

Kinetic simulation study of cerebellar long-term depression

Shinya Kuroda
Kawato Dynamic Brain Project
ERATO
Japan Science and Technology
619-0288
kshinya@erato.atr.co.jp

Nicolas Schweighofer
Kawato Dynamic Brain Project
ERATO
Japan Science and Technology
619-0288
nicolas@erato.atr.co.jp

Mitsuo Kawato
Kawato Dynamic Brain Project
ERATO
Japan Science and Technology
ATR
619-0288
kawato@isd.atr.co.jp

ABSTRACT

Cerebellar long-term depression (LTD) is believed to be the molecular and cellular basis of cerebellar learning. Because multiple molecular signal transduction pathways are involved, the systematic relationship between cerebellar LTD and the currently known signal transduction pathways remains obscure. To address this issue, we built a new diagram of the signal transduction pathways and developed a simulation of a computational model of kinetics for the phosphorylation of AMPA receptors, known as a key step for expressing cerebellar LTD. The phosphorylation of AMPA receptors in this model consists of an initial phase and an intermediate phase. We show that the initial phase is mediated by the activation of linear cascades of protein kinase C (PKC) whereas the intermediate phase is mediated by a mitogen-activated protein (MAP) kinase-dependent positive feedback loop pathway that is responsible for the transition from the transient phosphorylation of the AMPA receptors to the stable phosphorylation of the AMPA receptors. These phases are dually regulated by the PKC pathway and protein phosphatase pathway. Both phases also require nitric oxide (NO), although NO *per se* does not show any ability to induce LTD; this is consistent with a permissive role as experimentally reported. In addition, there are some discrepancies between the simulated and experimental results in the induction of the initial phase of LTD, suggesting that other mechanisms in addition to the phosphorylation of AMPA receptors are responsible for the initial phase of cerebellar LTD. Therefore, the kinetics simulation is a powerful tool for understanding and exploring the behaviors of complex signal transduction pathways involved in cerebellar LTD.

1. INTRODUCTION

Cerebellar long-term depression (LTD) is a process of decrease of synaptic strength between the parallel fibers (PF) and Purkinje cells (PC) induced by the conjunctive activation of PF and the climbing fiber (CF) [26,44]. Cerebellar LTD is thought to be a molecular and cellular basis for cerebellar learning [26,46,32]. Recent progress revealed that many signal transduction pathways are involved in the induction of cerebellar LTD [43,18,12]. PF is thought to transmit its signal through glutamate [28,45] and nitric oxide (NO) [60,12], and CF to transmit its signal through corticotropin-releasing factor (CRF) [53] and Ca^{2+} influx via voltage-gated Ca^{2+} channels [16,58,9,43,12]. Both PF- and CF-

mediated signals are transmitted into PC through multiple signaling pathways including mitogen-activated protein (MAP) kinase and Ca^{2+} , and finally lead to the phosphorylation of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors by protein kinase C (PKC) [18,12,48]. In addition, NO, by diffusing into PC, inhibits protein phosphatase through the cyclic GMP (cGMP) and soluble guanylyl cyclase (sGC) pathway, resulting in inhibiting the dephosphorylation of AMPA receptors [12]. Therefore, the signals from PF and CF dually regulate the phosphorylation of AMPA receptors through the PKC and protein phosphatase pathways. The phosphorylation of AMPA receptors has been shown to be a key step for cerebellar LTD expression through internalization [48,65]. However, due to the complex nature of the signaling pathways underlying the phosphorylation of AMPA receptors in cerebellar LTD, the systematic relationship between the synaptic inputs and the output responses remains obscure.

To understand and explore the behaviors of the complex signal transduction pathways, it is important to utilize the computational framework of kinetics simulation. Accordingly, by taking advantage of the recently developed program GENESIS/kinetikit [3], we here built a computational simulation model for the phosphorylation of AMPA receptors in cerebellar LTD based on kinetics parameters [37]. It was reasonable to incorporate only biochemical parameters of posttranslational biochemical reactions, but not those for gene expression and protein synthesis, into the simulation, simply because no kinetic parameters of gene expression and protein synthesis have thus far been available in cerebellar LTD. The phosphorylation of AMPA receptors in the kinetics model consisted of the initial and intermediate phases, the former was mediated by Ca^{2+} , DAG and AA-mediated PKC pathway and the latter by the MAP kinase-mediated positive feedback loop pathway. In addition, NO was required for both the initial and intermediate phases, consistent with a permissive role as experimentally shown. Therefore, the kinetics simulation of cerebellar LTD provided us with a novel method for understanding and exploring the complex nature of the signal transduction pathways involved in cerebellar LTD.

2. MATERIALS AND METHODS

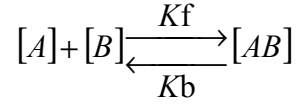
2.1 Block diagram showing the phosphorylation of AMPA receptors in cerebellar LTD

The conjunctive stimulation of PF and CF has been shown to elicit cerebellar LTD [26]. In addition, multiple signal transduction pathways have been shown to regulate cerebellar LTD [26,43,18,12]. According to the literature described below, a new block diagram of cerebellar LTD was reconstructed (Fig. 1). Cerebellar LTD has been shown to be mediated by the modulation of AMPA receptors [43,12] and the decrease in the excitatory postsynaptic potential (EPSP) has been thought to be mediated by the internalization of phosphorylated AMPA receptors [48,65]. PF mediates its signals to PC by releasing glutamate, resulting in the activation of AMPA receptors and type-1 G-protein-coupled metabotropic receptors (mGluR1) [1,8,12,25]. The activation of AMPA receptors leads to an influx of Ca^{2+} through the activation of a Na^+/Ca^{2+} pump [5,12] and to the activation of MAP kinase cascades through the activation of Lyn tyrosine kinase [22]. The activation of mGluR1 leads to the activation of GTP-binding proteins, Gq, and the subsequent activation of phospholipase C (PLC), resulting in the production of DAG and inositol-1,4,5-phosphate (IP3) [43,18,12]. IP3 mobilizes Ca^{2+} from the internal Ca^{2+} store through IP3 receptors [19,62]. The elevation of Ca^{2+} leads to the activation of cytosolic phospholipase A₂ (PLA₂), resulting in the production of arachidonic acid (AA) and the subsequent activation of PKC [3]. PKC has been shown to be involved in the induction of cerebellar LTD [10,42]. DAG, Ca^{2+} and AA activate protein kinase C [56].

In addition, PF has recently been shown to produce NO [60]. NO, diffusing into PC, activates sGC, and the activated sGC catalyzes GTP into cGMP [4,61,39,12]. Then, cGMP activates cGMP-dependent protein kinase (PKG) [64]. PKG phosphorylates its substrate, G-substrate [55,64]. Recently, cDNA of G-substrate was cloned [17,21] and it was shown that phosphorylated G-substrate preferentially inhibits protein phosphatase 2A (PP2A) rather than protein phosphatase 1 [17]. In this simulation, therefore, we assumed that the dephosphorylation of AMPA receptors is mediated by PP2A. CF has been known to depolarize PC, resulting in the influx of Ca^{2+} into PC through voltage-gated Ca^{2+} channels (Ica) [26,43,12]. In this study, therefore, Ca^{2+} elevation induced by the stimulation of PF and CF, or PF or CF alone were assumed based on the experimental results using Ca^{2+} indicators [51,50,34,63], not by the kinetics simulation. Recently, CRF found in CF was shown to play a permissive role in cerebellar LTD [53] and to activate MAP kinase without Ca^{2+} elevation [57]. In this simulation, therefore, MAP kinase was activated by PKC, Lyn and CRF through the activation of Raf and MEK. Activated MAP kinase phosphorylates and activates PLA₂, resulting in the production of AA and the subsequent activation of PKC [3]. Therefore, the PKC-MAP kinase pathways interact at this point. This connection should result in a positive feedback loop [3]. In this study, therefore, we attempted to set the parameters to fit the time course of the phosphorylation of the AMPA receptors to that of EPSP as previously reported [7]. We built the NO/cGMP pathway according to literature. All of the kinetic parameters are shown in [37].

2.2 Kinetic simulations

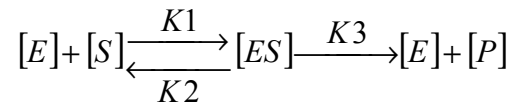
The phosphorylation of AMPA receptors in cerebellar LTD was simulated based on the following two biochemical reactions; protein-protein (molecule-molecule) interactions and enzymatic reactions. The protein-protein interactions included interactions such as NO-GS and cGMP-PKG. These reactions can be given by the following formulation:



Experimentally, in most cases, Kf and Kb are not available in the literature. However, Kd , the dissociation constant, has generally been reported. Therefore, based on the reported Kd values, Kf and Kb were calculated by the following definition:

$$Kd = \frac{Kb}{Kf}$$

The enzymatic reactions include phosphorylation and dephosphorylation. These reactions can be given by the following formulation of Michaelis-Menten:



where E, S and P denote enzyme, substrate and product, respectively.

As in the case of protein-protein interactions, the values of $K1$ and $K2$ are not generally given in the literature. However, $K3$ can be calculated by the experimentally derived $Kcat$ value, given by $Vmax$ divided by the concentration of the enzyme. The values of Km are also generally reported. Therefore, based on the Km values and $K3$ values, the values of $K1$ and $K2$ were calculated by the following definition:

$$Km = \frac{K2 + K3}{K1}$$

Unless apparent rate constants are available, we assume that $K2$ is about 2–20 times greater than $K3$ since $K2$ is generally greater than $K3$ in many enzymes.

Once the above parameters determined from the kinetics values were found to be robust enough to reproduce cerebellar LTD elicited under normal conditions [7], we simulated the following experiments. All of the numerical computations were performed with the kinetics library, which is an extension to GENESIS [3].

3. RESULTS

3.1 Building a kinetics simulation model for the phosphorylation of AMPA receptors in cerebellar LTD

To develop a computational kinetics simulation for the phosphorylation of AMPA receptors in cerebellar LTD, we first developed a block diagram for the phosphorylation of AMPA receptors in cerebellar LTD on the basis of reported data (Fig. 1). Because recent evidence has suggested that the phosphorylation of

AMPA receptors is a key step for the expression of cerebellar LTD through the internalization of the phosphorylated AMPA receptors [48,65,67], we measured concentrations of phosphorylated and non-phosphorylated AMPA receptors. According to literature, cerebellar LTD involves the phosphorylation of AMPA receptors of PC by both the activation of PKC [10,43,18,12,48], and the inhibition of protein phosphatase by the NO/cGMP pathway [55,27,2,12]. Raf, MAP kinase and PKC have been shown to form a potential positive feedback loop [3], and PKC has been shown to be regulated by the Ca^{2+} , DAG and AA-mediated linear pathway and by the positive feedback loop pathway. Since PP2A has been shown to be phosphorylated by PKG [17], we assume that the action of the NO/cGMP pathway is finally mediated by PP2A. Therefore, the phosphorylation of AMPA receptors is dually regulated by PKC and PP2A. Taking advantage of the kinetic parameters available in the literature with some assumptions, we built the computational kinetics simulation model of the phosphorylation of AMPA receptors in cerebellar LTD [37].

3.2 The conjunctive stimulation of PF and CF induces the stable phosphorylation of AMPA receptors in cerebellar LTD

Based on the kinetics parameters, we tried to make the kinetics simulation reproduce the experimental results, i.e., that the conjunctive stimulation of PF and CF induces cerebellar LTD [7]. Cerebellar LTD consists of an initial peak followed by a stable phase [7]. It has been shown that gene expression and protein synthesis are required for the late phase of cerebellar LTD [41]; however, these experimental conditions are different from those of the kinetics simulation because, under the experimental conditions, only PC was used and the non-involvement of NO was assumed [41]. By considering the fact that only posttranslational biochemical reactions were incorporated into the kinetics simulation, it is reasonable to assume that the stable phase of LTD can be divided into two phases; an intermediate phase maintained by the posttranslational biochemical reactions and a late phase maintained by the protein synthesis and gene expression. Moreover, since the time course of the phosphorylation of AMPA receptors in cerebellar LTD has not been determined and the phosphorylation of AMPA receptors is important for the expression of cerebellar LTD, we assumed here that the phosphorylation of AMPA receptors also consists of three phases (initial peak, intermediate phase and late phase), and built the simulation to reproduce the time course of EPSP in cerebellar LTD. In the kinetics simulation, the conjunctive stimulation of PF and CF was found to induce the phosphorylation of AMPA receptors and to reduce the concentration of non-phosphorylated AMPA receptors (Fig. 2A). The stimulation of either PF or CF alone failed to induce the stable phosphorylation of AMPA receptors in cerebellar LTD. However, an experimental stimulation similar to the CF stimulation alone in the kinetics simulation has been shown to induce an initial sharp decrease in the excitatory postsynaptic current (EPSC) [11,23]. The failure of the kinetics simulation to reproduce this observation raises the possibility that other mechanisms in addition to the phosphorylation of AMPA receptors underlie the initial phase of cerebellar LTD (see Discussion). The time course of the phosphorylation of AMPA receptors consists of three phases: an

initial phase including the initial peak of the phosphorylated AMPA receptors (0 – 10 min), an intermediate phase including the sustained phosphorylation of AMPA receptors (10 – 40 min), and a late phase including the disappearance of the AMPA receptor phosphorylation (> 40 min). The time course of the non-phosphorylated AMPA receptors in the initial and intermediate phases is very similar to that of the amplitude of EPSP evoked by the conjunctive stimulation of PF and CF [7] (Fig. 2A). In the late phase, however, the concentration of the non-phosphorylated AMPA receptors begins to increase and reaches the basal level at around 90 min due to the dephosphorylation of the AMPA receptors.

Consistently, the activation of PKC also consists of three phases (Fig. 2B): an initial phase mediated by the direct activation by Ca^{2+} , DAG and AA, an intermediate phase by the activation of the MAP kinase-mediated positive feedback loop (see below) and a late phase by the inactivation of PKC activity (see also Fig. 4). This positive feedback loop is essential for the transition from the transient phosphorylation of AMPA receptors to the stable phosphorylation of the AMPA receptors, and is responsible for the bistability of the AMPA receptor phosphorylation (see Figs. 4).

The PP2A activity markedly decreases by the stimulation and becomes lowest at 5 min, and then gradually reactivates (Fig. 2C). The inactivation of PKC and the reactivation of PP2A lead to the dephosphorylation of the AMPA receptors, resulting in the disappearance of the phosphorylated AMPA receptors in the late phase. The reactivation of PP2A is mediated by the degradation of NO. Although NO is degraded very fast and consequently the PKG activity returns to the basal level after the stimulation, the reactivation of PP2A is very slow because the dephosphorylation step of G-substrate, an inhibitor of PP2A, is assumed to be quite slow. Since PP2A activity is inhibited by G-substrate phosphorylated by PKG, it is interesting to test whether this prolonged phosphorylation of G-substrate really occurs *in vivo* if an antibody which specifically recognizes the phosphorylated G-substrate will be available in the future. The inactivation of PKC activity is mediated by the degradation of PKC itself and by the reactivation of PP2A activity. Although the reactivation of PP2A is very small, this small reactivation of PP2A blocks the activation of the positive feedback loop and the activity of the loop becomes lower than the threshold, which is a critical point for bistability, resulting in the transition from the activation state to the inactivation state of the loop. These results indicate that the current signal transduction pathways as shown in Fig. 1 are able to produce the initial and intermediate phases for the phosphorylation of AMPA receptors in cerebellar LTD elicited by the conjunctive stimulation of PF and CF.

3.3 Optimal duration of the stimulation to induce the stable phosphorylation of AMPA receptors in cerebellar LTD

We next examined what length of time of the conjunctive stimulation by PF and CF is required to induce the stable phosphorylation of AMPA receptors. In the simulation, the stable phosphorylation of AMPA receptors was not induced by a time length shorter than 3 min (Fig. 3A). Time lengths longer than 5 min induced the stable phosphorylation of AMPA receptors and the optimal duration of the stimulation was found to be 6 min.

The optimal duration in the simulation (6 min) of the phosphorylation of AMPA receptors was almost the same as that of the cerebellar LTD observed in an experiment (5 min) [29]. This result indicates that the phosphorylation of AMPA receptors shows bistability within a middle time scale of up to 40 min. The activation of PKC also showed a similar bistability (**Fig. 3B**). The PP2A activity first decreased linearly and then reached a plateau (**Fig. 3C**), indicating that the inhibition of PP2A does not exhibit bistability. Therefore, the bistability in the phosphorylation of AMPA receptors is mediated by the prolonged activation of PKC. The activation of the MAP kinase-mediated positive feedback loop has been reported to be responsible for the bistability (see below) [3]. In addition, we found that the inhibition of PP2A is required for this bistability (see also Fig. 4). Since bistability in the phosphorylation of AMPA receptors has not been shown experimentally, this result provides an interesting testable prediction of bistability in the phosphorylation of AMPA receptors in cerebellar LTD. A recently established anti-phosphorylated AMPA receptors antibody [49] should allow us to test this prediction in the future.

3.4 The role of each signaling molecule and pathway in the induction of the phosphorylation of AMPA receptor in the cerebellar LTD

We next explored the roles of signaling molecules and pathways in the phosphorylation of AMPA receptors (**Fig. 4**). Without PKC activation, the phosphorylation of AMPA receptors did not increase in the kinetic simulation (**Fig. 4 wide dashed line**) due to the lack of activation of PKC. This finding is consistent with an earlier observation that the inhibition of PKC activity results in the complete disruption of LTD in mice expressing inhibitory peptide of PKC in PC [13]. In contrast, cerebellar LTD has been shown to be unimpaired in mice lacking PKC γ gene, one of the isoforms of PKC [6]. This discrepancy may be due to the possibility that PKC isoforms function redundantly, or that there is a PKC-independent pathway. Various experiments suggest the involvement of PKC in the induction of LTD [42,27]. Taken together, the former possibility is more likely. Without NO production, no stable phosphorylation of AMPA receptors was observed (**Fig. 4 dotted line**) due to the lack of activation of PKG and that of inhibition of PP2A, despite the fact that PKC was fully activated. This finding is consistent with the observation that NO is essential for the induction of all phases of LTD, although NO itself shows no ability to elicit LTD [39]. In addition, these results support the idea that LTD is dually regulated by the PKC and NO pathways. Furthermore, without Ca²⁺ elevation, the stable phosphorylation of AMPA receptors did not occur with a lesser initial phase (**Fig. 4 dashed and dotted line**) due to the partial activation of PKC. This finding is also consistent with the fact that Ca²⁺ is essential for the induction of all phases of LTD [58,35]. Without DAG elevation, both the initial and intermediate phases of the AMPA receptor phosphorylation were observed, but the phosphorylation was less stable compared to that under the normal condition (**Fig. 4 dashed dotted and dotted line**) due to the partial activation of MAP kinase. This finding indicates that DAG contributes to the stable phosphorylation of AMPA receptors both in the initial phase and the intermediate phase.

Experimentally, it is difficult to analyze the role of DAG since no specific inhibitors of DAG are available. Moreover, the inactivation of PLC, which produces both DAG and IP₃, results in the depletion of both products. Consequently, the simulation is useful for analyzing the role of signal transduction pathways, which is experimentally difficult to analyze. Without PLA₂ activation, the intermediate phase of the AMPA receptor phosphorylation disappeared with a slight reduction in the initial phase (**Fig. 4 narrow dashed line**). The positive feedback loop was responsible for the intermediate phase because, without MAP kinase-mediated PLA₂ activation, the intermediate phase disappeared without affecting the initial phase (data not shown) due to the lack of the stable activation of MAP kinase. Lack of the activation of either Raf or MEK resulted in the same results as that without MAP kinase-mediated PLA₂ activation. This finding indicates that the positive feedback loop including PLA₂ is responsible for the intermediate phase of the AMPA receptor phosphorylation. This result is consistent with an earlier observation that PLA₂ regulates the intermediate phase of LTD [40]. Without the activation of PLA₂ by Ca²⁺ elevation, the initial phase came to be slightly reduced and the intermediate phase came to be less stable (data not shown).

4 DISCUSSION

The initial phase of the AMPA receptor phosphorylation in cerebellar LTD was dependent on the direct activation of PKC by linear cascades including DAG, Ca²⁺ and AA, while the intermediate phase was mediated by the activation of the positive feedback loop. The inactivation of PLA₂ resulted in the disappearance of the intermediate phase without a remarkable change in the initial phase, indicating that the positive feedback loop was responsible for the intermediate phase. Consistent with this result, the selective inhibition of PLA₂ has been reported to convert cerebellar LTD to short-term depression (STD), and the application of free unsaturated fatty acids has been shown to result in an apparent conversion from STD to LTD in cultured PC by the stimulation, where STD, not LTD, come to be normally induced [40]. Taken together, it is likely that PLA₂ and AA serve as regulators of the positive feedback loop and are responsible for the intermediate phase of LTD.

The lack of the activation of either Raf, MEK or MAP kinase led to the same result as that without the MAP kinase-mediated PLA₂ activation. MAP kinase and MEK have also been shown to be required for the induction of both the initial and intermediate phases of cerebellar LTD [31]. The reason why MAP kinase and MEK are not involved in the induction of the initial phase in the simulation may be due to the possibility that an unknown part of the MAP kinase cascade is missing in the simulation. MAP kinase has been shown to be required for the mGlu receptor activity [31]. Therefore, the molecular linkage between MAP kinase and mGlu receptors needs to be clarified and to be incorporated into the kinetics simulation.

In all phases, the inhibition of PP2A by NO is essential for the phosphorylation of AMPA receptors. This is consistent with an earlier observation that NO is essential for cerebellar LTD, but NO itself is insufficient to induce LTD [39], suggesting that PP2A acts as a gate signal of cerebellar LTD. This result indicates that significant amount of PF stimulation is required to induce

cerebellar LTD via inhibition of PP2A, and that spontaneous PF activity may be insufficient to inhibit PP2A activity enough to be required for the stable phosphorylation of AMPA receptors. If there is only small amount of PP2A which is easily inhibited by spontaneous PF activity, the PF activity together with spontaneous CF activity may cause unexpected and unwarranted cerebellar LTD. Therefore, the reason why such large amount of PP2A is needed may partially be due to the role of NO-PP2A pathway as a gate signal for the induction of cerebellar LTD. In this study, spontaneous PF activity was not included. However, if spontaneous PF activity is included, the PP2A may reach to the some basal level which may not be small enough to induce LTD, and should be further inhibited by NO signal, leading to induce LTD. Therefore, with or without spontaneous PF activity, the action of PP2A would be similar. In addition, PP2A has another role in the kinetics simulation; the dephosphorylation of Raf and MEK. Raf and MEK regulate the positive feedback loop and the inhibition of the dephosphorylation of these molecules was required for the activation of the positive feedback loop. Therefore, PP2A also has a permissive role in the phosphorylation of AMPA receptors as well as in the activation of the positive feedback loop. In contrast to PP2A action in the kinetics simulation, calcineurin, a different class of protein phosphatase, has been shown to regulate the intermediate phase, but not initial phase of hippocampal long-term potentiation (LTP) [47,66]. This observation suggests that calcineurin regulate the intermediate phase of hippocampal LTP through the possible feedback loop, but does not regulate the direct modification of receptors nor the activation of linear cascade induced by the agonist. Therefore, it is interesting to explore the possible role of calcineurin in the regulation of the possible positive feedback loop in the hippocampal LTP.

We compared the phosphorylation of AMPA receptors in the kinetics simulation and cerebellar LTD experimentally shown. In the initial phase, some results of the AMPA receptor phosphorylation in the kinetic simulation did not correlate with the experimental observations of cerebellar LTD. The stimulation of CF alone did not induce an apparent initial phase in the kinetics simulation, whereas a similar protocol can induce an initial sharp peak of EPSC decrease [11,23]. Additionally, without NO or Ca^{2+} elevation, a small peak in the phosphorylation of the AMPA receptors could still be observed in the kinetics simulation (Fig. 4), while this initial peak was not observed by the addition of blockers of Ca^{2+} [58,15] or NO [39]. These discrepancies raise the possibility that other mechanisms underlie the expression of the initial phase of cerebellar LTD. It has been shown that increasing Ca^{2+} in dendrite activates Ca^{2+} -dependent K^+ channels, resulting in shunting PF-induced EPSP [20,33,54]. Additionally, it has recently been shown that the activation of postsynaptic mGluR1 in PC dendrites transiently depresses synaptic transmissions at PF-PC synapses by presynaptic mechanism involving Ca^{2+} increase in PC dendrites and retrograde signaling [38], and that retrograde inhibition of Ca^{2+} influx occurs through endogenous cannabinoids at excitatory synapse on PC [36]. These mechanisms are likely to be the main mechanisms for the expression of the initial phase of cerebellar LTD. The kinetics parameters in these two mechanisms, although unknown at present, should allow us to test whether these mechanisms can explain the expression of the initial phase of cerebellar LTD by use of the kinetics simulation. Accordingly, the kinetics

simulation is a powerful tool for testing whether the phosphorylation of AMPA receptors can explain the expression of cerebellar LTD.

The phosphorylation of AMPA receptors has been shown to trigger the internalization of phosphorylated AMPA receptors [48,65]. A recent study has demonstrated that, in cortical neurons, the activation of AMPA receptors without the activation of NMDA receptors leads to the rapid and almost complete internalization of AMPA receptors within 5 min after the stimulation and the slow reinsertion of the AMPA receptors to the cell surface, resulting in a reduction in the number of AMPA receptors at the cell surface [14]. It is possible that a similar mechanism is involved in cerebellar LTD. Taken together with our results, it is likely that the phosphorylation of AMPA receptors is a key step for cerebellar LTD expression through the internalization of the AMPA receptors at least in the intermediate phase. However, the internalization of phosphorylated AMPA receptors was not incorporated into our model because of the absence of kinetics parameters. The absence of phosphorylated AMPA receptor internalization in the kinetics simulation may affect the results by affecting the balance between the phosphorylated and non-phosphorylated AMPA receptor concentrations; however, it is likely that the results would be similar if the internalization step was incorporated into kinetics simulation because the internalization process itself does not affect the signaling pathways. If the kinetics parameters of the phosphorylated AMPA receptor internalization will be determined, we can address the question of whether the phosphorylation of AMPA receptors and the subsequent internalization of the AMPA receptors can explain the intermediate phase as well as the initial phase of cerebellar LTD.

It has been shown that glial fibrillary acidic proteins [59], expressed in astrocytes but not in neurons, and the $\delta 2$ subunit of the glutamate receptor channels [24,30], are essential for the induction of LTD. The phosphorylation of AMPA receptors has been shown to lead to the internalization of the AMPA receptors [48,67]. Although the detailed mechanisms are still unknown, the molecules and mechanisms involved should be incorporated into the kinetics model in the future. Local Ca^{2+} release within the dendritic spines of PC has recently been shown to be required for LTD induction [52]. Therefore, the specific localization of molecules and accessibility should also be quantitatively determined and incorporated into the kinetics model. In addition to phosphorylation, Ca^{2+} -dependent K^+ channels [20,33,54], presynaptic retrograde signaling [38] and retrograde inhibition of presynaptic Ca^{2+} influx by endogenous cannabinoids [36] have been shown to underlie the expression of cerebellar LTD. Electrical processes, such as the channel activity of AMPA receptors and Ca^{2+} -dependent K^+ channels, coupled with biochemical processes should also be incorporated into the kinetics simulation provided the apparent kinetics parameters come to be determined. Moreover, the role of Raf in the induction of LTD has yet to be experimentally shown. Therefore, the present kinetics simulation should not be regarded as a definitive model, but as a complementary method for exploring cerebellar LTD in addition to experimental methods. It should also be emphasized that, even if the simulation can reproduce some experimental results, this does not exclude the possibility that unknown pathways or molecules are additionally needed for the induction of cerebellar LTD. In any case, experiments

together with the approach using kinetics simulation should greatly improve our understanding of the behaviors of the complex biochemical reactions underlying cerebellar LTD.

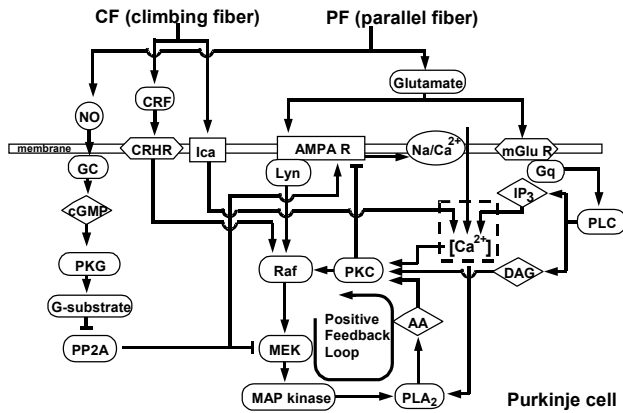


Figure 1. Block diagram of the phosphorylation of AMPA receptors in cerebellar LTD.

Block diagram of the phosphorylation of AMPA receptors in cerebellar LTD. The stimulation of PF and CF results in the elevation of the phosphorylation of AMPA receptors through the activation of PKC via the activation of both linear cascades including DAG, Ca^{2+} and AA, and a positive feedback loop including PLA_2 , and through the inhibition of protein phosphatase 2A. The dashed box indicates the generation of Ca^{2+} , which was reconstituted by the previous observation [51,50,34,63], but not by the kinetics simulation. The arrows and bars denote the stimulatory and inhibitory pathways, respectively.

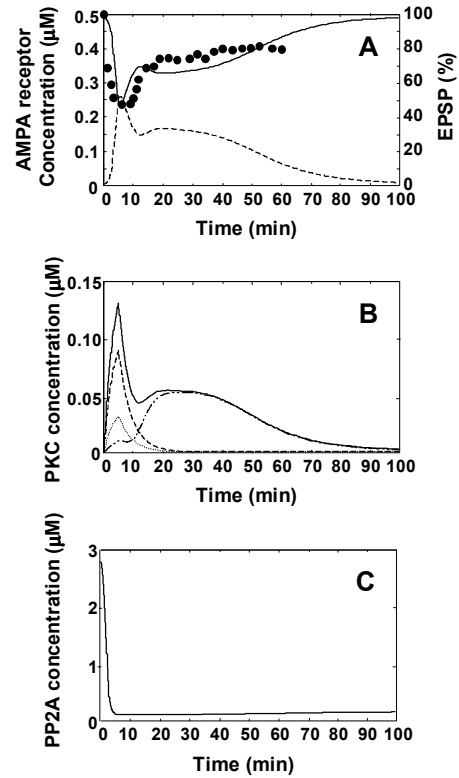


Figure 2. Conjunctive stimulation of PF and CF to induce the stable phosphorylation of AMPA receptors

The simulation was run at 1 Hz for a 5 min-conjunctive stimulation of PF and CF. The time course of the indicated molecule concentration is plotted. (A) The phosphorylation of AMPA receptors induced by the conjunctive stimulation of PF and CF. (dashed line): the concentration for the phosphorylated AMPA receptors. (straight line): the concentration of the non-phosphorylated AMPA receptors. The changes of EPSP induced by the 10 min-conjunctive stimulation of PF and CF is replotted (Circles) from the earlier observation [7]. Note that, in the kinetic simulation, the 5 min-stimulation of PF and CF is used because 5 min-stimulation is optimal according to the previous report [29]. (B) The activation of PKC by the stimulation. (straight line): the total PKC activity; (dashed line), Ca^{2+} -activated PKC activity; (dotted line), DAG-activated PKC activity; (dashed and dotted line), AA-activated PKC activity. (C) The inhibition of PP2A activity.

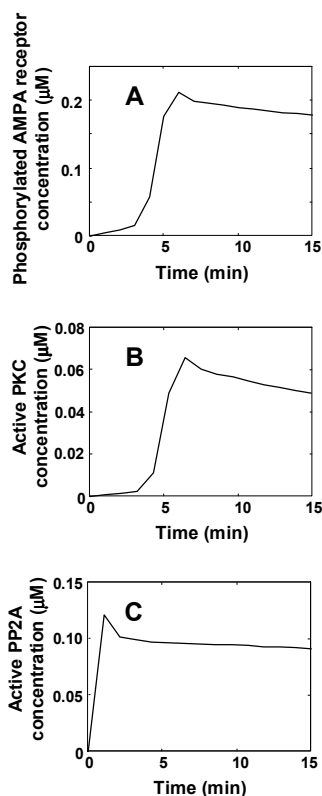


Figure 3. Optimal duration of the stimulation to induce the stable phosphorylation of AMPA receptors in cerebellar LTD. The duration of the conjunctive stimulation of PF and CF at 1 Hz was varied between 0 to 15 min with 1 min-intervals and the simulation was run. The concentrations of the phosphorylated AMPA receptors (A), active PKC (B), and active PP2A (C) at 30 min after the onset of the stimulation.

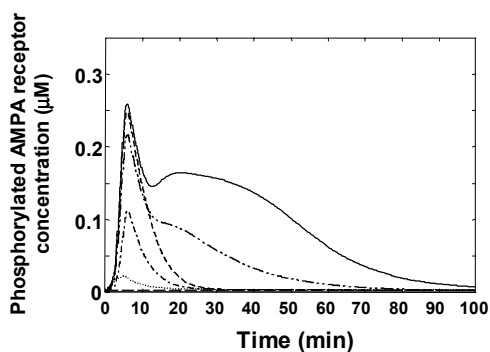


Figure 4. The role of each signaling molecule and pathway in inducing the phosphorylation of AMPA receptors in cerebellar LTD.

To explore the roles of the signaling molecules and pathways in inducing the phosphorylation of AMPA receptors in cerebellar LTD, the simulation was run under the same conditions as in Fig. 2A except that the concentration of the indicated molecules shown below was held at the basal level or the indicated pathway was deleted. Straight line, none; wide dashed line, PKC; dotted line, NO; dashed and dotted line, Ca^{2+} ; narrow dashed line, PLA₂; dashed dotted and dotted line, DAG.

5. REFERENCES

- [1] Aiba A, Kano M, Chen C, Stanton ME, Fox GD, Herrup K, Zwingman TA, Tonegawa S (1994) Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* 79:377-388.
- [2] Ajima A, Ito M (1995) A unique role of protein phosphatases in cerebellar long-term depression. *Neuroreport* 6:297-300.
- [3] Bhalla US, Iyengar R (1999) Emergent properties of networks of biological signaling pathways. *Science* 283:381-387.
- [4] Bredt DS, Snyder SH (1992) Nitric oxide, a novel neuronal messenger. *Neuron* 8:3-11.
- [5] Brorson JR, Manzolillo PA, Miller RJ (1994) Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J. Neurosci.* 14:187-197.
- [6] Chen C, Kano M, Abeliovich A, Chen L, Bao S, Kim JJ, Hashimoto K, Thompson RF, Tonegawa S (1995) Impaired motor coordination correlates with persistent multiple climbing fiber innervation in PKC gamma mutant mice. *Cell* 83:1233-1242.
- [7] Chen C, Thompson RF (1995) Temporal specificity of long-term depression in parallel fiber--Purkinje synapses in rat cerebellar slice. *Learn. Mem.* 2:185-198.
- [8] Conquet F, Bashir ZI, Davies CH, Daniel H, Ferraguti F, Bordi F, Franz-Bacon K, Reggiani A, Matarese V, Conde F, et al. (1994) Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. *Nature* 372:237-243.
- [9] Crepel F, Jaillard D (1991) Pairing of pre- and postsynaptic activities in cerebellar Purkinje cells induces long-term changes in synaptic efficacy in vitro. *J. Physiol. (Lond)* 432:123-141.
- [10] Crepel F, Krupa M (1988) Activation of protein kinase C induces a long-term depression of glutamate sensitivity of cerebellar Purkinje cells. An in vitro study. *Brain Res.* 458:397-401.
- [11] Daniel H, Hemart N, Jaillard D, Crepel F (1992) Coactivation of metabotropic glutamate receptors and of voltage-gated calcium channels induces long-term depression in cerebellar Purkinje cells in vitro. *Exp. Brain Res.* 90:327-331.
- [12] Daniel H, Levenes C, Crepel F (1998) Cellular mechanisms of cerebellar LTD. *Trends Neurosci.* 21:401-407.
- [13] De Zeeuw CI, Hansel C, Bian F, Koekkoek SK, van Alphen AM, Linden DJ, Oberdick J (1998) Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. *Neuron* 20:495-508.
- [14] Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28:511-525.

- [15] Eilers J, Takechi H, Finch EA, Augustine GJ, Konnerth A (1997) Local dendritic Ca²⁺ signaling induces cerebellar long-term depression. *Learn. Mem.* 4:159-168.
- [16] Ekerot CF, Kano M (1985) Long-term depression of parallel fibre synapses following stimulation of climbing fibres. *Brain Res.* 342:357-360.
- [17] Endo S, Suzuki M, Sumi M, Nairn AC, Morita R, Yamakawa K, Greengard P, Ito M (1999) Molecular identification of human G-substrate, a possible downstream component of the cGMP-dependent protein kinase cascade in cerebellar Purkinje cells. *Proc. Natl. Acad. Sci. USA* 96:2467-2472.
- [18] Fiala JC, Grossberg S, Bullock D (1996) Metabotropic glutamate receptor activation in cerebellar Purkinje cells as substrate for adaptive timing of the classically conditioned eye-blink response. *J. Neurosci.* 16:3760-3774.
- [19] Finch EA, Augustine GJ (1998) Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature* 396:753-756.
- [20] Gruol DL, Jacquin T, Yool AJ (1991) Single-channel K⁺ currents recorded from the somatic and dendritic regions of cerebellar Purkinje neurons in culture. *J. Neurosci.* 11:1002-1015.
- [21] Hall KU, Collins SP, Gamm DM, Massa E, DePaoli-Roach AA, Uhler MD (1999) Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate. A Purkinje cell substrate of the cyclic GMP-dependent protein kinase. *J. Biol. Chem.* 274:3485-3495.
- [22] Hayashi T, Umemori H, Mishina M, Yamamoto T (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. *Nature* 397:72-76.
- [23] Hemart N, Daniel H, Jaillard D, Crepel F (1994) Properties of glutamate receptors are modified during long-term depression in rat cerebellar Purkinje cells. *Neurosci. Res.* 19:213-221.
- [24] Hirano T, Kasono K, Araki K, Mishina M (1995) Suppression of LTD in cultured Purkinje cells deficient in the glutamate receptor delta 2 subunit. *Neuroreport* 6:524-526.
- [25] Ichise T, Kano M, Hashimoto K, Yanagihara D, Nakao K, Shigemoto R, Katsuki M, Aiba A (2000) mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. *Science* 288:1832-1835.
- [26] Ito M (1989) Long-term depression. *Annu. Rev. Neurosci.* 12:85-102.
- [27] Ito M, Karachot L (1992) Protein kinases and phosphatase inhibitors mediating long-term desensitization of glutamate receptors in cerebellar Purkinje cells. *Neurosci. Res.* 14:27-38.
- [28] Kano M, Kato M (1987) Quisqualate receptors are specifically involved in cerebellar synaptic plasticity. *Nature* 325:276-279.
- [29] Karachot L, Kado RT, Ito M (1994) Stimulus parameters for induction of long-term depression in in vitro rat Purkinje cells. *Neurosci. Res.* 21:161-168.
- [30] Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, Inoue Y, Kutsuwada T, Yagi T, Kang Y, et al. (1995) Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice. *Cell* 81:245-252.
- [31] Kawasaki H, Fujii H, Gotoh Y, Morooka T, Shimohama S, Nishida E, Hirano T (1999) Requirement for mitogen-activated protein kinase in cerebellar long term depression. *J. Biol. Chem.* 274:13498-13502.
- [32] Kawato M (1999) Internal models for motor control and trajectory planning. *Curr. Opin. Neurobiol.* 9:718-727.
- [33] Khodakhah K, Ogden D (1993) Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurones, cultured cerebellar astrocytes, and peripheral tissues. *Proc. Natl. Acad. Sci. USA* 90:4976-4980.
- [34] Kohda K, Inoue T, Mikoshiba K (1995) Ca²⁺ release from Ca²⁺ stores, particularly from ryanodine-sensitive Ca²⁺ stores, is required for the induction of LTD in cultured cerebellar Purkinje cells. *J. Neurophysiol.* 74:2184-2188.
- [35] Konnerth A, Dreessen J, Augustine GJ (1992) Brief dendritic calcium signals initiate long-lasting synaptic depression in cerebellar Purkinje cells. *Proc. Natl. Acad. Sci. USA* 89:7051-7055.
- [36] Kreitzer AC, Regehr WG (2001) Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto purkinje cells. *Neuron* 29:717-727.
- [37] Kuroda S, Schweighofer N, Kawato M (2001) Exploration of signal transduction pathways in cerebellar long-term depression by kinetic simulation. *J. Neurosci.* in press.
- [38] Levenes C, Daniel H, Crepel F (2000) Postsynaptic mGluR1 depress parallel fibers to Purkinje cells synaptic transmission by a retrograde presynaptic mechanism. *Soc. Neurosci. Abstr.* 332:4.
- [39] Lev-Ram V, Jiang T, Wood J, Lawrence DS, Tsien RY (1997) Synergies and coincidence requirements between NO, cGMP, and Ca²⁺ in the induction of cerebellar long-term depression. *Neuron* 18:1025-1038.
- [40] Linden DJ (1995) Phospholipase A2 controls the induction of short-term versus long-term depression in the cerebellar Purkinje neuron in culture. *Neuron* 15:1393-1401.
- [41] Linden DJ (1996) A protein synthesis-dependent late phase of cerebellar long-term depression. *Neuron* 17:483-490.
- [42] Linden DJ, Connor JA (1991) Participation of postsynaptic PKC in cerebellar long-term depression in culture. *Science* 254:1656-1659.
- [43] Linden DJ, Connor JA (1993) Cellular mechanisms of long-term depression in the cerebellum. *Curr. Opin. Neurobiol.* 3:401-406.
- [44] Linden DJ, Connor JA (1995) Long-term synaptic depression. *Annu. Rev. Neurosci.* 18:319-357.
- [45] Linden DJ, Dickinson MH, Smeyne M, Connor JA (1991) A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. *Neuron* 7:81-89.
- [46] Lisberger SG (1998) Cerebellar LTD: a molecular mechanism of behavioral learning? *Cell* 92:701-704.
- [47] Mansuy IM, Mayford M, Jacob B, Kandel ER, Bach ME (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell* 92:39-49.

- [48] Matsuda S, Laune T, Mikawa S, Hirai H (2000) Disruption of AMPA receptor GluR2 clusters following long-term depression induction in cerebellar Purkinje neurons. *Embo J* 19:2765-2774.
- [49] Matsuda S, Mikawa S, Hirai H (1999) Phosphorylation of Serine-880 in GluR2 by Protein Kinase C Prevents Its C Terminus from Binding with Glutamate Receptor-Interacting Protein. *J. Neurochem.* 73:1765-1768.
- [50] Midtgaard J, Lasser-Ross N, Ross WN (1993) Spatial distribution of Ca²⁺ influx in turtle Purkinje cell dendrites in vitro: role of a transient outward current. *J. Neurophysiol.* 70:2455-2469.
- [51] Miyakawa H, Lev-Ram V, Lasser-Ross N, Ross WN (1992) Calcium transients evoked by climbing fiber and parallel fiber synaptic inputs in guinea pig cerebellar Purkinje neurons. *J. Neurophysiol.* 68:1178-1189.
- [52] Miyata M, Finch EA, Khiroug L, Hashimoto K, Hayasaka S, Oda SI, Inouye M, Takagishi Y, Augustine GJ, Kano M (2000) Local calcium release in dendritic spines required for long-term synaptic depression. *Neuron* 28:233-244.
- [53] Miyata M, Okada D, Hashimoto K, Kano M, Ito M (1999) Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. *Neuron* 22:763-775.
- [54] Muller YL, Reitstetter R, Yool AJ (1998) Regulation of Ca²⁺-dependent K⁺ channel expression in rat cerebellum during postnatal development. *J. Neurosci.* 18:16-25.
- [55] Nairn AC, Hemmings HC, Jr., Greengard P (1985) Protein kinases in the brain. *Annu. Rev. Biochem.* 54:931-976.
- [56] Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607-614.
- [57] Rossant CJ, Pinnock RD, Hughes J, Hall MD, McNulty S (1999) Corticotropin-releasing factor type 1 and type 2alpha receptors regulate phosphorylation of calcium/cyclic adenosine 3',5'-monophosphate response element-binding protein and activation of p42/p44 mitogen-activated protein kinase. *Endocrinology* 140:1525-1536.
- [58] Sakurai M (1990) Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression. *Proc. Natl. Acad. Sci. USA* 87:3383-3385.
- [59] Shibuki K, Gomi H, Chen L, Bao S, Kim JJ, Wakatsuki H, Fujisaki T, Fujimoto K, Katoh A, Ikeda T, Chen C, Thompson RF, Itohara S (1996) Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. *Neuron* 16:587-599.
- [60] Shibuki K, Kimura S (1997) Dynamic properties of nitric oxide release from parallel fibres in rat cerebellar slices. *J. Physiol. (Lond)* 498:443-452.
- [61] Stone JR, Marletta MA (1996) Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* 35:1093-1099.
- [62] Takechi H, Eilers J, Konnerth A (1998) A new class of synaptic response involving calcium release in dendritic spines. *Nature* 396:757-760.
- [63] Wang SS, Denk W, Hausser M (2000) Coincidence detection in single dendritic spines mediated by calcium release. *Nat. Neurosci.* 3:1266-1273.
- [64] Wang X, Robinson PJ (1997) Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J. Neurochem.* 68:443-456.
- [65] Wang YT, Linden DJ (2000) Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* 25:635-647.
- [66] Winder DG, Mansuy IM, Osman M, Moallem TM, Kandel ER (1998) Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. *Cell* 92:25-37.
- [67] Xia J, Chung HJ, Wihler C, Haganir RL, Linden DJ (2000) Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. *Neuron* 28:499-510.