# Kainate receptor-mediated glutamate release facilitation of glutamate release at mossy fiber-CA3 synapses of the hippocampus involves $Ca^{2+}$-calmodulin and a high calcium threshold.

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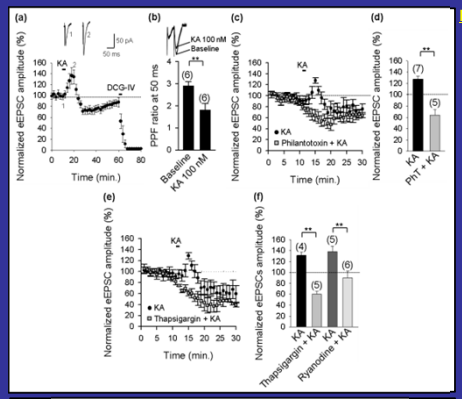
## INTRODUCTION

Glutamate receptors of the kainate-type participate postsynaptically as mediators of synaptic transmission and presynaptically modulate neurotransmitter release. At hippocampal MF–CA3 glutamatergic synapses, KA has a biphasic effect on glutamate release, with low KA concentrations (50–100 nM) producing an increase in glutamate release. In addition, glutamate release from hippocampal isolated nerve terminals (synaptosomes) is facilitated by the application of KA. This facilitation of glutamate release at MF–CA3 synapses or from synaptosomes is seen to involve an AC/ cAMP/PKA cascade. Here, we have resolved to determine the necessity for cytosolic $Ca^{2+}$ in the KAR-mediated facilitation of synaptic glutamate release and thereby elucidate how the AC might be activated after KAR activation in the absence of a canonical G-protein-mediated signal transduction. We confirm that the facilitation of glutamate release mediated by KAR activation at MF–CA3 synapse requires an increase of cytosolic $Ca^{2+}$. This increase is subserved by both extracellular $Ca^{2+}$ influx and intracellular $Ca^{2+}$ store mobilization. Crucially, we find that, mechanistically, this increase in $Ca^{2+}$ levels in the cytosol operates through the formation of $Ca^{2+}$–calmodulin complexes to activate AC. Correspondingly, with the $Ca^{2+}$–calmodulin dependence of KAR-mediated increase in glutamate release, we have also found that the induction of LTP at MF–CA3 synapse requires the activation of $Ca^{2+}$–calmodulin.

## METHODS

*Animals* The experiments were performed on adult (2-3 months old) C57Bl/6 male mice obtained from Harlan Laboratories (Spain).
*Electrophysiological recordings* Whole-cell patch-clamp recordings were made from cells located in the CA3 field of the hippocampus.. Perfusion solution contained D-AP5 (50 µM) to block NMDA receptors, and bicuculline (10-20 µM) to block $GABA_A$ receptors. To evoke eEPSCs, electrical pulses were delivered to mossy fibers. Neurons were voltage clamped, using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA, USA*Preparation of synaptosomes* Synaptosomes were prepared from the cerebral hippocampi of 2 month-old male mice. Synaptosomal pellets were stored on ice and used within 1-2 hours. *Glutamate release assay* Glutamate release was assayed by on-line fluorometry Release values quoted in the text are levels attained at "steady-state" after 4 min of depolarization (nmol/mg protein/4min).



## RESULTS

**Fig. 1. Facilitation of glutamate release mediated by presynaptic KAR activation at MF–CA3 synapses requires an increase of calcium in the cytosol.** (a) Time course of KA (100 nM) effect on a-amino-3-hydroxy- 5-methylisoxazole-4-propionate-mediated evoked excitatory postsynaptic currents (eEPSCs) amplitude. Inset show traces before and after 4-min KA perfusion. DCG-IV completely inhibited glutamate release (b) KA (100 nM) perfusion produces a decrease of the paired-pulse facilitation, inset shows scaled representative traces, calibration: x-axis, 50 ms; y-axis, 50 pA. (c) Time course of KA (100 nM) effect on eEPSCs amplitude in control condition (circles) and in the slices treated with philanthotoxin (PhTx, squares). (d) Quantification of data from (c). (e) Time course of the effect of KA on eEPSC amplitude in control slices (circles) and in thapsigargin-treated slices (squares). (f) In slices treated with thapsigargin or ryanodine, the increase of eEPSCs amplitude induced by KA is prevented. The number of experiments is indicated in parenthesis at the top of each bar. Results are expressed as means ± SE (**p < 0.01, Student's t-test).

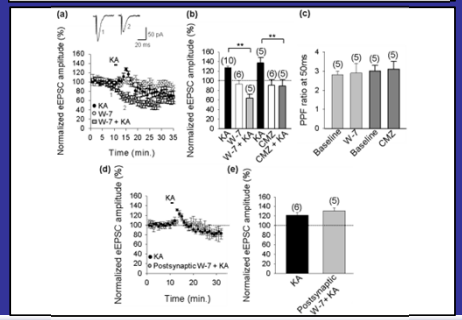


**Fig. 2. Facilitation of glutamate release mediated by presynaptic KAR activation requires $Ca^{2+}$-calmodulin at MF–CA3 synapses.** (a) Time course of KA (100 nM) effect on evoked excitatory postsynaptic currents (eEPSCs) amplitude in control condition (circles) and in the slices treated with 25 µM W-7 (squares). W-7 perfusion of slices does not affect basal synaptic transmission. Inset show traces before and after 4-min KA perfusion of W-7-treated slices. (b) KA effect is prevented in the presence of calmodulin antagonists (W-7 and CMZ). (c) The number of experiments is indicated in parenthesis at the top of each bar. Results are expressed as means ± SE (**p < 0.01, Student's t-test). (c) The treatment of slices with W-7 or CMZ does not affect paired-pulse ratio. (d) Time course of KA (100 nM) effect on eEPSCs amplitude in control conditions (black circles) and in cells treated with postsynaptic W-7 (gray circles). (e) Quantification of data from (d). The number of experiments is indicated in parenthesis at the top of each bar. Results are expressed as means ± SE (**p < 0.01, Student's t-test).

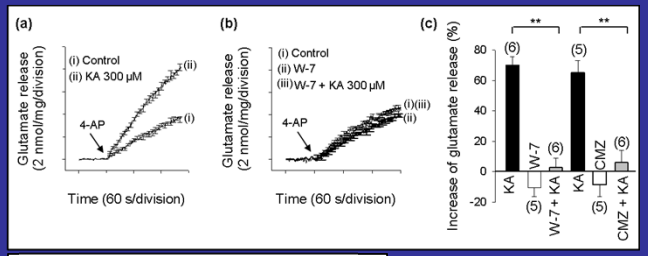


**Fig. 3 Facilitation of 4-AP-evoked glutamate release mediated by KAR activation requires $Ca^{2+}$/calmodulin in hippocampal synaptosomes.** (a) Glutamate release in the absence (i) and presence (ii) of 300 µM KA (added 1 min before the addition of 4-AP) in the presence of 100 µM of GYKI53655. (b) Effect of KA on glutamate release in control conditions (i) or following addition of W-7 (ii) or W-7 + 300 µM KA (iii). (c) Quantification of modulation using release levels achieved 4 min post 4-AP.

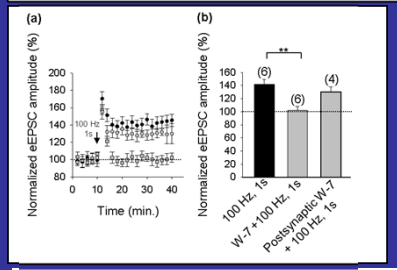


**Fig. 4 Long-term potentiation (LTP) induction requires $Ca^{2+}$-calmodulin at MF-CA3 synapses**. (a) LTP is prevented in W-7-treated slices. Graph shows time course of LTP in control slices (black circles) and in slices treated with W-7 (squares). The inclusion of W-7 in the postsynaptic cell does not prevent LTP induction (gray circles) (b) Quantification of data from (a). The number of experiments is indicated in parenthesis at the top of each bar. Results are expressed as means ± SE (**p < 0.01, Student's t-test).

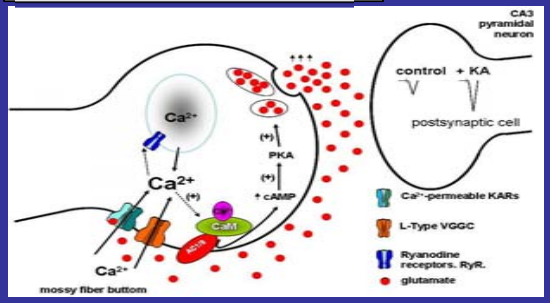


**Fig. 5. Proposed mechanism for KAR-mediated glutamate release facilitation.** Activation of presynaptic $Ca^{2+}$-permeable KARs promotes a cytosolic calcium increase. This increase is subserved by both extracellular $Ca^{2+}$ influx (through $Ca^{2+}$-permeable KARs and L-type voltage gated calcium channels) and intracellular $Ca^{2+}$ store mobilization triggered by $Ca^{2+}$-dependent $Ca^{2+}$ release mechanism. Cytosolic calcium activates AC via $Ca^{2+}$-calmodulin complex formation to produce an increase in cAMP and consequently the activation of PKA which finally leads to glutamate release facilitation. AC: Adenylate cyclase, CaM: $Ca^{2+}$-calmodulin complex , cAMP: cyclic Adenosine Monophospate, PKA: Protein Kinase A, VGGC: Voltage Gated Calcium Channels.

## CONCLUSION

The activation of presynaptic KARs by 100 nM KA at hippocampal MF–CA3 synapses results in the facilitation of glutamate release by a mechanism that involves an entry of $Ca^{2+}$ through permeable KARs and the triggering of release of $Ca^{2+}$ from internal stores in MF terminals. The $Ca^{2+}$ binds to calmodulin to form a $Ca^{2+}$–calmodulin complex, which then likely activates AC1 or AC8 to produce an increase in cAMP levels and an activation of PKA, thereby mediating the increase of glutamate release. Our results showing that LTP induction at these synapses requires $Ca^{2+}$–calmodulin, highlight the mechanistic congruence and possible interdependence of KAR-mediated facilitation of glutamate release and the induction of LTP.