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## **Failure to reproduce reversal of hippocampal LTP by the PKC-inhibitor, ZIP**

### **Abstract:**

At the cellular level, some patterns of excitation of neurons have been found to induce long-term increases in synaptic strength between neurons, through a process termed long-term potentiation (LTP). While the mechanism of LTP induction is well understood, the mechanisms that lead to the maintenance of synaptic strengthening are not as well understood. PKM $\zeta$  is an isoform of the PKC superfamily and is claimed to be necessary for late-phase LTP maintenance. The main evidence for the role of PKM $\zeta$  was inferred from reports that LTP can be reduced by the myristoylated  $\zeta$ -pseudosubstrate peptide (ZIP), claimed to inhibit PKC $\zeta$  specifically. Our objectives in this study were to produce stable LTP recordings for up to three hours after induction in an open chamber. This allowed us to test whether we could reproduce ZIP-induced LTP reversal. Measurements were made in the rat CA1 region of the hippocampus, as measured by field recordings. Our experiments have shown that we have successfully induced late phase long-term potentiation in the stratum radiatum of the CA1 region in the hippocampus. However, we were not been able to produce LTP reversal by adding ZIP one hour after LTP induction. We can therefore conclude that further research should focus on ZIP specificity and the reproducibility of ZIP induced LTP reversal.

Goldring 1988).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine protein kinase consisting of 12 identical subunits, each of which can be activated by the association of Ca<sup>2+</sup>-bound calmodulin. Phosphorylation at site T286 by an active subunit makes the phosphorylated subunit activity independent of Ca<sup>2+</sup>/calmodulin binding and therefore its activity can outlast a transient intracellular Ca<sup>2+</sup>/calmodulin signal. This property enables CaMKII to play an important role in the induction and expression of LTP (Lisman and Zhabotinsky 2001). CaMKII interacts with several ion channels including the NR2B subunit of NMDA receptors and voltage-sensitive calcium channels (Merrill *et al.* 2005; Hudmon *et al.* 2005). Furthermore, it has been suggested that CaMKII activation is important for the synapse specificity of LTP as CaMKII activation was found to be mainly restricted to stimulated spines when observing single dendritic spines during LTP (Lee *et al.* 2009). The importance of CaMKII in LTP induction is based on the evidence that it remains persistently activated after LTP induction; that when added postsynaptically it enhances synaptic transmission; and that genetic or pharmacological inhibition of CaMKII, blocks persistent activation of CaMKII, LTP and *in vivo* learning (Silva *et al.* 1992; Giese *et al.* 1998; Soderling, 2000; and Hudmon and Schulman 2002).

The induction of LTP accounts for the actual strengthening of synaptic transmission and is a strong model for the underlying neurobiology of memory formation. However, to be a strong model for subsequent memory consolidation, which can last hours, days, or even years, this mechanism needs to be maintained for long

catalytic domain thereby competitively inhibiting the binding of substrates to PKC $\zeta$ . In the presence of Ca<sup>2+</sup>, a protease cleaves the regulatory domain to form PKM a permanently turned on and soluble peptide that only contains the catalytic site of PKC $\zeta$  (Serrano *et al* 2005; Vlachos *et al* 2008; Yao *et al.* 2008; Gould and Newton 2008).

It was found that a splice variant PKC $\zeta$  coded for PKM $\zeta$ , exclusively. PKM $\zeta$  is similar to PKM in that is composed exclusively of the catalytic domain of PKC $\zeta$  (Sajikumar *et al.* 2005). Later it was proposed that while the formation of PKM from PKC $\zeta$  is a transient, activity dependent mechanism, the switch from PKM to PKM $\zeta$  was sustained via a positive feedback mechanism, in which PKM induces the self-sustaining transcription of PKM $\zeta$ . This theory is attractive in that, previous literature has shown experimentally that when protein synthesis is inhibited *in vivo*, memories cannot be consolidated (Shema *et al.* 2007; Sacktor 2008).

Ling *et al.* (2002), reported whole-cell recordings of excitatory postsynaptic currents (EPSCs) obtained from CA1 pyramidal cells, showed that diffusion of PKM $\zeta$  postsynaptically enhances EPSC amplitudes. To show that PKM $\zeta$  and LTP enhance synaptic transmission through the same mechanism, Ling *et al.* reported the occlusion of LTP by PKM $\zeta$  diffusion-induced potentiation, suggesting that LTP and PKM $\zeta$  enhance transmission through the same mechanism. Furthermore, the introduction of a dominant negative inhibitory form of PKM $\zeta$  (PKM $\zeta$ -K281W), while not changing baseline synaptic transmission or AMPAR and NMDAR currents, blocked LTP induction. The strongest evidence suggesting PKM $\zeta$  is involved in the maintenance of LTP comes from experiments that report PKM $\zeta$  inhibition reverses LTP (Pastalkova *et al.* 2006; Shema *et*

novel PKCs and CaMKII, while important for LTP induction, are not necessary for LTP maintenance (Ling *et al.* 2002; Liyanage *et al.* 1992; and McGlynn *et al.* 1992).

Chelerythrine is thought to be a highly selective PKC inhibitor found to act on the protein substrate-binding site of the enzyme's catalytic domain and has no inhibitory effect on CaMKII (Herbert *et al.*, 1990; Thompson 1996). Ling *et al.* (2002) reported that chelerythrine inhibits PKM $\zeta$  at low concentrations relative to conventional PKC $\alpha$ , the novel PKC- $\epsilon$  and CaMKII. This is of physiological significance because chelerythrine reverses LTP maintenance when applied 1 hour after tetanization (Ling *et al.* 2002). Chelerythrine specifically reversed potentiated synapses while the control, nontetanized pathway simultaneously recorded in each experiment was unchanged. While other PKC inhibitors have been found to prevent LTP induction, none had been found to reverse LTP maintenance. This finding supported the notion that conventional and novel PKCs along with CaMKII, while key to LTP induction, are not responsible for LTP maintenance and the role of PKM $\zeta$  in LTP maintenance (as reviewed by Sacktor 2008.)

ZIP is a synthesized pseudo-substrate that binds the catalytic site of PKM $\zeta$ , that had been made to be cell permeable, and is claimed to inhibit PKM $\zeta$  specifically (Ling *et al.* 2002; Braun and Mochly-Rosen 2003; Sacktor 2008; Bandyopadhyay *et al.* 1997). ZIP is claimed to inhibit PKM $\zeta$  at low concentrations relative to CaMKII; and ZIP was found to reverse LTP maintenance when applied 1 hour after tetanization (Ling *et al.* 2002). The control, nontetanized pathway, simultaneously recorded in each experiment, remained unchanged. This information led to the claim that PKM $\zeta$  is necessary for LTP

would disrupt LTP but would not be physiologically relevant. Third, while *in vitro* experiments report no changes in the baseline EPSC amplitude when ZIP is added, results reported in *in vivo* experiments, show a decreased potentiation in the control pathway (Pastalkova *et al.* 2006).

Our experiments will be performed in an open chamber, which has the advantage of permitting the rapid and definable measurement of peptide concentration changes in the chamber as well as providing compatibility with modern techniques of whole cell recording, which require cell visualization by a microscope with a water immersion objective. While long term LTP recordings are usually performed in interface chambers, these do not allow the use of a microscope with a water immersion objective. There are two objectives in our investigation: Our first objective is to produce stable LTP recordings for up to three hours after LTP induction, as this type of long term recordings are usually performed in interface chambers. We will check we are producing true late phase LTP by adding a protein synthesis inhibitor before LTP induction as the effect of protein synthesis inhibitors is a standard test of late phase LTP. Our second objective will be to assess the reproducibility of ZIP-induced LTP reversal experiments by adding ZIP and the control scrambled ZIP one hour after LTP induction.

minutes before LTP induction. The tetanization protocol consisted of four 100Hz, 1-second train delivered at 5 minute intertrain intervals (Scharf *et al.* 2002).

## Solutions

Anisomycin (Sigma, St. Louis, MO) was prepared as a stock solution (10 mM, in DMSO) and stored at  $-80^{\circ}$  C until used at final concentration of 50  $\mu$ M in the ACSF bath. ZIP and scrambled ZIP (scr-ZIP) (Tocris, Ellisville, MO) was prepared as a stock solution (10 mM in DMSO) was stored at  $-80^{\circ}$  C until used at final concentration of 5 $\mu$ M. This concentration was chosen because it was optimal in inhibiting LTP induction (Sacktor 2008). These were dissolved in 50  $\mu$ L of DMSO, and added to ACSF. For each experiment, the proteins were administered before starting the baseline and no washouts were performed.

## Statistics

Initial data analysis was performed using a custom-made program written in AXOBASIC, followed by analysis using Microsoft Excel and Microcal Origin. Plots were made in Origin. Every data point represents an average of all collected data points in a 2-min interval (unless noted otherwise). Statistical differences of values after drug application. Data are presented as means  $\pm$  SE.

to about 30% over the course of the three hours following LTP induction. In these experiments, the control pathway was found to remain at the baseline level following the LTP induction, and to stay stable at baseline for the three hours following LTP induction. To check that we were in fact dealing with LTP and not an increased excitability of the hippocampal neurons, the fiber volley for the LTP and control pathways were recorded. The fiber volley is a measure of the depolarization of presynaptic neurons, and is proportional to the number of fibers that fire an action potential.

The goal of inducing LTP after the addition of the peptide synthesis inhibitor anisomycin was to test the claim that our experiments are producing true late-phase LTP. Compared to DMSO experiments, anisomycin experiments show a smaller average increase in normalized EPSP peak of 50% directly following LTP induction as seen in figure 2.a. This initial potentiation was found to decrease to a 10-15% potentiation and to be maintained for least three hours after LTP induction. The normalized EPSP slope was also recorded and was found to be very similar to the EPSP peak results as the EPSP slope amplitude increase to about 60%. This initial potentiation was found to decrease to about 10% over the course of the three hours following LTP induction. In these experiments, the control pathway was found to remain at the baseline level following the LTP induction, and to stay stable at baseline for the three hours following LTP induction. To check that we were in fact dealing with LTP and not an increased excitability of the hippocampal neurons, the fiber volley for the LTP and control pathways were recorded. As seen in figure 2.c. while there is some increase in fiber volley amplitudes, this increase is only about 5% above baseline and does not appear to be temporally related to

In the next set of experiments we added ZIP and scrambled ZIP one hour after LTP induction in an attempt to reproduce hippocampal LTP reversal in an open chamber. This set of experiments (figure 3.a,b and c) show that sustained potentiation was observed in both when ZIP and when scr-ZIP were applied one hour after LTP induction. The average initial increase and subsequent decrease in normalized EPSP peak for both sets of experiments were very similar as they exhibited an initial EPSP increase by about 75% followed by a decrease to only a 10% potentiation which was maintained for least three hours after LTP induction. The normalized EPSP slope was also recorded and was found to be very similar to the EPSP peak results as the EPSP slope amplitude increased by about 65%. This initial potentiation was found to decrease to about 30% over the course of the three hours following LTP induction. In these experiments, the control pathway was found to remain at the baseline level following the LTP induction, and to stay stable at baseline for the three hours following LTP induction. To check that we were in fact dealing with LTP and not an increased excitability of the hippocampal neurons, the fiber volley for the LTP and control pathways were recorded. As seen in figure 3.c. while there is some variation in fiber volley amplitudes ( $\pm 15\%$ ) most of the increase in EPSP slope and peak appears attributable to LTP and not general excitability of the neurons. All in all, we can conclude that we have not been able to reproduce hippocampal LTP reversal by adding ZIP and scrambled ZIP one hour after tetanic stimulation.

## **Discussion:**

Our experiments have shown that we have successfully induced long-term potentiation in the stratum radiatum of the CA1 region in the hippocampus. As seen in Figure 2c, while there is some increase in fiber volley amplitudes, this increase is only about 10% above baseline and does not appear to be temporally related to the time of LTP induction. This suggests that most of the increase in EPSP peak and slope are due to LTP and not general excitability of the axons, or increased mobilization of presynaptic fibers. Indeed, it is important to measure the depolarization of these presynaptic fibers as an increase in EPSP should be independent of fiber volley size, otherwise instead of EPSP increases representing potentiation, these EPSPs might be accounted for by an increase mobilization of presynaptic fibers. Our experiments were performed in an open chamber, which has the advantage of providing compatibility with modern techniques of whole cell recording, as these techniques require cell visualization by a microscope with a water immersion objective, while interface chambers, usually used for long-term LTP recordings do not allow the use of a microscope with a water immersion objective. However, we have not been able to reproduce previous experiments that reported LTP reversal when ZIP was added one hour after LTP induction.

ZIP is a synthesized cell permeable pseudo-substrate that binds the catalytic site of PKM $\zeta$  and is claimed to inhibit PKM $\zeta$  specifically (Ling *et al.* 2002; Braun and Mochly-Rosen 2003; Sacktor 2008). The fact that PKM and PKM $\zeta$  are identical to the catalytic domain of PKC $\zeta$ , with the only difference being the cleavage of the regulatory domain, then ZIP should in principle also inhibit PKC $\zeta$ . While previous literature

of these three crucial steps as they only report that the application of an inhibitor (ZIP) decreases LTP .

As seen in figure 2 a. and b., in the presence of anisomycin that was added before the induction of LTP, the potentiation observed was significantly smaller (about 20%) than the potentiation observed in the absence of anisomycin. This finding is partly in line with previous literature that reported a significantly lower potentiation of translation-dependent late LTP in the presence of anisomycin administered before LTP induction (Frey *et al.* 1988; Fonseca *et al.* 2006). The results do differ to some extent in that in the literature. The initial phase of potentiation, following LTP induction was not found to be different in the presence or absence of anisomycin. We found the difference in potentiation is observed as soon as LTP induction is initiated. This suggests that the LTP pathway in the absence of anisomycin might be more excitable, which appears to be supported by figure 2, c. as this figure shows that this pathway had a greater fiber volley on average.

Our finding that the peptide inhibitor application does not decrease late LTP, might suggest we did not produce true LTP. This however, does not seem to be supported by figure 2c, which shows that the FV does not increase after the induction of LTP therefore showing that the potentiation is not due to an increased mobilization of more presynaptic fibers or a general increase in excitability of the axons.

In the second part of our experiments, we compared the effect of ZIP and scrambled ZIP on late LTP. Neither ZIP nor scr-ZIP were found to reverse LTP as seen in Figure 3, a. and b. This finding is further supported by experiments that show both ZIP

between the concentration of ZIP in the ACSF and in the chamber. Interestingly, previous experiments use a different sort of chamber called “interface chambers” in which the slice is kept moist, but its top surface is in contact with oxygen and the ACSF is not oxygenated by bubbling a 95%O<sub>2</sub>-5%CO<sub>2</sub> mixture. It is also possible the myristoylated peptides could have stuck to the tubing we used for the circulation of the ACSF. To remediate this limitation, the concentration of the peptides should be monitored inside the chamber itself.

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