

# Kainate Receptor–Mediated Inhibition of Glutamate Release Involves Protein Kinase A in the Mouse Hippocampus

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**José V. Negrete-Díaz, Talvinder S. Sihra, José M. Delgado-García, and Antonio Rodríguez-Moreno.** Kainate receptor–mediated inhibition of glutamate release involves protein kinase A in the mouse hippocampus. *J Neurophysiol* 96: 1829–1837, 2006. First published June 28, 2006; doi:10.1152/jn.00280.2006. The mechanisms involved in the inhibition of glutamate release mediated by the activation of presynaptic kainate receptors (KARs) at the hippocampal mossy fiber–CA3 synapse are not well understood. We have observed a long-lasting inhibition of CA3 evoked excitatory postsynaptic currents (eEPSCs) after a brief application of kainate (KA) at concentrations ranging from 0.3 to 10  $\mu$ M. The inhibition outlasted the change in holding current caused by the activation of ionotropic KARs in CA3 pyramidal cells, indicating that this action is not contingent on the opening of the receptor channels. The inhibition of the eEPSCs by KA was prevented by G protein and protein kinase A (PKA) inhibitors and was enhanced after stimulation of the adenylyl cyclase (AC) with forskolin. We conclude that KARs present at mossy fiber terminals mediate the inhibition of glutamate release through a metabotropic mechanism that involves the activation of an AC-second messenger cAMP-PKA signaling cascade.

## INTRODUCTION

Neurotransmitter receptors are located on the presynaptic, as well as the postsynaptic, side of the synapse. Kainate receptors (KARs) are a family of glutamate receptors that participate in normal synaptic transmission and in some forms of long-term potentiation (LTP; proposed as a cellular mechanism for learning and memory), and their aberrant function may underlie neuropathological states including epilepsy (Huettner 2003; Lerma 2003; Lerma et al. 2001 for reviews). The actions of the defining agonist kainate (KA) at these receptors are mediated postsynaptically, to regulate excitatory synaptic transmission, and presynaptically, to modulate neurotransmitter release. In the latter context, KARs have been implicated in the modulation of both glutamate and GABA release in the hippocampus (Huettner 2003; Lerma 2003 for reviews).

Interestingly, KAR activation has a biphasic effect on glutamate release in the hippocampus, such that low (20–50 nM) KA concentrations produce an increase in glutamate release in the mossy fiber–CA3 region (Contractor et al. 2001; Lauri et al. 2001a,b; Rodríguez-Moreno and Sihra 2004; Schmitz et al. 2001), whereas higher (>50 nM) concentrations produce a decrease of the evoked excitatory postsynaptic currents (eEPSCs) in CA1 (Chittajallu et al. 1996; Frerking et al. 2001; Kamiya and Ozawa 1998; Vignes et al. 1998) and in mossy fiber–CA3 regions of the hippocampus (Contractor et al. 2000;

Kamiya and Ozawa 2000; Schmitz et al. 2000). Because the exact mechanism by which KARs produce an inhibition of glutamate release remains unclear, here we examined how KAR activation induces a long-lasting depression of glutamate release from hippocampal mossy fibers (MFs) synapsing onto CA3 neurons in the hippocampus. We found that this inhibition involves the activation of adenylyl cyclase (AC) and protein kinase A (PKA) through a pertussis toxin (PTX)-sensitive G protein.

## METHODS

### *Hippocampal slices*

Hippocampal slices were prepared from adult C57Bl/6 mice as described in detail elsewhere (Rodríguez-Moreno et al. 1997, 1998). The whole brain containing the two hippocampi was removed under ice-cold solution consisting of (in mM) 124 NaCl, 2.69 KCl, 1.25  $\text{KH}_2\text{PO}_4$ , 2  $\text{MgSO}_4$ , 1.8  $\text{CaCl}_2$ , 26  $\text{NaHCO}_3$ , and 10 glucose (pH 7.3, 300 mOsm) and positioned on the stage of a vibratome slicer and cut to obtain 350- $\mu$ m-thick transverse hippocampal slices, which were maintained continuously oxygenated for  $\geq 1$  h before use.

### *Electrophysiological recordings*

Whole cell recordings were performed from neurons visually identified by IR-DIC microscopy using a  $\times 40$  water immersion objective. All experiments were carried out at room temperature (26–29°C). For experiments, slices were continuously perfused at a rate of 2–3 ml/min with the solution mentioned above (2 mM  $\text{Mg}^{2+}$  and 1.8 mM  $\text{Ca}^{2+}$ ). Some previous results about actions of KAR at this synapse were obtained by using 4 mM of divalent cations ( $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) in the extracellular solution just to reduce global excitability and minimize polysynaptic activation; we also performed experiments using this kind of solution (4 mM  $\text{Ca}^{2+}$ /4 mM  $\text{Mg}^{2+}$ ). Very similar data were obtained with both types of extracellular solutions, suggesting that the relative contribution of polysynaptic activation was minimal in our experimental conditions, and experimental data were pooled together. In a few experiments, to prevent the activation of KARs situated in interneurons and their subsequent firing, we used higher divalent cations in the extracellular solution (8 mM  $\text{Ca}^{2+}$  and 17 mM  $\text{Mg}^{2+}$ ). This high divalent solution has no effect on the probability of transmitter release, because no change in pair pulse facilitation (PPF) was observed.

Drugs were applied by gravity-feed, switching between four perfusion lines. Unless otherwise stated, all solutions contained SYM2206 (100  $\mu$ M), bicuculline (50  $\mu$ M), and SCH50911 (50  $\mu$ M) to block AMPA,  $\text{GABA}_A$ , and  $\text{GABA}_B$  receptors, respectively, in experiments involving *N*-methyl-D-aspartate (NMDA) EPSCs and

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D-AP5 (100  $\mu\text{M}$ , to block NMDA receptors), bicuculline (50  $\mu\text{M}$ ), and SCH50911 (50  $\mu\text{M}$ ) in those involving AMPA EPSCs. To be sure that we were recording currents monosynaptically evoked from mossy fibers, we used several criteria. 1) Experiments were included for data analysis only if L-CCG-1 (10–30  $\mu\text{M}$ ), a group II mGluR agonist that selectively blocks synaptic transmission at mossy fiber terminals but not from commissural/associational connections (Kamiya et al. 1996), applied at the end of the experiment, caused a >85% reduction in the synaptic response. 2) We analyzed a typical characteristic of MF-CA3, i.e., the ability of this synapse to display rapid and pronounced frequency facilitation with paired stimuli (PPF). No differences in PPF ratios were observed in slices treated with either PTX or cAMP analogues (Rp-Br-cAMP and Sp-8-CPT-cAMP; Supplemental Fig. S1<sup>1</sup>). 3) Key experiments were repeated using high divalent concentrations (4 mM  $\text{Ca}^{2+}$ /4 mM  $\text{Mg}^{2+}$ ); in this situation, the excitability of the slice was clearly diminished. 4) We measured the rise-time of the eEPSCs and only considered responses that were typical in terms of the rise-time described for mossy fiber responses (i.e., <1.5 ms, 20–80%) as described by Kapur et al. 1998; and Yeckel et al. 1999. When protocol 1) could not be applied, i.e., in PTX experiments or when use some analogs of cAMP that also affect L-CCG-1 mediated responses, we used the other criteria. In PTX experiments, slices were incubated with the G protein inhibitor PTX (6–8 h at 37°C before use). For these experiments, control slices were also incubated for 6–8 h at 37°C before use. To evoke mossy fiber EPSCs, electrical pulses were applied by a bipolar electrode placed in the dentate granule cell layer or stratum lucidum. Tight-seal (>1 G $\Omega$ ) whole cell recordings were obtained from the cell body of neurons situated in the CA3 pyramidal layer. Patch electrodes were fabricated from borosilicate glass and had a resistance of 5–10 M $\Omega$  when filled with (in mM) 120 CsCl, 8 NaCl, 1 MgCl<sub>2</sub>, 0.2 CaCl<sub>2</sub>, 10 HEPES, and 2 EGTA (pH 7.3, 287 mOsm). We recorded synaptic responses mediated by these receptors at a membrane potential of –30 mV to relieve  $\text{Mg}^{2+}$  blockade. In current-clamp experiments, K-gluconate substituted for CsCl. In voltage-clamp experiments, 20 mM QX-314 (intracellular blocker of Na<sup>+</sup> channels) was included in the pipette solution to avoid firing of unclamped cell compartments. A 40-ms paired pulse stimulation protocol was used for pair pulse ratio (PPR) analysis. In experiments determining synaptic efficiency, we defined a “failure” as a current record in which no detectable EPSC was observed above the baseline noise. Neurons were voltage or current clamped using an Axopatch 200B amplifier (Axon Instruments). Access resistance (8–30 M $\Omega$ ) was regularly monitored during recordings and cells were rejected if it changed >15% during the experiment. Data were filtered at 2 kHz, digitized, and stored on a computer using pClamp (Axon Instruments).

### Data analysis

Data are presented as means  $\pm$  SE. Significance was assessed at  $P < 0.05$ , using the Student's unpaired *t*-test.

The noise-free CV was calculated (described in detail in Rodríguez-Moreno et al. 1997) as

$$\text{CV} = \frac{\sqrt{\sigma^2(\text{EPSC}) - \sigma^2(\text{noise})}}{\text{Amplitude}_{(\text{EPSC})}}$$

where  $\sigma^2(\text{EPSC})$  and  $\sigma^2(\text{noise})$  are the variance of the EPSC and baseline, respectively. For each cell,  $\text{CV}_{\text{KA}}/\text{CV}_{\text{Control}}$  was obtained.

### Compounds

Bicuculline methobromide, kainate, naloxone, pertussis toxin (PTX), and salts were purchased from Sigma; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-(2S)-5,5-dimethyl-2-morpho-

lineacetic acid (SCH50911), ( $\pm$ )-4-(4-aminophenyl)-1,2-dihydro-1-methyl-*z*-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM2206), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), D-2-amino-5-phosphonovaleric acid (D-APV),  $\alpha$ -methyl-4-phosphonophenylglycine (MPPG),  $\alpha$ -methyl-4-carboxyphenylglycine (MCPG), (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (LCCG-1), were obtained from Tocris. Forskolin, dideoxyforskolin, 3-isobutyl-1-methylxanthine (IBMX), H-89, and 8-bromoadenosine-3',5'-cyclic monophosphothioate (Rp-Isomer, Rp-8-Br-CAMPs) were purchased from Calbiochem.

## RESULTS

### Long-lasting inhibition of mossy fiber-CA3 eEPSCs by presynaptic KAR activation

We examined whether KA was able to depress NMDA receptor-mediated synaptic transmission at MF-CA3 synapses. We performed the experiments in the presence of the AMPA receptor antagonist SYM2206 (100  $\mu\text{M}$ ), which, at this concentration, completely blocks AMPA-mediated EPSCs (Fig. 1A). We added bicuculline (50  $\mu\text{M}$ ) and SCH50911 (50  $\mu\text{M}$ ) to isolate excitatory responses and glycine (10  $\mu\text{M}$ ) to allow for NMDA receptor activation. To be sure that our stimulating electrode was activating mostly MFs, we added the group II mGluR agonist L-CCG-1 to the bath. L-CCG-1 has been shown to block synaptic transmission at MF terminals, but not at commissural/associational connections (Kamiya et al. 1996). More than 90% of the evoked eEPSCs was blocked by 10–30  $\mu\text{M}$  L-CCG-1 ( $92.2 \pm 4.8\%$ ,  $n = 35$ ), indicating that most of the synaptic current that we were recording originated from MF activation. In agreement with previous reports (Contractor et al. 2000; Kamiya and Ozawa 2000; Schmitz et al. 2000), high KA concentrations (0.3–10  $\mu\text{M}$ ) produced a decrease of the mean eEPSC amplitude (Fig. 1, A–C). In several cells, a slight, transient, and short timed increase of the eEPSC was observed previously to the depression of the eEPSCs (data not shown). At 1  $\mu\text{M}$ , KA produced a decrease of  $63.2 \pm 3.7\%$  ( $n = 35$ ) of the NMDA-mediated eEPSC (Fig. 1, A and B). In parallel experiments, 0.3–10  $\mu\text{M}$  KA produced a similar degree of reduction of the AMPA receptor-mediated eEPSCs (recorded in the absence SYM2206, but presence of 50  $\mu\text{M}$  D-APV, bicuculline, and SCH50911, to block NMDA, GABA<sub>A</sub>, and GABA<sub>B</sub> receptors, respectively; Fig. 1B). This similar degree of depression of both NMDA and AMPA receptor-mediated EPSCs indicates that kainate most probably reduced the release of glutamate. The  $63.2 \pm 3.7\%$  decrease of NMDA-mediated eEPSC amplitude ( $n = 35$ ) produced by 1  $\mu\text{M}$  KA was almost completely antagonized by the nonselective AMPA/KA receptor antagonist CNQX (100  $\mu\text{M}$ ; +CNQX,  $12.1 \pm 1\%$  decrease; Fig. 1D). These data indicate that the effect is mediated by the activation of KARs.

In the foregoing experiments, KA produced a change in holding current of  $27.6 \pm 4.8$  pA in 13 out 35 neurons recorded; this change in holding current lasted only 6 min (Fig. 1C). Notably, however, the KA-mediated depression of the eEPSCs outlasted the change in holding (H) current because of the activation of KAR on the postsynaptic membrane (Fig. 1C). We recorded cells until the complete recovery of the eEPSC amplitude after KA effect and found that KA-mediated depression of eEPSCs was  $63.2 \pm 3.7\%$  in the peak depression (at 6 min after KA application);  $41 \pm 2$ , 25 min after KA application;  $17.3 \pm 2.3\%$ , 45 min after KA application; and recovered

<sup>1</sup> The online version of this article contains supplemental data.

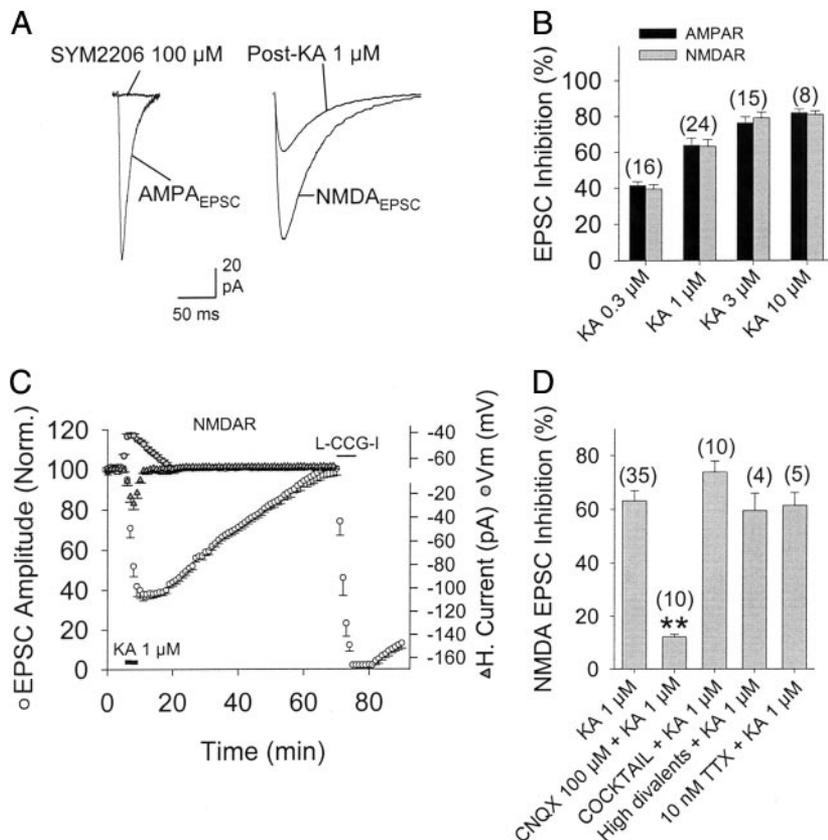


FIG. 1. Long-lasting inhibition of mossy fiber-CA3 evoked excitatory postsynaptic currents (eEPSCs) by kainate receptor (KAR) activation. *A*: effect of 1  $\mu\text{M}$  kainate (KA) on *N*-methyl-D-aspartate (NMDA) receptor-mediated eEPSCs. Responses are shown before and 4 min after KA perfusion; 100  $\mu\text{M}$  SYM2206 completely blocks AMPA EPSCs. *B*: concentration dependency of KA effects on NMDA (in the presence of 100  $\mu\text{M}$  SYM2206) and AMPA eEPSC amplitude. Note that KA produced a similar decrease in NMDA and AMPA-mediated eEPSCs. *C*: inhibition of MF eEPSCs (open circles) induced by KA application (1  $\mu\text{M}$ ) outlasted the variation in holding current (H current; triangles) or  $V_m$  (gray circles) induced at postsynaptic CA3 neurons. The group II mGluR agonist, L-CCG-I, which selectively blocks mossy fiber responses, suppresses eEPSCs. *D*: CNQX (100  $\mu\text{M}$ ) antagonized the effect of 1  $\mu\text{M}$  KA on the NMDA receptor-mediated component. In the presence of a cocktail containing metabotropic glutamate receptor and  $\text{GABA}_A^-$ ,  $\text{GABA}_B^-$ , opioid-, muscarinic-,  $\beta$ -adrenergic-, and adenosine-receptor antagonists, KA produced a similar decrease of the amplitude of eEPSCs. In the presence of high divalent cations, KA inhibition of eEPSC was not affected. The depression produced by KA was also present in the presence of 10 nM TTX. The number of experiments is indicated in brackets. Results are expressed as means  $\pm$  SE (\*\* $P < 0.01$ , Student's *t*-test).

completely 60 min after KA application, indicating that the KA effect last for  $\sim 1$  h. Confirming the different time-courses of the depolarizing effect of KA and the effect of this agonist (KA) on the eEPSCs, experiments in current-clamp mode showed that the KA effect on the eEPSCs clearly outlasted the changes in membrane potential ( $V_m$ ; Fig. 1C). To learn whether the KA concentrations used have some effect on the time-course of the recovery, we also studied the time-course of the action of 0.3  $\mu\text{M}$  KA. We found that that this KA concentration produced a decrease of mean eEPSC amplitude ( $39.3 \pm 2.1$ ,  $n = 16$ ) at 6 min, and the complete recovery was also long-lasting and complete at 45 min, thus indicating a slightly fast recovery than when using 1  $\mu\text{M}$  KA. However, similar to the first experiment, the effect of 0.3  $\mu\text{M}$  KA on the EPSCs clearly outlasted the holding current changes, which recovered completely in 6 min (data not shown).

Although the observed effects of KA on the EPSC produced MFs may be attributable to the direct activation of presynaptic KARs on terminals, the inhibition may alternatively involve an indirect pathway such that activation of somatodendritic KARs release one or more neuromodulators that alter release from the terminal. To address the latter possibility, we examined the sensitivity of the KA-induced depression to antagonists for a number of different neuromodulatory receptors known to affect synaptic transmission. We found that after treating slices with a cocktail containing the mGluRs antagonists MCPG/MPPG (2.0 mM each), naloxone (100  $\mu\text{M}$ ), bicuculline (50  $\mu\text{M}$ ), SCH 50911 (50  $\mu\text{M}$ ), atropine sulfate (50  $\mu\text{M}$ ), propranolol (100  $\mu\text{M}$ ), and DPCPX (0.1  $\mu\text{M}$ ) to block metabotropic glutamate-, opioid-,  $\text{GABA}_A^-$ ,  $\text{GABA}_B^-$ , muscarinic-,  $\beta$ -adrenergic-, and

adenosine  $1_A^-$  receptors, respectively, KA still inhibited the eEPSCs effectively (Fig. 1D).

It remains possible that KA causes release of a neuromodulator not blocked by the cocktail. Hippocampal interneurons, in particular, release a wide variety of neuromodulatory substances on activation and are also known to possess somatodendritic KARs (Rodríguez-Moreno et al. 2000), which indirectly affect excitatory transmission (Schmitz et al. 2000). To eliminate the possibility that the observed KA effect was mediated by interneuron activation, we performed additional experiments using high divalent cations in the extracellular solution (8 mM  $\text{Ca}^{2+}$  and 17 mM  $\text{Mg}^{2+}$ ). These conditions are known to prevent the KAR activation of interneuron discharge, without altering release probability (Frerking et al. 2001). Although, the KA (1  $\mu\text{M}$ ) induction of interneuron discharge [770  $\pm$  185%,  $n = 5$ , increase of the spontaneous inhibitory postsynaptic currents (sIPSCs)] in normal extracellular solution was prevented in the presence of high divalent concentrations ( $99 \pm 16\%$ ,  $n = 4$ ), this treatment had no effect on the KA-mediated depression of the eEPSCs ( $59.6 \pm 6.3\%$  of decrease of eEPSC;  $n = 4$ ; Fig. 1D). Finally, to more assiduously attenuate the excitability of the entire network, we performed experiments in the presence of 10 nM TTX (Frerking et al. 1998). In this situation, KA (1  $\mu\text{M}$ ) still depresses excitatory synaptic transmission (Fig. 1D). Together, these results suggest that the depression of MF-CA3 eEPSC by KA administration is unlikely to be mediated indirectly through the release of any of the widely reported neuromodulators in the hippocampus and is indeed independent of interneuron activity. The implication from this is that a direct action of KA on

the presynaptic terminal underlies the inhibitory effect of KAR activation on glutamate release.

To confirm that the observed effects of KA on the NMDA and AMPA eEPSCs were indeed presynaptic, we used several analyses to delineate presynaptic regulation. First, we examined the change in the CV of synaptic responses versus the change in their averaged (mean) amplitude (M). Consistent with a presynaptic effect of KA, the decrease in the mean eEPSC (NMDA and AMPA receptor-mediated) amplitude was paralleled by a decrease in  $1/CV^2$ , a parameter known to vary as a function of quantal size (Thomson and Deuchars 1995; Fig. 2A). Second, we used paired stimuli to MFs to facilitate the amplitude of the second evoked EPSCs (Fig. 2B) and determined whether this PPF was altered by KA. The PPR for our recordings (measured with an interval of 40 ms) was  $2.6 \pm 0.3$  ( $n = 10$ ) for NMDA-mediated EPSCs. During KA application, the PPR significantly increased to  $4.3 \pm 0.7$  ( $n = 10$ ; Fig. 2,  $B_1$  and  $B_2$ ), suggesting that the release probability at the

MF synapse had decreased (Manabe et al. 1993). Similar results were obtained for AMPA-mediated EPSCs (Fig. 2B<sub>2</sub>). Third, we determined synaptic failure rates in the cells recorded. The synaptic failure of the NMDA-mediated EPSCs was  $5 \pm 1\%$  ( $n = 9$ ) under control conditions. When KA was present, the proportion of these failures was clearly increased (to  $45 \pm 7\%$ ,  $n = 9$ ; Fig. 2C). Similar results were obtained for the AMPA-mediated EPSCs (Fig. 2B). These data indicate that a change in the synaptic reliability may be the cause of the EPSC reduction obtained with KAR activation.

#### KAR inhibition of glutamate release involves a PTX-sensitive G protein

From the foregoing results, it is clear that application of KA depresses the release of glutamate at MF terminals, a phenomenon that reverses slowly on KA washout. This contrasts with the depolarizing action of KA (reflected in the  $V_m$  and H current), which reverses rapidly (Fig. 1C). These results are clearly incompatible with the simple idea that effects contingent on the depolarization of MF nerve terminals—through conduction block or inactivation of  $Ca^{2+}$  channels (Kamiya and Ozawa 2000)—are solely responsible for the reduction in glutamate release seen here. Indeed, rather than a classical ionotropic action of KA mechanism, our data suggest a metabotropic action as previously proposed (Rodríguez-Moreno and Lerma 1998; Rodríguez-Moreno et al. 2000).

To test this, we examined the effects of the specific G protein inhibitor, PTX, on the KA-induced depression of the NMDA-mediated eEPSCs (Fig. 3). In slices treated with PTX ( $5 \mu\text{g/ml}$ , 6–8 h, at  $37^\circ\text{C}$ ), the eEPSCs showed similar amplitude and shape to the eEPSCs obtained in untreated slices (also incubated for 6–8 h, at  $37^\circ\text{C}$ ; Fig. 3A<sub>1</sub> cf. Fig. 3A<sub>2</sub> minus PTX), but PTX prevented the inhibitory effect of KA. Figure 3B shows the time-course of KA action in untreated and PTX-treated slices. As we stated before, the changes in holding current can be used to monitor ionotropic KAR activity. On studying the changes in holding current in slices treated with PTX, we observed that whereas KA-mediated effect on eEPSC amplitude was prevented, the agonist continued to produce a change in holding current similar that observed in PTX-untreated slices ( $27 \pm 7.2 \text{ pA}$ ,  $n = 6$  in untreated slices vs.  $29 \pm 6 \text{ pA}$ ,  $n = 8$  in PTX-treated slices). These data indicate that PTX blocks the G protein-mediated metabotropic effect of KA, but not the ionotropic stimulation caused by KA receptor activation (Fig. 3B). Overall, in slices treated with PTX, the inhibitory effect of KA ( $1 \mu\text{M}$ ) on NMDA eEPSCs was entirely abolished (control,  $67.0 \pm 8.1\%$ ,  $n = 6$  vs. +PTX,  $11.2 \pm 6.2\%$ ,  $n = 8$ ; Fig. 3C). These data indicate that activation of a PTX-sensitive G protein is required for the KA-induced depression of glutamate release.

#### KAR inhibition of glutamate release involves a cAMP-dependent cascade

KA-induced G protein activation could lead to a depression of synaptic transmission by a direct interaction with calcium channels (i.e., acting through a pathway intrinsic to the membrane; De Waard et al. 1997) or, alternatively, through a soluble messenger, as we have proposed for GABA release modulation (Rodríguez-Moreno and Lerma 1998; Rodríguez-

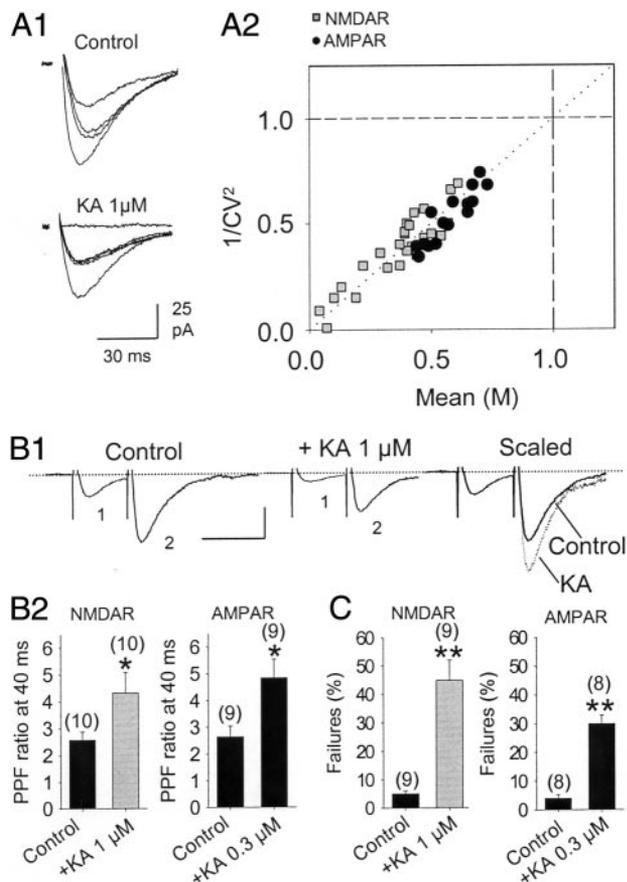


FIG. 2. The KA-mediated depression of eEPSCs at mossy fiber-CA3 synapse involves a presynaptic mechanism.  $A_1$  and  $A_2$ : effect of KA on the trial-to-trial fluctuation of EPSCs. Mean (M) eEPSC amplitudes and their CV were measured during KA application and normalized by the respective control values for each cell. The fractional variation in  $1/CV^2$  is plotted against the mean (M) amplitude for all neurons. Experimental data follow the predicted relation for a purely presynaptic (diagonal line) rather than postsynaptic (horizontal line) site of action. For AMPA-mediated currents, data were plotted for the effect of  $0.3 \mu\text{M}$  KA, a concentration at which the agonist activates KARs preferentially over AMPA receptors.  $B_1$ : representative traces showing paired-pulse (1,2) facilitation at 40-ms interval. KA ( $1 \mu\text{M}$ ) perfusion produces an increase of the paired-pulse facilitation. Calibration: x-axis, 40 ms; y-axis, 60 pA.  $B_2$ : quantified paired-pulse ratios (PPRs) for NMDA and AMPA-mediated currents.  $C$ : effect of KA on the number of failures for NMDA- (left) and AMPA- (right) mediated currents.

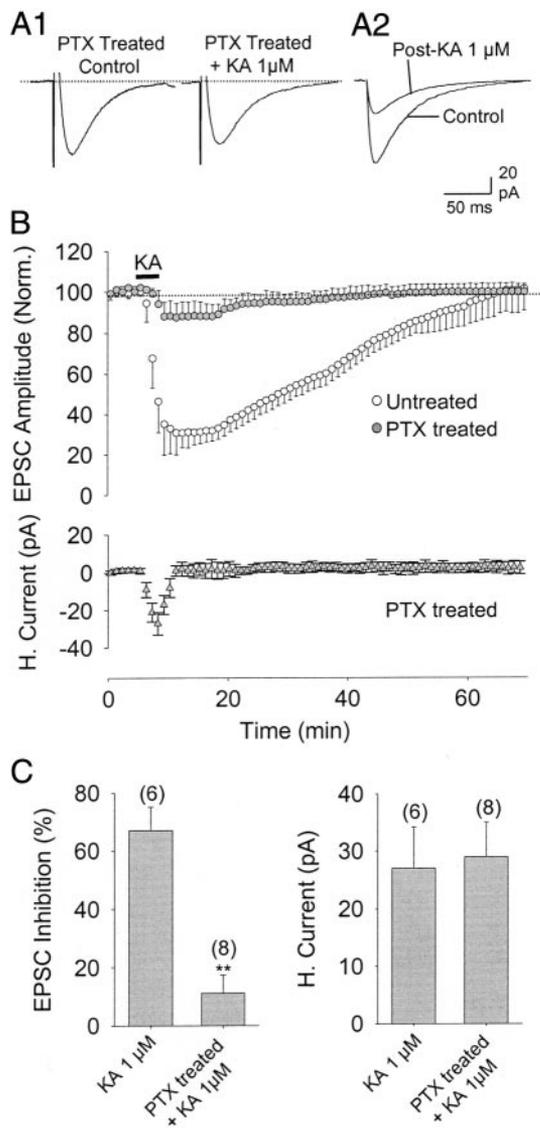


FIG. 3. KA-induced inhibition of glutamate release involves a pertussis toxin-sensitive G protein. *A*<sub>1</sub>: representative experiments showing NMDA-mediated eEPSCs recorded from CA3 pyramidal neurons in slices treated with pertussis toxin (PTX, 5 μg/ml, 6–8 h, 37°C). KA (1 μM) did not produce any eEPSC inhibition in the presence of this G protein inhibitor (cf. with the effect of KA observed in untreated slices, *A*<sub>2</sub>, control). *B*: time-course of the eEPSC amplitude before, during, and after bath application of KA in untreated (open circles) and in PTX-treated (filled circles) slices. Effect of KA on holding current in PTX-treated slices, Note that KA continues to produce a change in the holding current as observed in untreated slices. *C*: effects of 1 μM KA on eEPSC amplitude and holding current in normal slices and slices treated with PTX. PTX prevented the inhibitory action of KA (1 μM) on glutamate release but not the change in holding current. Holding current is expressed as an absolute value. Bars represent means ± SE (\*\**P* < 0.01, Student's *t*-test).

Moreno et al. 2000). The inhibition of GABA release in the CA1 region by KA has been shown to involve the activation of a PTX-sensitive G protein and protein kinase C (PKC) (Rodríguez-Moreno and Lerma 1998). To test for a similar mechanism at these synapses, we determined the effects of KA on eEPSCs after incubation (2–4 h) with calphostin C, a highly specific inhibitor of PKC. However, this treatment resulted in no change in the activity of KA (50.2 ± 10.3, *n* = 8; Fig. 4*B*). To be sure that calphostin C treatment was working in these experiments, we recorded in interleaved slices the IPSC in

CA1 pyramidal neurons evoked by stimulating the stratum oriens in the presence of AMPA and NMDA receptors blockers (100 μM SYM2206 and 50 μM APV). As reported previously (Rodríguez-Moreno and Lerma 1998; Rodríguez-Moreno et al. 2000), KA 3 μM depressed the IPSC amplitude (61 ± 3.6%, *n* = 5) in untreated slices, but this KA-induced depression of GABA release was significantly reduced in calphostin C-treated slices (6.4 ± 4.3%, *n* = 5). We therefore concluded that modulation of glutamate release by KA receptors involves a different mechanism than that described for depression of GABA release. The cAMP cascade is one of the major second messenger systems regulating glutamate release at MF terminals (Huang et al. 1996; Weisskopf et al. 1994). Also, our recent observations that KAR-mediated facilitation of glutamate release at MF terminals is contingent on PKA activation (Rodríguez-Moreno and Sihra 2004) led us to test whether the cAMP/PKA transduction system was involved in the depression of MF-CA3 synapse effected by KAR activation.

First, we prevented activation of cAMP-dependent PKA by treating slices with either Rp-Br-cAMP, a competitive inhibitor of cAMP-mediated activation of the regulatory subunit of PKA, or H-89, which directly inhibits the catalytic subunit of the kinase. As shown in Fig. 4, these inhibitors prevented the action of KA at MF-CA3 synapses. Rp-Br-cAMP produced a small decrease of the eEPSC amplitude (16 ± 6.6%, *n* = 4;

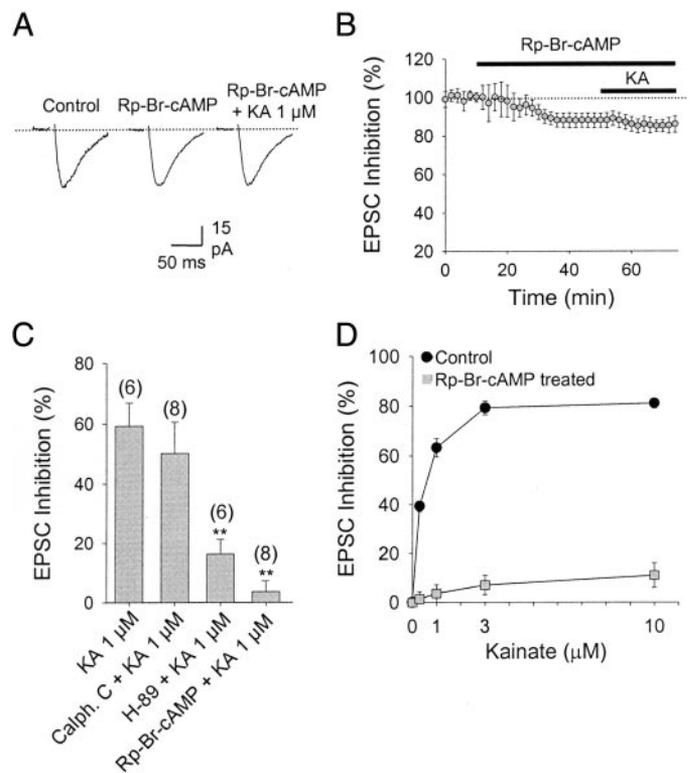


FIG. 4. Inhibition of protein kinase A (PKA) prevents the inhibitory effect of KA. *A*: a representative experiment showing that KA (1 μM) does not affect the amplitude of NMDA-mediated eEPSCs in Rp-Br-cAMP treated slices. *B*: time-course of the effect of Rp-Br-cAMP on eEPSC amplitude. Subsequent application KA have insignificant or not effect on eEPSCs amplitude. *C*: inhibition of PKA by H-89 (10 μM) and Rp-Br-cAMP (100 μM) prevented the action of KA (\*\**P* < 0.01). The inhibition of PKC by calphostin C had no effect. *D*: effect of Rp-Br-cAMP across the full range of KA concentrations that produced inhibition. In slices treated with Rp-Br-cAMP, KA did not produce any decrease of the NMDA-mediated eEPSC amplitude with any of the KA concentrations used.

Fig. 4, *A* and *B*), but did not affect the shape of the responses. In Rp-Br-cAMP-treated slices, KA ( $1 \mu\text{M}$ ) produced a decrease of the mean NMDA-mediated eEPSCs of  $3.6 \pm 3.6\%$  ( $n = 8$ ; Fig. 4, *A-C*), indicating a complete abolition of the effect of KA on eEPSCs. In H-89 ( $10 \mu\text{M}$ )-treated slices, KA ( $1 \mu\text{M}$ ) produced a decrease of the eEPSC amplitude of  $16.3 \pm 4.9\%$  ( $n = 6$ ; Fig. 4*C*). These inhibitory effects persisted across the full range of KA concentrations used to produce inhibition (Fig. 4*D*).

Second, if a decrease of cAMP levels is necessary for KAR-mediated decrease of glutamate release, preventing this decrease should block this effect. We treated slices with the membrane-permeable cAMP analog Sp-8-CPT-cAMPs ( $100 \mu\text{M}$ ) that should "clamp" PKA activity at a high constant level. We performed experiments by applying Sp-8-CPT-cAMPs in the perfusion. In this situation, the cyclic nucleotide analog produced a clear increase of the eEPSC amplitude ( $218 \pm 46\%$ ,  $n = 3$ , data not shown). We also performed the experiment in slices incubated with the cAMP analog for 2 h. In both situations, KA produced a similar and small and statistically insignificant decreases of eEPSC mean amplitude, and data were pooled together ( $5.3 \pm 3.7\%$ ,  $n = 6$ ; Fig. 5, *A* and *B*).

To further test the hypothesis that the PKA pathway mediates the action of KA on glutamate release at the MF-CA3 synapse, we increased PKA activity by enhancing cAMP production through AC activation using forskolin. Forskolin was applied to the bath while measuring the synaptic transmis-

sion (Fig. 5*A*). Forskolin ( $30 \mu\text{M}$ ) + IBMX ( $5 \mu\text{M}$ ) caused a  $350 \pm 33\%$  increase of NMDA-mediated eEPSCs ( $n = 6$ ), an effect dependent on the increase in cAMP and the subsequent activation of PKA (Weisskopf et al. 1994). In forskolin-stimulated slices, the action of KA ( $1 \mu\text{M}$ ) was enhanced, producing a decrease of  $85 \pm 4\%$  ( $n = 14$ ) on the NMDA eEPSCs amplitude versus  $63.3 \pm 3.7\%$  ( $n = 35$ ) in slices not treated with forskolin + IBMX (Fig. 5, *A* and *B*). With lower concentrations of KA ( $0.3 \mu\text{M}$ ), the effect was more apparent. KA ( $0.3 \mu\text{M}$ ) produced a decrease of  $76 \pm 7\%$  ( $n = 6$ ) of the eEPSC amplitude versus  $39.4 \pm 2.1\%$  ( $n = 16$ ) in untreated slices. Forskolin-induced potentiation of synaptic transmission in CA3 is long-lasting (Tong et al. 1996; Weisskopf et al. 1994). We also performed experiments where KA was applied after slice incubation with forskolin for  $>1$  h, and similar results were obtained. Notably, the effect of forskolin application was specific to PKA activation, because the application of 1,9-dideoxyforskolin ( $30 \mu\text{M}$ ), an inactive isomer of forskolin, had no action on the effect of KA (Fig. 5*B*). Finally, in keeping with the involvement of an AC/PKA signaling cascade in the KA-mediated regulation reported herein, other paradigms to stimulate AC resulted in a similar potentiation of the KA action. For instance, KA ( $1 \mu\text{M}$ ) depressed the MF eEPSCs more potently after application of isoproterenol ( $1 \mu\text{M}$ ;  $83 \pm 8\%$  of inhibition,  $n = 4$ ), an agonist of  $\beta$ -adrenergic receptors known to activate AC (Huang and Kandel 1996).

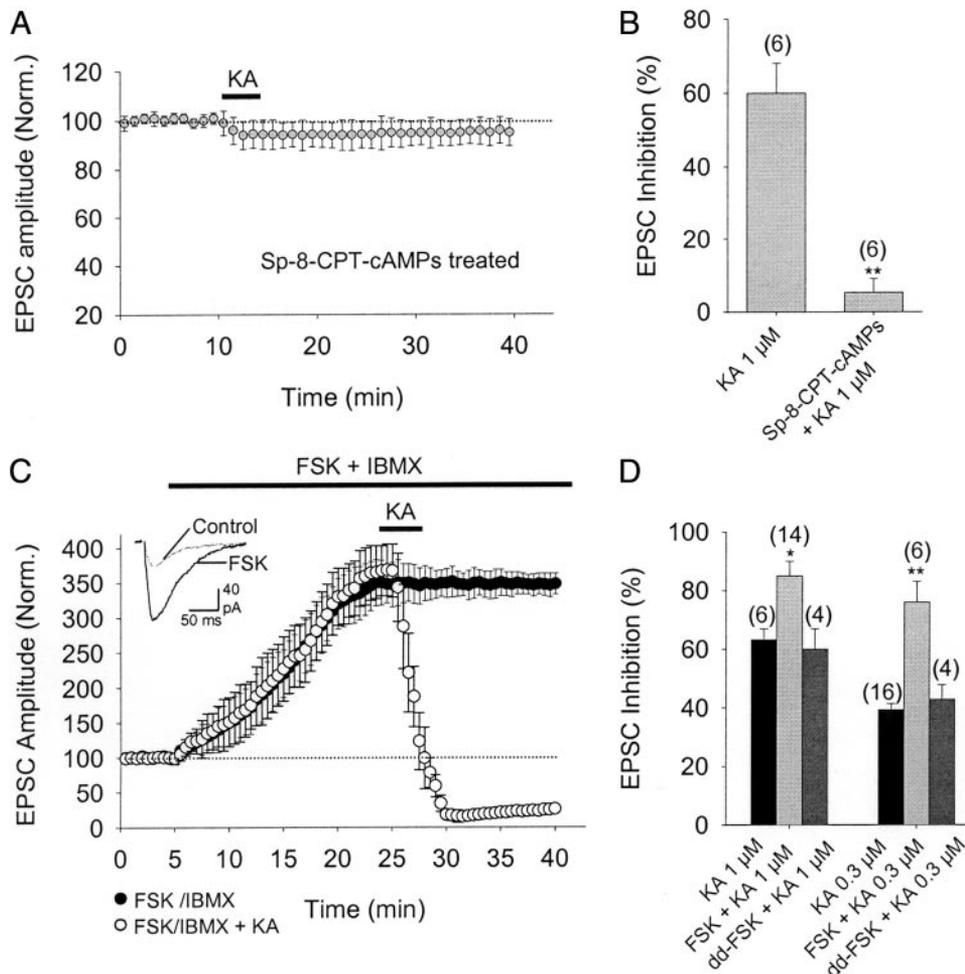


FIG. 5. Suppression of KA-mediated depression of eEPSC by elevation of intracellular cAMP levels. *A*: time-course of the effect of  $1 \mu\text{M}$  KA on eEPSC amplitude in slices treated with Sp-8-CPT-cAMP. Note that KA in this situation has insignificant or not effect on eEPSCs amplitude. *B*: quantification of the effect of KA in Sp-8-CPT-cAMP-treated slices. Number of experiments is indicated in brackets. Results are expressed as means  $\pm$  SE ( $*P < 0.05$ ;  $**P < 0.01$ , Student's *t*-test). *C*: forskolin ( $30 \mu\text{M}$ ) + IBMX ( $5 \mu\text{M}$ ) were added to the bath while measuring NMDA receptors-mediated synaptic transmission. Filled circles represent the summary of experiments ( $n = 6$ ) in which forskolin was applied continuously. After the effect of forskolin reached an approximate plateau, KA ( $1 \mu\text{M}$ ) was introduced in the recording chamber (open circles). *D*: summary of experiments in which KA ( $1$  or  $0.3 \mu\text{M}$ ) was applied to  $30 \mu\text{M}$  forskolin- or  $30 \mu\text{M}$  dideoxyforskolin (dd-FSK)-treated slices. Number of experiments is indicated in brackets. Results are expressed as means  $\pm$  SE ( $*P < 0.05$ ;  $**P < 0.01$ , Student's *t*-test).

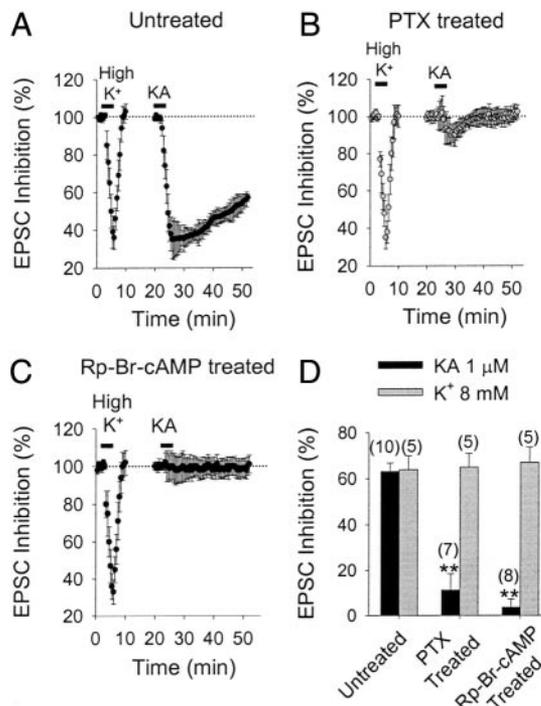


FIG. 6. KA-induced inhibition is not mediated by an effect on depolarization. *A*: high  $K^+$  concentration (8 mM) produces a decrease of the NMDA-mediated eEPSCs amplitude that recovers rapidly after  $K^+$  washout. In the same slice, KA 1  $\mu$ M produced a decrease of eEPSC amplitude similar to naïve slices. *B*: in PTX-treated slices,  $K^+$  (8 mM) had the same effect as that in untreated slices but KA failed to produce a decrease of NMDA-mediated eEPSCs amplitude. *C*: in Rp-Br-cAMP-treated slices,  $K^+$  (8 mM) produced a similar decrease of the NMDA-mediated eEPSCs as that in untreated slices, but the effect of KA was prevented. *D*: summary of results obtained for control untreated slices, PTX, and Rp-Br-cAMP-treated slices. Values in brackets indicate number of neurons (slices) recorded.  $**P < 0.01$ , Student's *t*-test.

Some authors (Schmitz et al. 2000) have invoked a role for presynaptic depolarization in the inhibitory mechanism of KA action, based on the observation that  $K^+$  ions also produce a decrease of eEPSCs. We therefore performed experiments comparing the nature of the effects of  $K^+$  on eEPSC amplitude to those produced by KA application (Fig. 6*A*). We found that while 8 mM  $K^+$  produced a decrease of the eEPSCs similar to KA (1  $\mu$ M), in slices that had previously been treated with Rp-Br-cAMP or PTX, the effects of  $K^+$  persisted (Fig. 6, *B–D*). This is in stark contrast to the abolition of the inhibitory effects of KA by the inclusion of PKA and G protein inhibitors. These observations are consistent with the view that the KA mediated inhibition reported herein differs from a depolarization-dependent inhibition of the eEPSCs and that the two processes are likely independent.

## DISCUSSION

Our results show that the activation of presynaptic KARs at mossy fibers produces a long-lasting inhibition of glutamate release. We also establish that the KAR-mediated effect is produced through  $G_{i/o}$  protein activation. Furthermore, we showed that manipulations that modify the cAMP-mediated signaling in mossy fiber terminals interfere with the inhibitory action of KA. These results imply the coupling of KARs to a second messenger cascade involving regulation of PKA. Although the role of the PKC pathway in the metabotropic

actions of KA are now well recognized (Cunha et al. 1999; Fisahn et al. 2005; Melyan et al. 2002, 2004; Rodríguez-Moreno and Lerma 1998; Rodríguez-Moreno et al. 2000; Rozas et al. 2003), Frerking et al. (2001) found that at the CA3–CA1 synapse, KARs have a metabotropic action depressing glutamate release that is not mediated by protein kinases. Rodríguez-Moreno and Sihra (2004) described the increase in glutamate release observed by low KA concentrations to be mediated by the activation of a AC(cAMP)PKA cascade but not through by the activation of a G protein by the agonist. Here we show a new mechanism for the action of KARs involving a G protein and AC(cAMP)PKA cascade, which is arguable novel in the CNS.

We used three different approaches at the outset to establish that the KAR-mediated inhibitory effect on eEPSCs was indeed presynaptic. First we examined the change in the CV of synaptic responses versus the change in their averaged (mean) amplitude (*M*). Consistent with a presynaptic effect of KA, the decrease in the mean eEPSC (NMDA and AMPA receptor-mediated) amplitude was paralleled by a decrease in  $1/CV^2$ , a parameter known to vary as a function of quantal size. Second, the PPR for our recordings during KA application was significantly increased, suggesting that the release probability at the MF synapse had decreased. Third, we determined synaptic failure rates in the cells recorded. When KA was present, the proportion of these failures was clearly increased. Altogether, three independent parameters to determine the precise locus for KA action all confirmed a presynaptic mode of action for KA at MF-CA3 synapses. The fact that the effects observed for NMDA- and AMPA-mediated currents (at KA concentration 0.3  $\mu$ M, which mostly activates KA receptors over AMPA receptors) were congruent also effectively suggests that the mode of action for KA observed herein is presynaptic, because a postsynaptic effect might not have been expected to display such equivalence between NMDA and AMPA EPSCs.

The next key question arising pertains to the mechanism of action of KARs in the present context. We characterized completely the recovery of eEPSCs amplitude after 1  $\mu$ M KA application and notably found that this recovery lasted for 1 h. Similar to the results obtained by Contractor et al. 2000 (they applied 3  $\mu$ M KA), we found a long-lasting effect of KA that outlasted the change in holding current. While other authors studied the effects of KA on eEPSC amplitude, in most cases, they do not (or only partially) studied the recovery of the responses (the majority just to see that they are going to recover but not the complete recovery). Different time-courses for the recoveries was observed (Contractor et al. 2000; Kamiya and Ozawa 2000; Schmitz et al. 2000). One possible explanation for differences might be the perfusion rates used; whereas some authors do not show this data, most authors use very similar perfusion rates in slices, our rate (2–3 ml) is a widely used perfusion rate. We think these differences may most probably be explained by the different KA concentration used in different studies, in fact, we observed that 0.3  $\mu$ M KA produced a decrease of the eEPSC amplitude that recovers completely in 45 min. Kamiya and Ozawa (2000) previously concluded that KAR activation enhanced the excitability of MFs, most probably by axonal depolarization. This depolarization seemed to cause a use-dependent decrease in action potential-induced  $Ca^{2+}$  influx, thereby reducing transmitter release. In our experiments, this type of mechanism is unlikely

to underlie the modulation seen, given that the KA-induced depression of the eEPSCs clearly outlasted the depolarizing action of KA observed as the change in postsynaptic holding current recorded on KA perfusion. Because the transient change in holding current reveals the time-course of the presence of KA in the bath in an accurate manner (this rapid recovery of membrane current toward resting values in fact served as monitor of our ability to washout KA from the experimental cell surroundings), our results rule out the depolarization of MF terminals as the only cause of reduction of transmitter release (Kamiya and Ozawa 2000). If the depression of transmitter release by KA was the exclusive result of the depolarizing action of the agonist, the effect on the eEPSCs would have been predicted to have recovered with a time-course similar to the voltage changes. This is patently not the case (Fig. 1C).

Notwithstanding, the aforementioned discussion, we considered the contention by other authors (Schmitz et al. 2000) who have invoked a role for presynaptic depolarization in the inhibitory mechanism of KA action, based on the observation that  $K^+$  ions also produce a decrease of eEPSCs. Having reproduced this inhibition with 8 mM  $K^+$ , similar to that produced by KA (1  $\mu$ M), in contrast to the KA effect, we found that the inhibitory effect produced by 8 mM  $K^+$  could not be regulated by treatments that attenuated  $G_{i/o}$  function [PTX or PKA activity (Rp-Br-cAMP)]. This contrast is consistent with the view that the KA-mediated inhibition reported herein differs from a depolarization-dependent inhibition of the eEPSCs and that the two paradigms are not directly comparable. This also suggests that, in the situations studied, KA does not substantially depolarize mossy fiber terminals and that, of the potential actions mediated by KAR present in the terminals, the metabotropic action prevails over the ionotropic one.

The inhibitory effects of KA reported here are similar to the metabotropic mechanism of action we have described previously (Rodríguez-Moreno and Lerma 1998; Rodríguez-Moreno et al. 2000). In these earlier studies, we attributed the inhibitory effects of KA on GABAergic neurons to a metabotropic mechanism involving the inhibitory G proteins  $G_{i/o}$ . Since then, several authors have ascribed various metabotropic roles to KA receptors. With respect to excitatory glutamatergic synaptic transmission, Frerking et al. (2001) have reported G protein involvement in KA-induced depression at the CA3–CA1 synapse. Accordingly, we aimed to establish the G protein dependency of the KA-induced depression in eEPSCs described here.

The sensitivity of the KAR-induced depression to PTX implicates the  $G_i/G_o$  subtype of G protein in the modulation. Previous studies have suggested a coupling between G protein and KARs in hippocampal membranes (Cunha et al. 1999). There is precedence for this in that several authors have indicated the possibility of ion channel forming receptors coupling to G protein-mediated signaling systems. Indeed, AMPA receptors suggested to couple to  $G_{i/o}$  proteins to regulate AC activity (Wang et al. 1997) and nonreceptor protein tyrosine kinases (Hayashi et al. 1999), and may also interact with stimulatory G proteins to regulate phosphodiesterase activity in the retina (Kawai and Sterling 1999). Despite the growing congruence between metabotropic actions of KA- and AMPA-type glutamate receptors, the exact mechanism as to how these receptors might activate G proteins remains unre-

solved. Given that there is little structural similarity of KARs (or AMPA receptors) to classical metabotropic receptors, the involvement of linking accessory proteins is tenable (Rodríguez-Moreno and Lerma 1998).

The long-lasting effect of KA on glutamate release we show evinces a metabotropic action of KAR that is mediated by cAMP as a soluble messenger given that modulation by the agonist was either attenuated by PKA inhibition (using Rp-Br-cAMP or H-89), occluded by manipulations that keep PKA activity constant (using Sp-8-CPT-cAMPs), or enhanced by AC activation (using forskolin or  $\beta$ -adrenergic agonist). Our interpretation of the latter result is that KA more readily inhibits glutamate release after the forskolin-induced increase in cAMP level. Indeed, a similar potentiation of eEPSC reduction has been already noted for L-CCG-1, a group II mGluR agonist that inhibits AC at MFs (Tzounopoulos et al. 1998). We therefore tender the possibility that MF KARs are negatively coupled to the production of cAMP by AC inhibition at MF terminals. This inhibition would translate as a lower level of PKA-mediated phosphorylation, known to lead to a depression of  $Ca^{2+}$  entry into MF boutons, thereby decreasing glutamate release from the terminals. Indeed, action potential-induced presynaptic  $Ca^{2+}$  influx is reduced by KA at MFs (Kamiya and Ozawa 2000). These results are reminiscent of what has been described for group II mGluRs (Pin and Duvoisin 1995; Tzounopoulos et al. 1998), which suppress AC activity through  $G_{i/o}$  protein coupling. However, because of the persistence of KA action in the presence of the inhibitor cocktail designed to block such receptors at this synapse, the effect of KA could not be attributed to an indirect effect through this or other class of presynaptic receptors supporting a similar signal transduction. Thus KA was equally effective after blocking all types of mGluRs, opioids, GABA<sub>A</sub>, GABA<sub>B</sub>, muscarinic,  $\beta$ -adrenergic, and adenosine 1<sub>A</sub> receptors. Likewise, suppression of interneuron activity (that might lead to neuromodulatory crosstalk) by the use of high divalent cation concentration or TTX had no effect on the KA-mediated suppression of synaptic transmission at MF-CA3 terminals. It will be interesting to determine in a future work the exact physiological relevance of this action of KA through the cAMP/PKA signaling pathway, because endogenous glutamate has been shown to gain access to and activate these presynaptic autoreceptors (Schmitz et al. 2000).

In conclusion, our results show that the activation of presynaptic KARs at MF terminals results in a long-lasting inhibition of glutamate release. Furthermore, this action of KARs seems to be mediated by the activation of a PTX-sensitive G protein, with paradigms that modify cAMP-mediated signaling in MF terminals, interfering with the action of KA. These findings together suggest the coupling of KARs receptors to a second messenger cascade involving the regulation of PKA activity and extend the metabotropic actions of KA receptors in the brain to include the AC(cAMP)/PKA signaling cascade.

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