitively identified the action that these presumed presynaptic receptors have on the synaptic terminals, nor have they definitively shown that the presynaptic actions of KAR activation are directly mediated by presynaptic receptors.

The hippocampal mossy fiber synapses provide an ideal system to study the possible presynaptic actions of KARs. High levels of the GluR6, GluR7, KA1, and KA2 KAR subunits are expressed in dentate granule cells (Wisden and Seeburg, 1993; Bahn et al., 1994; Bureau et al., 1999; Paternain et al., 2000), the cells that give rise to the mossy fibers. In addition, the high-affinity kainate binding sites that are present in s. lucidum (Monaghan and Cotman, 1982), the termination zone for mossy fibers, are lost following destruction of granule cells (Represa et al., 1987). Recently, it has been reported that a KAR agonist can depress mossy fiber excitatory postsynaptic currents (EPSCs), although the mechanism was not explored (Vignes et al., 1998). We have used hippocampal slices to determine the possible functional consequence of activating these presumed presynaptic KARs and whether synaptically released glutamate can access these receptors. After the completion of the present study, a paper appeared by Kamiya and Ozawa (2000) that also presents evidence for presynaptic KARs on mossy fibers.

*To whom correspondence should be addressed (e-mail: nicoll@phy.ucsf.edu).

Results

Kainate Receptors Directly Depolarize Mossy Fibers

We first looked for effects of kainate on the presynaptic compound action potential, or fiber volley, generated by mossy fiber stimulation. These experiments were all carried out in the presence of the the AMPA receptor–selective antagonist GYKI 53655 (100 μM) (Paternain et al., 1995; Wilding and Huettner, 1995), D-APV (100 μM), and picrotoxin (100 μM) to ensure that any effects would be due to KAR activation. Kainate had highly reproducible effects on the orthodromic fiber volley evoked by mossy fiber stimulation in the hilus/granule cell layer and recorded in s. lucidum. Low concentrations (0.1–1 μM) caused an increase in the amplitude, a faster rise time, and a variable decrease in the latency. Figure 1A plots the increase in amplitude for six experiments (0.5 μM) and shows that, as expected for the involvement of KARs, the response was reversibly blocked by the application of the non-NMDA receptor (NMDAR) antagonist 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX) (30 μM). Such an increase in the fiber volley suggests that the excitability of these fibers is increased by kainate.

To test more directly the effects of kainate on the excitability of mossy fibers, antidromic action potentials were recorded with whole-cell recording from granule cells (Figure 1B). The stimulus to the axons was set to straddle threshold. As shown in the offset traces in Figure 1B, a stimulus that only intermittently activated the axon in the absence of kainate does so in every trial in its presence. The summary of the results for five cells (Figure 1B) shows that on average, an action potential was evoked 26% ± 4% of the time in control conditions and increased to 98% ± 2% in the presence of kainate (0.5 μM), directly demonstrating an increase in axonal excitability (Wall, 1958). During the application of kainate, the granule cells depolarized ~3 mV. However, this somatic effect was not responsible for the appearance of antidromic action potentials, because hyperpolarizing the cells back to their resting potential did not block the action potential invasion. In addition, depolarization of the granule cells prior to the application of kainate had no effect on the success rate of antidromic invasion, indicating that the increase in success rate was due to the action of kainate at the site of stimulation and not to the ability of the spike to invade the soma.

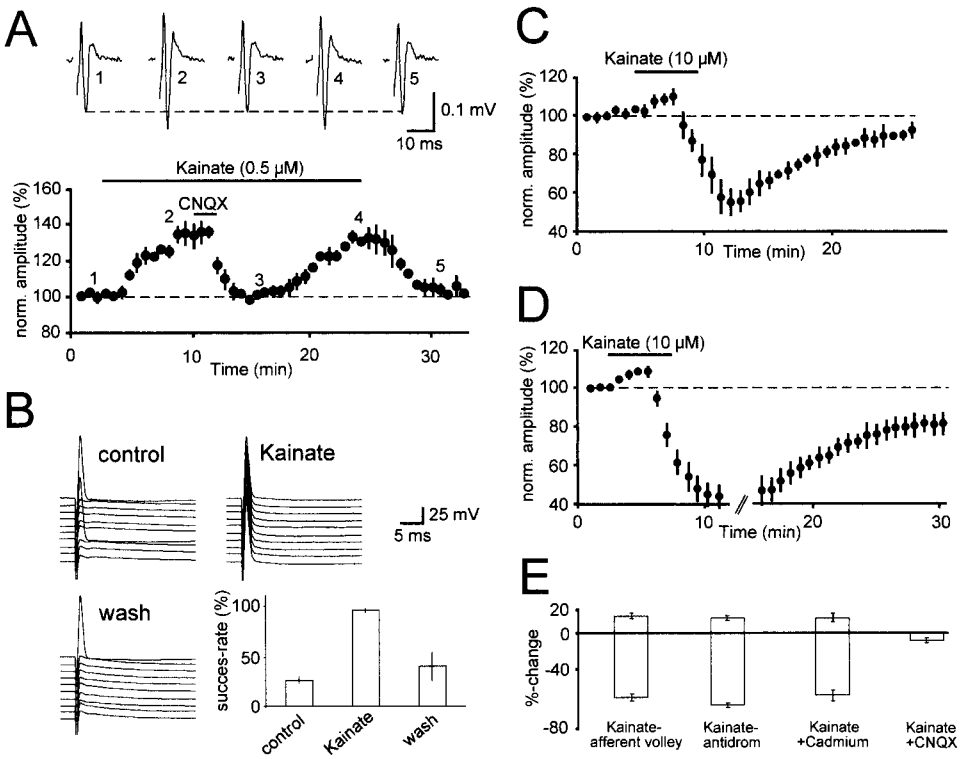

Figure 1. Kainate Affects Orthodromic and Antidromic Action Potentials

(A) Averaged traces of afferent volleys recorded in the presence of GYKI 53655, picrotoxin, and APV (100 μM each) are shown at top. Summary graph (below) of six experiments shows a kainate-induced increase in the amplitude that was reversibly suppressed by the non-NMDAR antagonist CNQX (30 μM).

(B) Superimposed traces of antidromic action potentials recorded with a whole-cell patch electrode from a dentate granule cell. The stimulus was applied to the mossy fiber in s. lucidum and was adjusted to straddle threshold. In two of the ten trials, an action potential was elicited. In the presence of kainate (0.5 μM), all ten stimuli elicit action potentials, and this goes down to one of ten after the washout of kainate. A summary graph of five experiments is plotted. On average, across cells the stimulus gave an action potential on about 26% ± 4% of the trials in the control.

(C) Higher concentrations of kainate (10 μM) cause a transient increase followed by a decrease in the amplitude of the fiber volley (n = 7).

(D) Averaged traces and summary graph of kainate (10 μM) on antidromically elicited compound action potentials (n = 15). Note the similar sequence, as seen on the fiber volley. The records were interrupted for input/output curves, which are not shown.

(E) Summary bar graph of the effects of kainate (10 μM) on the afferent volley and the antidromic population spike, as well as the action of cadmium and CNQX on these effects.

High concentrations of kainate (10 μM) caused a transient increase followed by a large decrease in the amplitude of the fiber volley (n = 7) (Figure 1C). The decrease in amplitude was associated with a marked slowing of the fiber volley (data not shown). A similar sequence of events was observed when the antidromic compound action potential was recorded in the granule cell layer (n = 15) (Figure 1D). The results with kainate (10 μM) are summarized in Figure 1E, in which the peak enhancement and depression are plotted, as well as the blockade of these effects by CNQX. One possible explanation for the effects of kainate is that KAR activation of other cells in the region leads to the release of an indirect modulator, which then acts on the mossy fibers. To test this possibility, we examined the effects of kainate after blockade of transmitter release by the Ca^{2+} channel antagonist cadmium and found that it had no effect on the action of kainate (Figure 1E) (n = 6).

By analogy to classical studies on axons (Merrill et al., 1978; Raymond, 1979; Kocsis et al., 1983), the effects of kainate could be due to a presynaptic depolarization, in which low concentrations of kainate would cause a modest depolarization of the axons, which increases excitability, resulting in an enhanced amplitude and conduction velocity of the compound action potential. High concentrations would then cause a larger depolarization, resulting in Na^+ channel inactivation and a decrease in the amplitude of the compound action potential. If this model is correct, the effects of kainate on the fiber volley should be mimicked by raising extracellular K^+ to directly depolarize the presynaptic fibers. As predicted by the model, when K^+ was increased in a stepwise fashion (from 2.5 mM to 7.5, 9.5, 11.5, and 13.5 mM), there was an increase in the fiber volley, followed by a decrease (Figure 2A). These findings and those presented in Figure 1 argue strongly that the effects of kainate on mossy fibers are mediated by a depolarizing action.

Although these results are consistent with a direct ionotropic action of presynaptic KARs, it is still possible that activation of other cells releases K^+, which, in turn, depolarizes mossy fibers, as suggested previously in other systems (Weight and Erulkar, 1976; Malenka et al., 1981). We therefore used ion selective K^+ electrodes

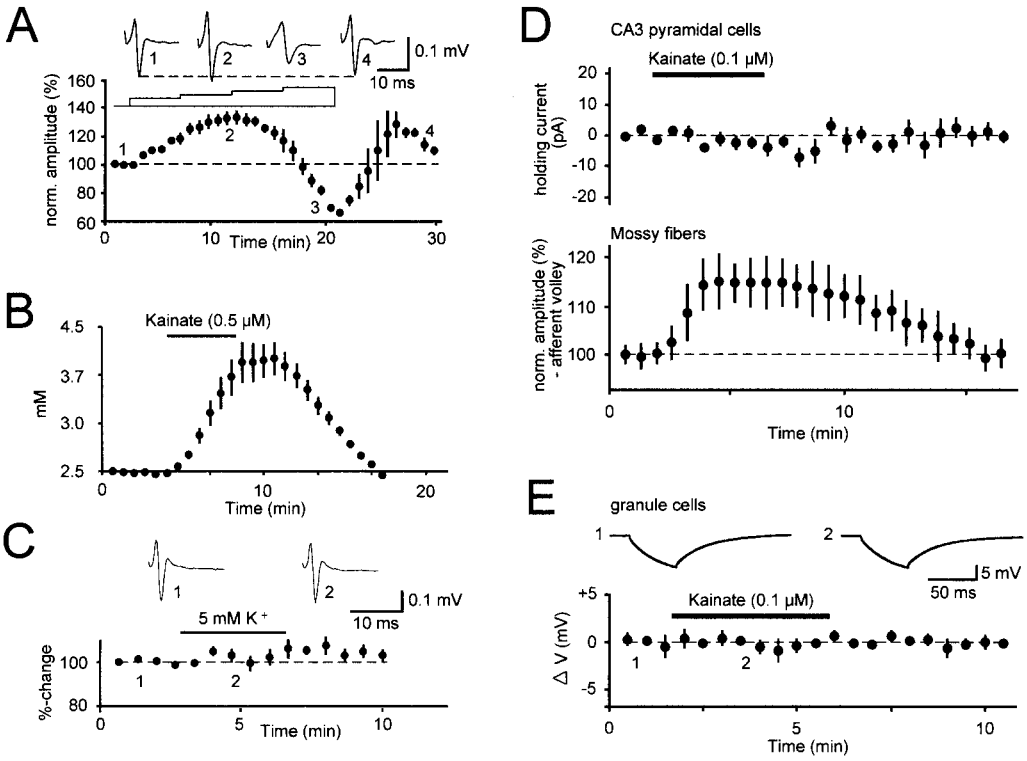

Figure 2. Extracellular K^+ Mimics, but Does Not Mediate, the Kainate Receptor Effects on Mossy Fibers

(A) Summary graph of the effects of increasing concentrations of K^+ on the fiber volley. The total K^+ in the bath was increased from 2.5 mM to 7.5, 9.5, 11.5, and 13.5 mM and then returned to 2.5 mM.

(B) Extracellular K^+ measurement following the application of 0.5 μM kainate. Note that kaniate increases the extracellular K^+ by only 1.3 mM.

(C) The addition of 2.5 mM K^+ (total K^+ = 5.0 mM) has no effect on the afferent volley.

(D) A low concentration of kainate (0.1 μM) has no effect on the holding current of CA3 pyramidals cells (upper graph) but still increases the amplitude of the fiber volley (lower graph) (n = 5).

(E) This same low concentration of kainate had no effect on the membrane potential or input resistance of dentate granule cells (n = 4).

to monitor the extracellular K^+ in s. lucidum during the application of kainate (Figure 2B). The application of kainate (0.5 μM) caused, on average, a 1.3 ± 0.3 mM (n = 5) increase in extracellular K^+. To test the sensitivity of mossy fibers to K^+, we added 2.5 mM K^+ to the solution (total K^+ = 5 mM) (Figure 2C) and found that it had no effect on the fiber volley. In addition, lower concentrations of kainate (0.1 μM) had no effect on the holding current of CA3 pyramidal cells (Figure 2D) or on the membrane potential of dentate granule cells (Figure 2E) but still evoked an increase in the fiber volley (Figure 2D). These findings, as well as results to be presented below, indicate that increases in extracellular K^+ cannot explain the kainate-induced changes in the fiber volley.

Synaptic Release of Glutamate Activates Presynaptic Kainate Receptors

The results thus far were obtained with exogenous application of kainate, and it is unclear whether the release of glutamate from mossy fiber synapses can also gain access to these presynaptic receptors. To test this hypothesis, we set up an experiment in which we activated two independent populations of mossy fibers and recorded the fiber volleys in s. lucidum (Figure $3A_1$). One pathway was stimulated repetitively to initiate glutamate release, and the other pathway was used to test the effects of the synaptically released glutamate. The test electrode was positioned first to evoke a fiber volley recorded in s. lucidum. Then, the conditioning electrode was inserted and a position found by trial and error, which evoked an enhancement of the test fiber volley.

Traces of a representative experiment are shown in Figure $3A_2$. Repetitive mossy fiber stimulation elicited a slow negative field potential in s. lucidum, which is due to the KAR-mediated slow excitatory postsynaptic potential (EPSP) generated in CA3 pyramidal cells (Castillo et al., 1997). When a test stimulus was applied to the second electrode 80–120 ms after the onset of the conditioning tetanus to the first electrode, an increase in the amplitude and a shift in the latency of the fiber volley are clearly apparent in the enlarged superimposed traces in Figure $3A_2$ (traces 1 and 2). Importantly, application of CNQX reverses all of the effects of the preceding tetanus (traces 2 and 3). The results of this experiment are plotted in Figure $3A_3$, which also shows that kynurenate (5 mM) (n = 3), another antagonist of KARs, and cadmium (100 μM) (n = 3), which blocks transmitter release, block the changes in the presynaptic fiber volley. The size of the first response in the tetanus is also plotted below and is not changed by the antagonists, indicating an activity dependence to the drug effects. A summary (n = 6) of the tetanus-induced change in the fiber volley and its reversal by CNQX is shown in Figure 3B. Using the identical setup, we also monitored extracellular K^+ in

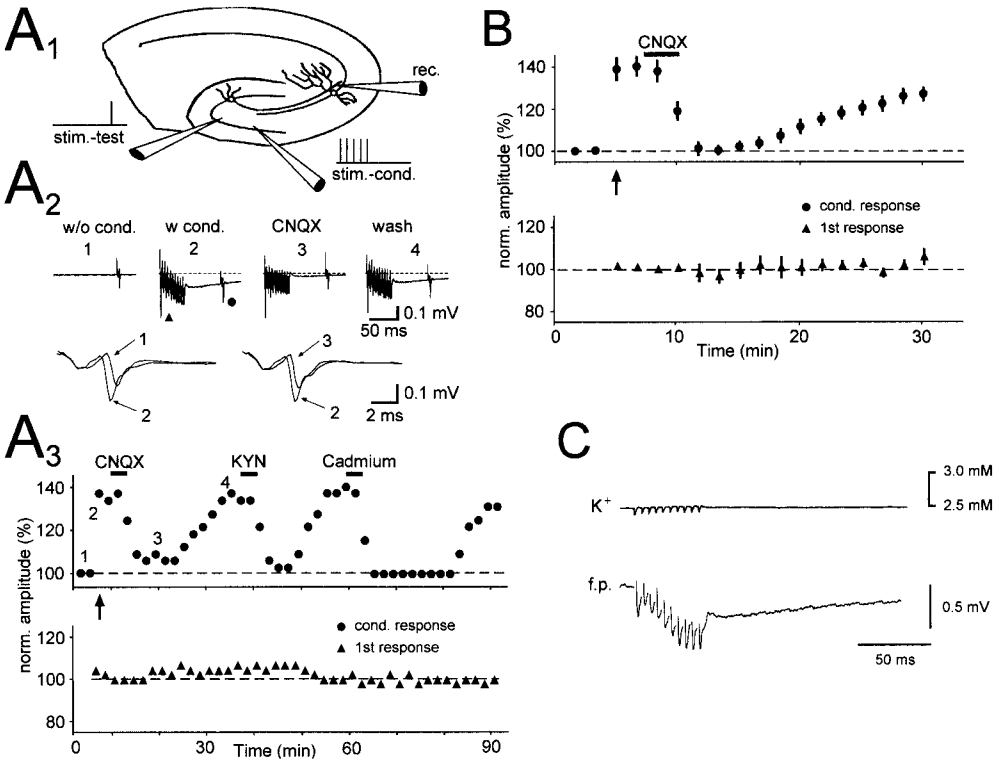

Figure 3. Synaptic Release of Glutamate by Brief Stimulus Trains to Mossy Fibers Causes the Heterosynaptic Activation of Presynaptic Kainate Receptors

(A_1) Schematic drawing of the experimental setup. Two independent sets of mossy fibers were stimulated. The independence was verified by the lack of a refractory period when the two pathways were stimulated at a close interval. One set (stim.-cond.) was stimulated repetitively (ten pulses at 200 Hz) to release glutamate, while the other set (stim.-test) was used to test the effects of synaptically released glutamate.

(A_2) Traces from a representative experiment are shown. A conditioning train caused a decrease in the latency and an increase in the amplitude of the test afferent volley, as clearly shown in the expanded superimposed traces. All of these effects are reversed following a short application of CNQX.

(A_3) Time course of the entire experiment partially shown in (A_1) and (A_2), and the effects of kynurenate and cadmium. The first two traces are responses without conditioning, and the arrow indicates the onset of the conditioning stimulus, which was continued for the rest of the experiment. Note that the first response during the train (closed triangle in trace 2 of [A_2]) was not affected by the drugs, indicating the activity dependence of the drug effects on the conditioned response (closed circle in trace 2 of [A_2]).

(B) Summary graph of six experiments done in the same way as shown in (A).

(C) Recording with a K^+-sensitive microelectrode shows no change in the extracellular K^+, but a normal KAR-mediated field EPSP (f.p.) is present.

response to the tetanus (Figure 3C). Despite the generation of a KAR-mediated field EPSP, in no case (n = 5) did we see a rise in K^+, supporting the conclusion that the changes in the mossy fiber volley were not an indirect effect of raised extracellular K^+.

We also carried out experiments at 36°C to address the possible physiological relevance of this heterosynaptic increase in fiber volley. At 36°C, the 200 Hz conditioning tetanus caused a 31% ± 7% (n = 4) increase in the fiber volley, and reducing the tetanus into a more physiological range (four pulses at 25 Hz) at 36°C still evoked a substantial enhancement of the fiber volley (23% ± 8%, n = 3) that was blocked by CNQX (data not shown).

An alternative explanation for the results illustrated in Figures 3A and 3B is that tetanic stimulation to the conditioning electrode induces a KAR-mediated EPSP in the granule cell bodies at the site stimulated by the test stimulus, resulting in the activation of more granule cells. Two experiments were performed to address this possibility. First, we set up an experiment in which a conditioning stimulus enhanced the test fiber volley (Figure 4A). We then recorded from the granule cell layer at the test stimulus site to determine if these granule cells received a KAR-mediated EPSP. The tetanus failed to induce a slow field potential at this site (Figure $4B_1$). We then recorded from granule cells at this site with whole-cell recording (Figure $4B_2$). The tetanus failed to induce a CNQX-sensitive change in membrane potential (n = 4). Second, we used the local application of CNQX to determine the site of KAR activation (Figure 4C). Application of CNQX to the dentate gyrus (DG) had no effect on the tetanus-induced fiber excitability changes, but an identical application of CNQX in the same experiment to the CA3 region (CA3) did block the changes (n = 4).

In an additional set of experiments designed to test for the synaptic activation of presynaptic KARs, we stimulated in s. lucidum and recorded antidromic action potentials with a whole-cell patch electrode from dentate granule cells (Figure 5), similar to the experiment in Figure 1B. The stimulus strength to the mossy fibers was adjusted so that it either was just below threshold or

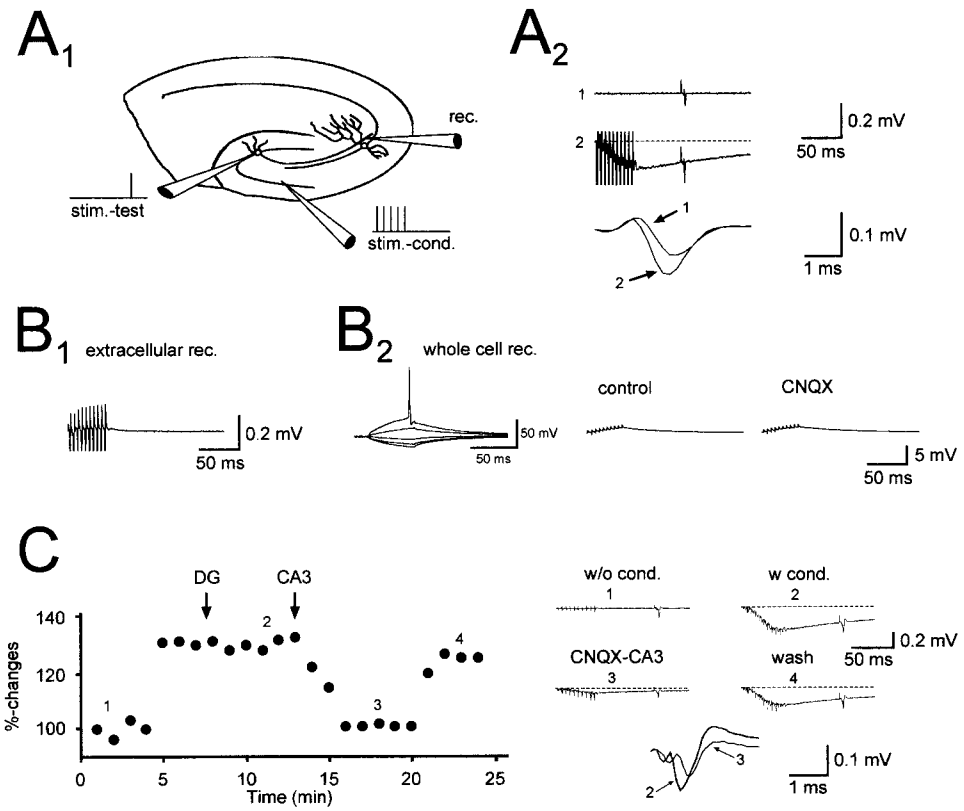

Figure 4. The Site of Action of Synaptically Released Glutamate on the Mossy Fiber Volley Is in S. Lucidum

(A_1) Diagram of the experimental setup.

(A_2) A conditioning stimulus at one electrode (stim.-cond.) in the granule cell layer enhances the fiber volley evoked by a second stimulating electrode (stim.-test) in the granule cell layer and recorded in s. lucidum.

(B) A recording electrode was then placed at the stim.-test site to determine if the conditioning stimulus evoked a response (B_1). No response was observed. In B_2, a patch electrode was inserted to record from individual granule cells at the same site. Note that the conditioning stimulus fails to exert a CNQX-sensitive depolarization.

(C) The same experimental setup as that shown in A_1 was used except that a local perfusion apparatus was included that allowed CNQX to be locally applied to the DG or to the CA3 region (see Experimental Procedures). At 5 min into the experiment, the conditioning stimulus was switched on, causing the enhancement of the fiber volley. CNQX was then applied (30 s) to the dentate gyrus but had no effect on the conditioning-induced enhancement. An identical application of CNQX was then made to the CA3 region, and this completely and reversibly blocked the enhancement. Sample traces are shown to the right.

intermittently generated antidromic action potentials. Repetitive stimuli were then applied. As can be seen in Figure 5A, while the stimulus was initially below threshold, toward the end of the tetanus, the axon of the recorded cell reaches threshold. Most importantly, this change in threshold is blocked by the application of CNQX, indicating that the change in excitability results from the synaptic activation of KARs. A summary of five experiments is graphed in Figure 5B, in which the average success rate of the last five stimuli in the tetanus is plotted. These results are in accord with the experiments on the orthodromic fiber volley, indicating that repetitive stimulation of mossy fibers causes the synaptic release of glutamate, which spreads to neighboring mossy fibers and increases their excitability.

Is this spread limited to mossy fibers or might the glutamate released from the neighboring associational/commisural (A/C) synapses spread into s. lucidum to activate the presynaptic KARs? To test this possibility, the conditioning electrode was placed in s. radiatum (Figure 6A). We verified that the A/C electrode did not inadvertently activate mossy fibers by the lack of a field KAR–mediated EPSP (Figure $6B_1$). Nevertheless, a tetanus to the A/C synapses evoked an enhancement of the mossy fiber volley, and CNQX reversed this enhancement (Figures $6B_1$ and $6B_2$). A summary of five experiments is shown in Figure 6C.

Activation of Presynaptic Kainate Receptors Inhibits Synaptic Transmission

What consequence does the activation of presynaptic KARs have on mossy fiber transmission, and might this effect be related to the depolarization? Since kainate (0.5 μM) application in the absence of GYKI 53655 causes an inward current of ~1 nA in CA3 pyramidal cells (see also Castillo et al., 1997), AMPA receptor-mediated synaptic transmission could not be studied. We therefore recorded pharmacologically isolated NMDA EPSCs by holding the membrane potential at a positive holding potential to remove the Mg^{2+} block and compared the effects of KAR activation with those of K^+-induced depolarization. All experiments examining the effects of kainate on the EPSC were performed in the

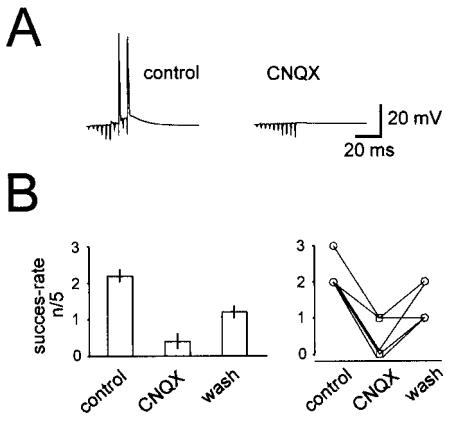

Figure 5. Synaptic Release of Glutamate Increases the Excitability of Single Mossy Fiber Axons

(A) A representative experiment showing that repetitive stimulation of mossy fibers in s. lucidum in CA3 causes changes in the threshold for antidromically elicited action potentials. This effect is blocked by CNQX.

(B) The results of five experiments are summarized in the bar graph, and the same results from each cell are also shown in the plot to the right.

continuous presence of the $GABA_B$ receptor antagonist SCH50911. Figure 7A shows NMDA EPSCs from a typical experiment and their reversible depression by high K^+ and kainate. Superimposition and scaling of the traces indicated that no change in the kinetics occurred during the KAR-induced depression. A summary of five experiments (Figure 7B) in which the actions of high K^+ and kainate were compared in the same cells shows that the inhibition is highly reproducible. K^+ and kainate at these concentrations actually increased the presynaptic afferent volley (Figure $7B_1$).

These effects were associated with no significant change in the passive membrane properties for high K^+, and only a 9% ± 3% decrease in the input resistance and an outward current of 27 ± 9 pA for kainate. It should be noted that in a total of 12 cells in which the effects of kainate on the EPSC were examined, in 4 of these cells no change in holding current could be observed. The small change in holding current induced by kainate in the presence of GYKI 53655 is surprising given the large change in holding current in the absence of GYKI 53655 (~1 nA at a holding potential of −70 mV in the absence of GYKI 53655). This reduction is presumably due to some contribution of AMPA receptors to the response generated by 0.5 μM kainate, as well as an antagonism of the KARs by 100 μM GYKI 53655 (Wilding and Huettner, 1995; Frerking et al., 1998). In those cells that showed a small postsynaptic effect of kainate, the recovery was considerably faster than the effect on the NMDA EPSC.

Lower concentrations of kainate (0.1–0.2 μM) more clearly separated the pre- and postsynaptic effects of kainate. At these low concentrations, kainate still caused an increase in the fiber volley, as well as an inhibition of the NMDA EPSC (28% ± 9%, n =4), but

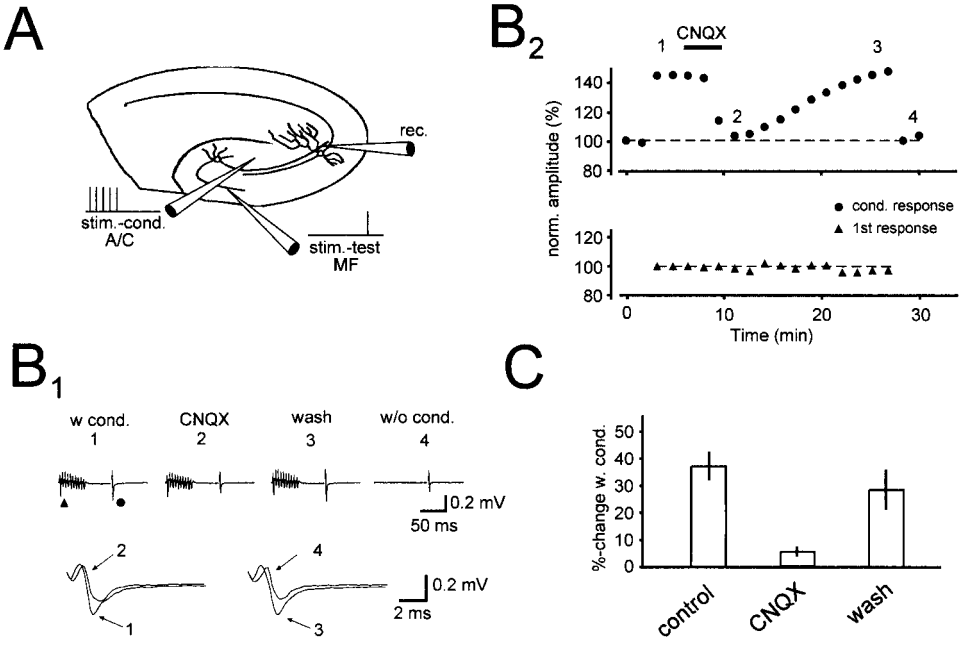

Figure 6. Synaptic Release of Glutamate from A/C Synapses Enhances the Mossy Volley by Activating Kainate Receptors

(A) Diagram of the experimental setup. The conditioning stimulating electrode (stim.-cond. A/C) was placed in the s. radiatum, near to the s. lucidum. The other stimulating electrode (stim.-test MF) was placed in the granule cell layer to activate the mossy fibers. The mossy fiber volley was recorded with an electrode (rec.) placed in s. lucidum.

(B) Sample traces from a typical experiment showing that A/C conditioning enhances mossy fiber responses in a CNQX-reversible manner are illustrated in B_1. The time course of the entire experiment is plotted in (B_2). The top graph plots the responses to the test stimulus (closed circle in trace 1 in [B_1]), while the bottom graph plots the first response to the conditioning tetanus (closed triangle in trace 1 in [B_1]). The first two data points and the last two data points are unconditioned.

(C) A summary graph of five experiments.

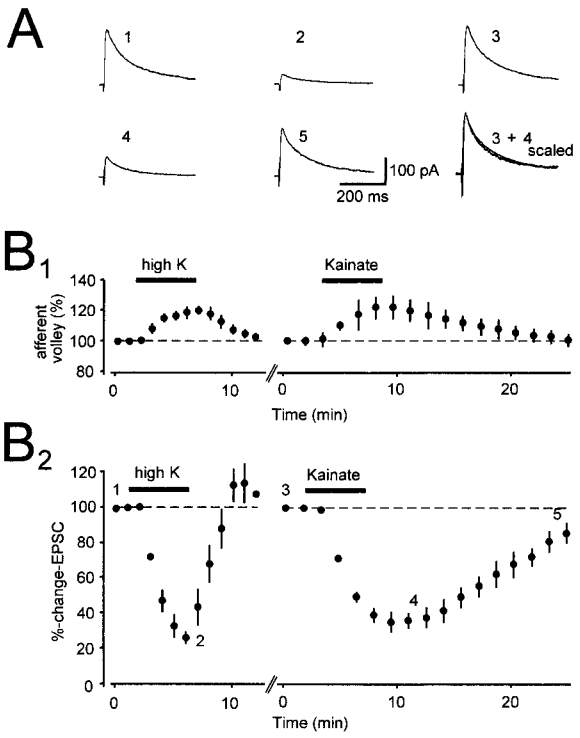

Figure 7. Kainate Suppresses Mossy Fiber–Evoked NMDA EPSCs

(A) Representative experiment showing that the application of high K^+ (7 mM) and kainate (0.5 μM) causes a dramatic decrease in the NMDA EPSC. The scaled superimposed traces show that no change in the kinetics of the EPSC accompanied the decrease. During this experiment, kainate induced a small (<25 pA) outward current that was shorter lasting than the effect on the synaptic currents.

(B) (B_1) shows the effects of 7 mM K^+ and 0.5 μM kainate on the fiber volley (n = 5). Note that the increase in the fiber volley induced by 0.5 μM kainate is considerably less than that shown in Figure 1A. This is presumably because the present experiments were carried out in the presence of elevated divalents, to prevent polysynaptic activity. (B2) shows the effects of the same concentration of K^+ and kainate applied for the same period of time on evoked NMDA EPSCs (n = 5). The membrane potential of the CA3 pyramidal cells was held between +20 and +40 mV.

had no effect on the holding current (see also Figure 2D). This decrease of the NMDA EPSC with no change in the membrane properties of the pyramidal cell suggests that the reduction of the EPSC by kainate is due to a decrease in the release of glutamate from the mossy fiber synapses, rather than a change in the postsynaptic cell. To confirm that there was no postsynaptic effect of kainate, we examined the effect of bath-applied kainate on the NMDAR response evoked by iontophoresis of NMDA in s. lucidum. The response was unaltered by the bath application of kainate (98% ± 6%, n = 4). Also consistent with a presynaptic locus, an analysis of the coefficient of variation of the NMDA EPSC showed a decrease in $1/CV^2$ that was similar to the decrease in the mean amplitude induced by kainate. Kainate (0.5 μM) reduced the amplitude to 41% ± 5% and the $1/CV^2$ to 33% ± 7% of control values ($p < 0.001$) (n = 12). High K^+ also had similar effects, reducing the amplitude to 25% ± 8% and the $1/CV^2$ to 32% ± 15% of control values ($p < 0.02$) (n = 4). These findings support a presynaptic site for the inhibition of synaptic transmission.

The fact that the presynaptic inhibition and the changes in presynaptic fiber volley had the same dose–response relationship and that high K^+ mimicked both effects suggests that the two are causally related. However, it is possible that the presynaptic inhibiton could be mediated by an action separate from that affecting the fiber volley. We reasoned that, were this the case, the two effects might be separated by the use of subtype-selective agonists of KARs. We found, as reported, that the GluR5-selective agonist ATPA (2 μM) has no effect on holding current (n = 4) or membrane potential (n = 5) but does cause a depression of the field EPSP (Vignes et al., 1998); however, much to our surprise, we found that the inhibition of synaptic responses occurred in the complete absence of any change in the fiber volley. An example of the inhibition of the field EPSP in the absence of any change in the fiber volley is shown in Figure 8A, and an average of all of the experiments on the synaptic response is shown in Figure $8B_1$. To investigate more closely for changes in the fiber volley, a series of experiments were carried out in the presence of GYKI. ATPA had no effect on the fiber volley (Figure $8B_2$). The inhibition of synaptic transmission by ATPA was also observed with the NMDAR EPSC (53% ± 7%), and 2,3-hydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) blocked this depression (Figure 8C), indicating that the effect is mediated by KAR activation. This indicates that at least for ATPA, the effects on synaptic transmission can be separated from the effects on the fiber volley. ATPA is known to activate the GluR5 receptors on CA1 interneurons (Cossart et al., 1998), and we found that ATPA both depolarized CA3 interneurons and greatly increased the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) in CA3 pyramidal cells (n = 4) (Figure 8D). We therefore wondered if the effects on synaptic transmission might be mediated indirectly by the release of GABA onto presynaptic $GABA_B$ receptors on the mossy fiber terminals. Indeed, the presynaptic inhibitory effects of ATPA could be prevented by the $GABA_B$ antagonist SCH50911 (Figure 8E). These results suggest that the GluR5-specific agonist ATPA has little, if any, direct effect on fiber excitability, excitatory synaptic transmission, or postsynaptic membrane properties at mossy fiber synapses.

Presynaptic Kainate Receptors Mediate a Heterosynaptic Inhibition

Two technical issues confound experiments designed to examine the possibility of a presynaptic KAR-mediated heterosynaptic inhibition. First, tetanic stimulation of mossy fibers evokes a KAR-mediated EPSC that overlapped the time course of the effects on the fiber volley, thus making it difficult to distinguish clearly between pre- and postsynaptic effects. Secondly, the use of the NMDAR-mediated synaptic current suffers from the same problem, because the time course of the NMDAR EPSC is overlapping with that of the KAR-mediated effects. We circumvented the first problem by placing the conditioning electrode in s. radiatum (Figure 9A), which does not activate a KAR EPSC. To deal with the second problem, we selectively blocked NMDAR EPSCs evoked by A/C stimulation while preserving the mossy fiber NMDAR EPSC by briefly exposing the slice to the irreversible, use-dependent blocker MK 801 and tetanizing the A/C fibers (Scanziani et al., 1996; see Experimental Procedures). Under these conditions, tetanic stimulation

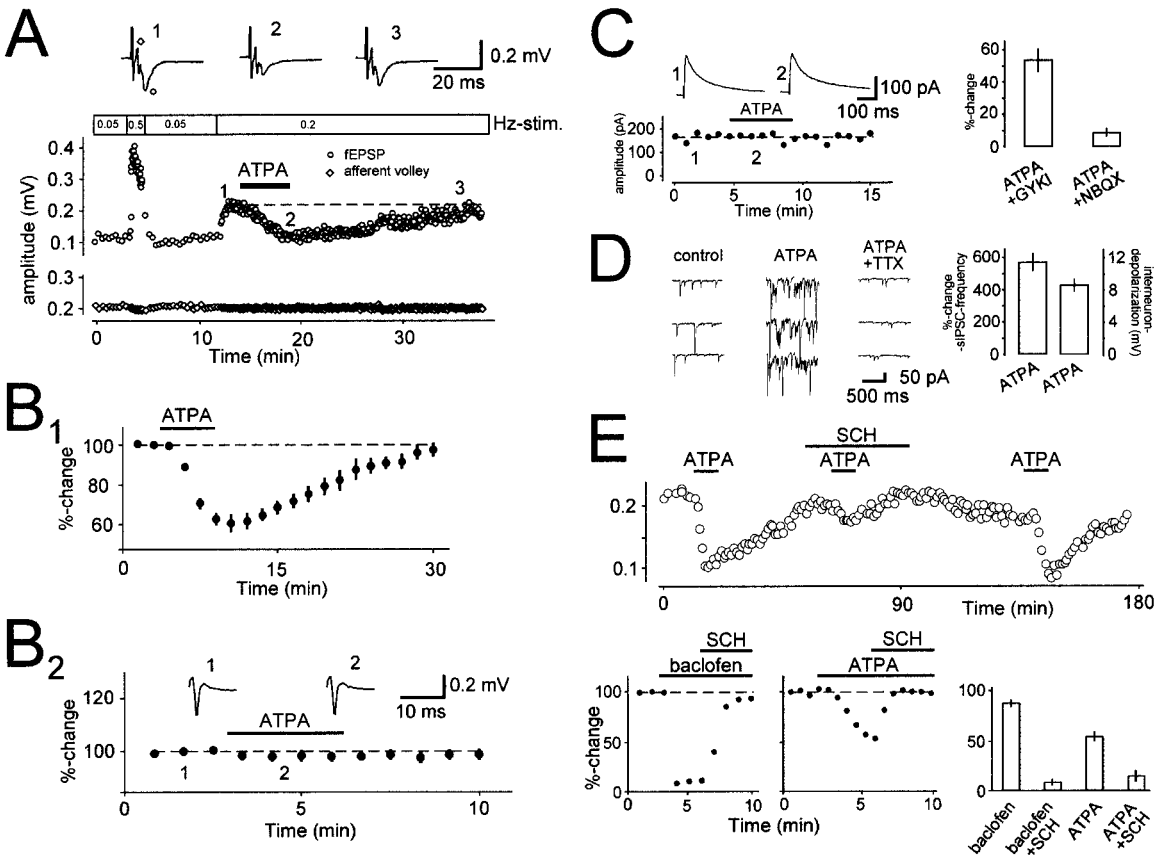

Figure 8. The GluR5-Selective Agonist ATPA Inhibits Mossy Fiber Transmission Indirectly via $GABA_B$ Receptors

(A) An individual field potential experiment showing the frequency facilitation, which is a prominent feature of mossy fiber synapses, and the inhibition of synaptic responses (upper graph) without any change in the fiber volley (lower graph).

(B) Summary graphs showing the inhibition of mossy fiber field potential responses (B_1). In a separate set of experiments, GYKI was used to isolate more clearly the fiber volley. ATPA had no effect on the fiber volley (B2) (n = 6).

(C) The inhibition, as recorded using the NMDAR EPSC, is blocked in the presence of NBQX but not GYKI, confirming that it is due to KAR activation (n = 4; representative experiment in NBQX shown on left, summary shown on right).

(D) ATPA causes a dramatic increase in the frequency of tetrodotoxin-sensitive spontaneous IPSCs, indicating that it excites inhibitory interneurons.

(E) The inhibitory action of ATPA is reversibly antagonized by the $GABA_B$-selective antagonist SCH50911 (upper graph). SCH50911 also reversed the inhibitory action of the $GABA_B$ agonist baclofen (lower left graph) and the inhibitory action of ATPA (lower middle graph). A summary bar graph of all the SCH50911 experiments is shown to the right (baclofen, n = 4; baclofen + SCH, n = 4; ATPA, n = 7; ATPA + SCH, n = 7).

of A/C fibers elicits little or no postsynaptic response but does cause a reduction in the mossy fiber–evoked NMDAR EPSC, which is entirely reversed by NBQX (Figure $9B_1$). Superimposition and scaling of the control and inhibited responses indicated that there was no change in the kinetics of the EPSC during the inhibition. The time course of this experiment is shown in Figure $9B_2$, which also demonstrates that the response is mediated by mossy fibers, since it is blocked by the group 2–selective, mGluR-selective agonist L-CCG1 (Kamiya et al., 1996). A summary of five experiments is illustrated in Figure 9C.

Discussion

The present results show that KAR activation depolarizes hippocampal mossy fiber axons, in agreement with the conclusions of Kamiya and Ozawa (2000). We have also demonstrated that glutamate released from mossy fiber synapses can activate these presynaptic KARs. This action of glutamate is not limited to the synapses that release glutamate but also occurs on neighboring mossy fibers due to the spread of glutamate from the activated synapses. Remarkably, this spread is not confined to the mossy fibers but can occur from nearby A/C synapses. In addition, we present evidence that activation of these presynaptic KARs strongly inhibits transmitter release from mossy fiber synapses and that this can be mimicked by depolarizing the synapses with K^+. Finally, this presynaptic inhibition can be engaged by the release of glutamate from neighboring synapses.

It has recently been reported that kainate (Kamiya and Ozawa, 2000) and an agonist ATPA (Vignes et al., 1998) can also have presynaptic effects on mossy fibers. However, these studies do not exclude possible postsynaptic effects such as a decrease in the sensitivity of AMPARs, nor do they rule out the possibility of indirect effects. These concerns are particularly relevant given

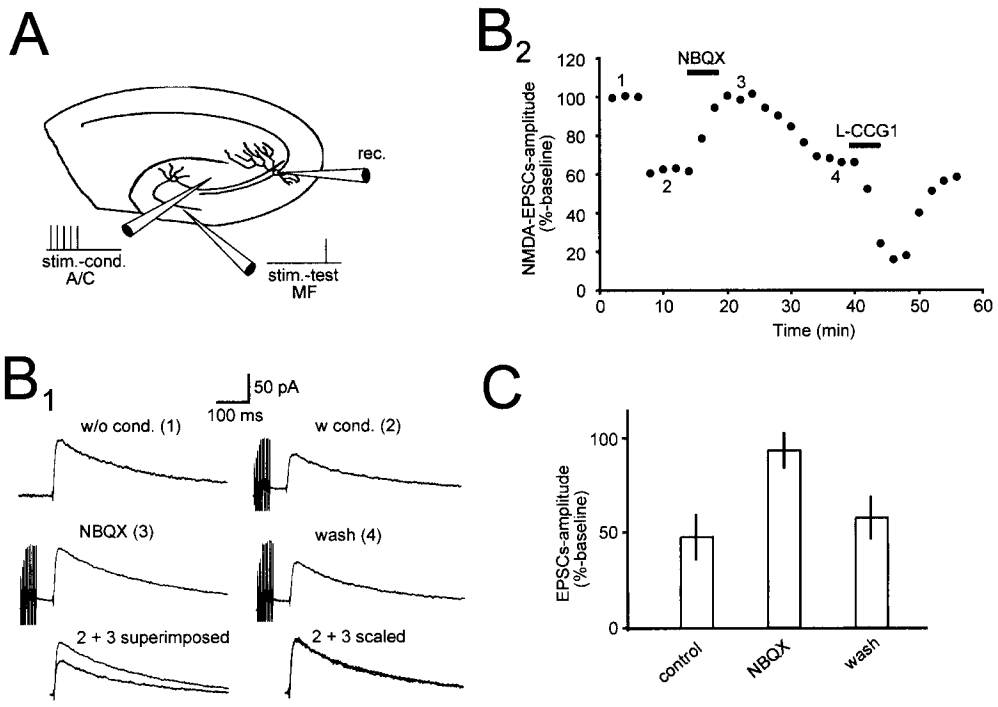

Figure 9. The Synaptic Release of Glutamate from A/C Synapses Causes a Kainate Receptor–Mediated Presynaptic Inhibition of the Mossy Fiber NMDAR EPSC

(A) Diagram of the experimental setup. The conditioning stimulating electrode (stim.-cond. A/C) was placed in the s. radiatum, near to the s. lucidum. The other stimulating electrode (stim.-test MF) was placed in the granule cell layer to activate mossy fibers. The mossy fiber EPSC was recorded with a whole-cell electrode (rec.) placed in s. pyramidale.

(B) Traces from a typical experiment are shown in (B_1). This entire experiment is plotted in (B_2). The A/C NMDAR EPSC was previously blocked with a brief application of MK 801 (see Experimental Procedures). The A/C conditioning stimulus inhibits the mossy fiber NMDAR EPSC. This inhibiton is reversibly blocked by NBQX. Application of the group 2 mGluR agonist L-CCG1 blocks the NMDAR EPSC, indicating that it is mediated by mossy fiber synapses.

(C) A summary graph of all five experiments.

recent studies demonstrating that KAR activation can lead to an indirect presynaptic inhibition (Frerking et al., 1999; Chergui et al., 2000). In the present experiments, the fact that the response to kainate is unaltered by the blockade of transmitter release with cadmium indicates that the KAR-induced release of an unidentified transmitter cannot account for the effects.

However, a rise in extracellular K^+, secondary to the postsynaptic depolarization, remains a real possibility, especially since the application of high K^+ concentrations was shown to mimic all of the effects of KAR activation. A number of observations indicate that this cannot account for the presynaptic effects. First, the rise in K^+ measured with K^+-sensitive microelectrodes caused by kainate application was well below that required to alter the fiber volley, and the conditioning tetanus used to activate KARs failed to alter extracellular K^+. Second, concentrations of kainate below that required to depolarize CA3 pyramidal cells still increase the excitability of mossy fibers. Third, tetanic stimulation of A/C fibers, which fails to activate KARs on CA3 pyramidal cells, nonetheless increased the excitability of mossy fibers via KAR activation. Fourth, the possibility that the excitability change in mossy fibers is mediated by KAR activation of interneurons and the release of K^+ is most unlikely since these cells are relatively sparse in the CA3 subfield; in any case, such spread would have been detected by a rise in K^+ in s. lucidum sufficient to depolarize mossy fibers, which was not observed. Thus, the KARs are present on the mossy fibers themselves, and their effect is similar to the effect of kainate on isolated peripheral nerve preparations (Agrawal and Evans, 1986).

The precise localization of these receptors on mossy fibers remains unclear. Since these receptors can be activated by synaptically released glutamate and affect transmitter release, it seems plausible that they would be present on the synaptic boutons, but we cannot exclude their presence also or exclusively on the axon. However, because mossy fibers form en passant synapses throughout the length of s. lucidum, the distinction is somewhat semantic, and it is expected that action potential propagation will be progressively more affected as it travels down the fiber, independent of the exact location of the KARs. In support of this conclusion is the observation that the effects on conduction velocity were more pronounced when the distance between the stimulating and recording electrodes was large (data not shown). Interestingly, low concentrations of kainate that increased fiber excitability had no effect on the membrane potential of granule cells, suggesting that KARs are preferentially targeted to the axon or presynaptic terminal. Immunohistochemical results have also reported the presence of KARs on fibers in s. lucidum (Petralia et al., 1994). Curiously, in a previous study, local application of kainate to mossy fibers, which was

sufficient to activate a postsynaptic inward current, failed to alter the frequency of miniature (m) EPSCs (Castillo et al., 1997). Either the contribution of mossy fiber mEPSCs to the overall recorded frequency was small, and, therefore, an effect was overlooked, or the magnitude of the presynaptic depolarization is not sufficient to appreciably alter mEPSC frequency.

Are the KARs expressed on CA3 pyramidal cells the same or different from those expressed on the mossy fiber, and are the KARs that increase the excitability of mossy fibers the same as those that inhibit transmitter release? Studies on mRNA in situ hybridization indicate that messages for GluR6, KA1, and KA2 are strongly expressed in CA3 pyramidal cells, while GluR6, GluR7, KA1, and KA2 are strongly expressed in dentate granule cells from which mossy fibers originate (Wisden and Seeburg, 1993; Bureau et al., 1999). We have found that concentrations of kainate that have no effect on the somata of CA3 pyramidal cells or granule cells are still capable of increasing the excitability of mossy fibers and inhibiting transmitter release, suggesting that the affinity of the presynaptic receptors is higher than that of the postsynaptic receptors. In addition, the increase in fiber excitability and the inhibition of synaptic transmission outlasted the postsynaptic conductance change, consistent with a higher affinity of the presynaptic receptors. The fact that the release of glutamate from A/C synapses activates the presynaptic mossy fiber receptors but not the postsynaptic CA3 pyramidal cell receptors also supports this difference in affinity and indicates that the difference holds for the endogenous agonist glutamate. Presumably it is the very high affinity of these presynaptic receptors that accounts for the remarkably widespread influence that mossy fibers receive from surrounding synapses, including the anatomically distinct A/C synapses in s. radiatum. The finding that the increase in the excitability of the mossy fibers and the inhibition in transmitter release have the same high-affinity sensitivity to kainate suggests that these two effects may be mediated by the same presynaptic KAR; however, we cannot unequivocally rule out the possibility that two presynaptic receptors exist with similarly high affinities, that have independent actions on the fiber volley and EPSC.

Another issue that remains to be worked out is the mechanism by which KARs affect fiber excitability and inhibit transmitter release. The fact that a concentration of K^+ that increases the presynaptic fiber volley to the same extent as kainate also exerts a similar depression of the NMDAR EPSC strongly suggests that KARs exert their effect on release by depolarizing the mossy fibers. Alternatively, it is conceivable that the action of KARs on fiber excitability and the presynaptic inhibition of synaptic transmission are mediated by independent ionotropic and/or metabotropic actions (Lerma, 1997; Rodriguez-Moreno and Lerma, 1998; Frerking and Nicoll, 2000; Rodriguez-Moreno et al., 2000). However, this scenario fails to explain why depolarizing the synapses with high K^+ mimics both actions of kainate, unless one were to propose the release of another substance that exactly matches the presynaptic inhibition mediated by KARs. It has recently been reported (Kamiya and Ozawa, 2000) that kainate decreases the action potential–evoked increase in Ca^{2+} in mossy fiber terminals. Thus, it appears that either via an inactivation of Na^+/Ca^{2+} channels secondary to depolarization or via a more direct metabotropic action, the end result is a decrease in voltage-dependent Ca^{2+} entry into the terminals. It would be of interest to examine the effect of presynaptic depolarization on this Ca^{2+} transient. Final determination of the degree to which ionotropic mechanisms can explain the effects of KAR activation will have to await direct recordings from mossy fiber boutons.

A result that, at first glance, supports the idea that the regulation of the fiber volley is independent of regulation of the EPSC is that the GluR5-specific agonist ATPA caused a selective depression of the EPSC without affecting the fiber volley. However, this effect of ATPA is secondary to an ATPA-induced increase in interneuronal GABA release, as it is blocked by the $GABA_B$ receptor antagonist SCH50911. Because the effects of kainate in this study are all monitored in the presence of SCH50911, the ATPA-induced depression of the EPSC is fundamentally unrelated to the kainate-induced depression that we have described. However, we would predict that the indirect effects of GluR5 activation would participate in the synaptic depression induced by kainate in the absence of SCH50911. Our observation that GluR5 agonists activate the interneuronal KARs but not pre- or postsynaptic KARs at mossy fiber synapses provides further support for the idea that GluR5 is involved predominantly in regulation of interneurons, as suggested by the expression data (Paternain et al., 2000).

Our results also suggest that endogenously released glutamate can activate the presynaptic KARs to cause both an increase in excitability and a decrease in synaptic transmission. Most of these experiments were performed at room temperature, and a frequency of 200 Hz was used for the conditioning tetanus, and, thus, the physiological relevance of these findings could be questioned. However, the heterosynaptic effects were still present at a temperature of 36° C and with a total of four pulses at 25 Hz tetanus, a frequency that has been recorded in vivo for dentate granule cells (Penttonen et al., 1997). It has also been reported in the CA1 region that a conditioning tetanus can cause a KAR-induced disinhibition (Min et al., 1998), although it remains unclear whether this effect is due to spillover, as described here, or a KAR-induced disynaptic EPSC on interneurons.

A presynaptic inhibitory action of KARs has been proposed for other excitatory (Chittajallu et al., 1996; Kamiya and Ozawa, 1998; Vignes et al., 1998) and inhibitory (Clarke et al., 1997; Rodriguez-Moreno et al., 1997; Bleakman and Lodge, 1998; Rodriguez-Moreno and Lerma, 1998; Frerking and Nicoll, 2000) synapses. While the mechanism at inhibitory synapses remains controversial, the fact that kainate does not increase the frequency of mIPSCs, even under high K^+ conditions in which mIPSC frequency is strongly influenced on voltage-dependent Ca^{2+} entry (Frerking et al., 1999), would argue against a direct depolarization of the inhibitory synapses. It will be of interest to determine whether other excitatory synapses use a mechanism similar to the one characterized for mossy fiber synapses in the present study.

Experimental Procedures

Hippocampal slices were prepared from young adult (postnatal day 18–29 [P18–P29]) Sprague-Dawley rats. In brief, the animals were deeply anesthetized with halothane and decapitated, and the brains were removed. Tissue blocks containing the subicular area and hippocampus were mounted on a Vibratome (Leica) in a chamber filled with ice-cold artificial cerebrospinal fluid containing (in mM):

NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1; $CaCl_2$, 2.5; $MgSO_4$, 1.3; and glucose, 10, saturated with 95% O_2–5% CO_2 (pH 7.4). Transverse slices were cut at 300–400 μm thickness and were stored in a submerged chamber, where they were held for 1–7 hr, until slices were transferred to the recording chamber, where they were perfused at a high rate of 3–4 ml/min. Unless otherwise stated, all solutions contained GYKI (100 μM) and picrotoxin (100 μM). D-APV (100 μM) was also present in all experiments except those involving the NMDA EPSC. All experiments examining presynaptic inhibitory effects of kainate were carried out in the presence of SCH50911 (20 μM) to prevent any possible effects of $GABA_B$ receptors. In addition, in those experiments examining heterosynaptic inhibition, MCPG (0.5 mM) was also present to block mGluRs.

The recording chamber was mounted on an Olympus microscope equipped for IR-DIC microscopy. The slices were allowed to rest for at least 1 hr after the preparation before recordings were performed. For all voltage-clamp whole-cell recordings investigating synaptic transmission, the $CaCl_2$ and $MgSO_4$ had to be increased to 4 mM to prevent polysynaptic activation. Whole-cell recording electrodes for current-clamp recordings were filled with (in mM): K-gluconate, 130; KCl, 5; $MgCl_2$, 1; HEPES, 10; and Na_2ATP, 2 (pH adjusted to 7.3 with KOH). For whole-cell voltage-clamp recordings, the internal solution contained (in mM): Csgluconate, 117.5; CsCl, 2.5; NaCl, 8; TEACl, 10; EGTA, 10; MgATP, 4; Na_3GTP, 0.3; HEPES, 10; and QX-314, 5 (pH adjusted to 7.3 with CsOH). Electrode resistances ranged from 4 to 7 MΩ for granule cell recordings and from 1.5 to 3 MΩ for CA3 pyramidal cell recordings. Access resistances ranged from 4 to 20 MΩ, and they were continuously checked during the recording and were not allowed to vary more than 15% during the course of the experiment. No series resistance compensation was used.

Field potential recordings were performed with low-resistance patch pipettes filled with HEPES-buffered external solution placed in s. lucidum in CA3 or in the DG granule cell layer. Bipolar tungsten electrodes were placed in the granule cell layer or in the hilus region to stimulate mossy fibers or in s. lucidum for antidromic stimulation. Continuous local application was performed as previously described (Veselovsky et al., 1996), but with bigger tip openings (~200–300 μm). K^+-sensitive microelectrodes were manufactured and tested as described previously (Lux and Neher, 1973; Heinemann et al., 1977). Iontophoresis of NMDA (1 M, adjusted to a pH of 9) was accomplished with electrodes pulled from borsilicate glass and having resistances of 70–100 MΩ. NMDA was ejected using a constant current iontophoresis instrument (WPI) with pulses ranging from 200 to 400 nA for 5–30 ms. The electrode was lowered to the surface of s. lucidum under visual control. The iontophoretic currents and the position of the electrode were adjusted until a satisfactory response was obtained. In some experiments, a holding current of 10–20 nA was used to prevent leakage of NMDA. The NMDARs on the A/C pathway were selectively blocked using the irreversible, use-dependent antagonist MK 801 (see Scanziani et al., 1996). A stimulating electrode was placed in s. radiatum to stimulate A/C fibers. MK 801 (50 μM) was applied for 5 min, and at the end of the application the A/C fibers were tetanized (100 Hz for 1 s, repeated four times). The electrode was left in place, and the slice was washed for at least 3 hr to remove MK 801 from the slice. This procedure was found to block the NMDAR EPSC on the A/C input, while the mossy fiber NMDAR EPSC remained stable for the time course of the experiment, confirming that MK 801 had been successfully washed from the slice. Average values are expressed as mean ± SEM.

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