Involvement of Basal Protein Kinase C and Extracellular Signal-Regulated Kinase 1/2 Activities in Constitutive Internalization of AMPA Receptors in Cerebellar Purkinje Cells

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AMPA receptor (AMPA) internalization provides a mechanism for long-term depression (LTD) in both hippocampal pyramidal neurons and cerebellar Purkinje cells (PCs). Cerebellar LTD at the parallel fiber (PF)–PC synapse is the underlying basis of motor learning and requires AMPAR activation, a large Ca^{2+} influx, and protein kinase C (PKC) activation. However, whether these requirements affect the constitutive AMPAR internalization in PF–PC synapses remains unclarified. Tetanus toxin (TeTx) infusion into PCs decreased PF-EPSC amplitude to 60% within 20–30 min (TeTx rundown), without change in paired-pulse facilitation ratio or receptor kinetics. Immunocytochemically measured glutamate receptor 2 (GluR2) internalization ratio decreased at the steady state of TeTx rundown. TeTx rundown did not require AMPAR activity nor an increase in intracellular Ca^{2+} concentration. TeTx rundown was suppressed partially by the inhibition of either conventional PKC or mitogen-activated protein kinase kinase (MEK) and completely by the inhibition of both kinases. The background PKC activity was shown to be sufficient, because a PKC activator did not facilitate TeTx rundown. The inhibition of protein phosphatase 1/2A (PP1/2A) enhanced TeTx rundown slightly, and both inhibition of PP1/2A and activation of PKC maximized it, but one-half of AMPARs at PF–PC synapses remained in the TeTx-resistant pool. The inhibition of actin depolymerization suppressed TeTx rundown and decreased the GluR2 internalization ratio. In contrast, the inhibition of actin polymerization enhanced TeTx rundown and increased the GluR2 internalization ratio. We suggest that the regulation of actin polymerization is involved in the surface expression of AMPARs and the surface expressing AMPARs are constitutively internalized through both basal PKC and MEK–ERK1/2 (extracellular signal-regulated kinase 1/2) activities at PF–PC synapses.

Key words: AMPA receptor; trafficking; PKC; ERK1/2; actin; LTD

Introduction

Most of the excitatory synaptic transmission in the mammalian CNS is mediated by AMPA receptors (AMPA). In the hippocampus, AMPARs containing glutamate receptor 1 (GluR1) subunits are inserted into the synaptic membrane in an activity-dependent manner (Shi et al., 1999), whereas AMPARs containing GluR2/3 subunits are inserted constitutively (Lüscher et al., 1999) and recycled rapidly (Man et al., 2000; Shi, 2001; Bredt and Nicoll, 2003; Collingridge et al., 2004). The regulation of the constitutive AMPAR trafficking provides mechanisms for the activity-dependent long-term depression (LTD) of synaptic efficacy (Malinow and Malenka, 2002), which is elicited by the activation of NMDA receptors (NMDARs) (Liu et al., 2004) or metabotropic glutamate receptor 1 (mGluR1) (Xiao et al., 2001) in the hippocampus. The downstream mechanism of NMDAR-dependent AMPAR trafficking is regulated by Ca^{2+} -monomeric GTP-binding proteins-MAPK (mitogen-activated protein kinase) signaling (Rush et al., 2002; Zhu et al., 2002, 2005; Morishita et al., 2005), and actin polymerization (Okamoto et al., 2004; Morishita et al., 2005).

In the cerebellum, LTD at parallel fiber (PF)–Purkinje cell (PC) synapses, whose AMPARs are mainly composed of a combination of GluR2/3 subunits, is induced by the associative activation of PF and climbing fiber synapses, which contributes to a certain form of motor learning and motor coordination (for review, see Ito, 2001). The internalization of AMPAR through clathrin-dynamin-dependent endocytosis is an underlying mechanism of cerebellar LTD (Wang and Linden, 2000). Although there are similarities between hippocampal LTD and cerebellar LTD, NMDARs are lacking in cerebellar PCs; therefore, how clathrin-dynamin-dependent AMPAR endocytosis is modulated remains unclear. Cerebellar LTD induction requires AMPAR activation (Linden et al., 1991), a large Ca^{2+} concentration

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tion increase (Konnerth et al., 1992), and the mGluR-dependent activation of protein kinase C (PKC) (Xia et al., 2000; Chung et al., 2003). Indeed, cerebellar LTD requires extracellular signal-regulated kinase 1/2 (ERK1/2) activity (Kawasaki et al., 1999). The regulation of rapid recycling of AMPARs at PF–PC synapses could be a possible mechanism underlying cerebellar LTD induction, similarly to hippocampal LTD. However, intracellular signals controlling constitutive AMPAR trafficking is not well understood in cerebellar PCs. In the present study, we demonstrated that tetanus toxin (TeTx) [a blocker of exocytosis mediated by vesicle-associated membrane protein (VAMP)] infusion into PCs caused the rundown of PF-EPSC amplitude through the constitutive AMPAR internalization, and then addressed the question of how intracellular signals involved in cerebellar LTD induction modify the constitutive AMPAR internalization at PF–PC synapses.

Materials and Methods

Sagittal slices (300 μ m) of the cerebellar vermis were prepared from rats [postnatal day 15 (P15) to P24] and kept in artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 1 MgSO_4 , 2 CaCl_2 , 10 D-glucose, and 0.1 picrotoxin, bubbled with 95% O_2 and 5% CO_2 . Whole-cell voltage-clamp recordings were performed at 30–31°C using an Axopatch-200B amplifier and pCLAMP 9.0 software (Molecular Devices, Foster City, CA). Patch pipettes (2.5–4 M Ω) were filled with a solution containing the following (in mM): 110 K-gluconate, 30 KCl, 10 HEPES, 4 MgCl_2 , 0.3 EGTA, 4 Na_2ATP , 0.4 Na_3GTP , and 1 glutathione; the pH was adjusted to 7.3 with KOH. Inactive TeTx was prepared by boiling it for 10 min at 100°C. An electrode for PF stimulation was placed in the molecular layer. Primary dissociated cerebellar culture was prepared from embryonic day 20 rat embryos. After 21–25 d *in vitro*, electrophysiological recordings were performed in ACSF buffered with HEPES (10 mM), and bubbled with O_2 . For recording from cultured PCs, a single granule cell (GC) was stimulated to elicit GC–PC EPSC.

EPSC amplitude was normalized by basal EPSC amplitude recorded within 3–5 min after establishing the whole-cell recording configuration. To obtain paired-pulse facilitation ratio (PPFR), paired stimulations were applied (interstimuli interval of 50 ms). Series resistance was not compensated, but data were discarded when series resistance varied by more than $\pm 10\%$. Signals were filtered at 1 kHz and digitized at 10 kHz. All values are shown as mean $\pm$ SEM. Statistical comparisons were performed using the Mann–Whitney *U* test. Drugs added to the pipette solution were purchased from commercial sources: 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis, MO), tetanus toxin (List Biological, Campbell, CA), botulinus toxin C (Wako, Osaka, Japan), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976), (–)-indolactamV, okadaic acid, cyclosporin A, 2-(2-diamino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), jasplakinolide, and latrunculinA (Calbiochem, San Diego, CA). The final DMSO or EtOH concentration in the pipette solution was 0.01%.

TeTx was activated with 10 mM DTT and mixed with 120 ng of the recombinant VAMP2 protein in cleavage buffer (10 mM NaPO_4 and 150 mM NaCl, pH 7.4). After incubation (1 h; 37°C), cleavage reaction was stopped by the addition of SDS sample buffer. Cleaved proteins were separated on a 12.5% polyacrylamide gel and detected using a silver stain kit (Wako).

Surface GluR2 was first labeled with a monoclonal antibody against the N terminus of GluR2 (Chemicon, Temecula, CA) (30 min; room temperature). After incubation (30 min; 37°C), cells were fixed in 4% paraformaldehyde. An Alexa 546-conjugated secondary antibody was applied under nonpermeant condition, and then the Alexa 488-conjugated secondary antibody was applied under permeant condition (Man et al., 2000). A polyclonal antibody against calbindin D was also used to identify PCs, which were then stained with the secondary antibody conjugated with Alexa 633. Fluorescent images were acquired and

Figure 1. Suppression of PF-EPSC by TeTx in PCs. **A**, Effects of TeTx (150 nM; $n = 11$) or inactivated TeTx ($n = 8$) on PF-EPSC. Interruption of PF stimulation (Stim) had no effect on TeTx rundown ($n = 8$). Current traces show EPSCs at 3–5 min (black) or 26–30 min (gray). **B**, Dose-dependent effects of TeTx (50 nM, $n = 5$; and 300 nM, $n = 8$) on PF-EPSC. **C**, Effect of BoTx (1 μ M; $n = 6$) on PF-EPSC. Error bars indicate SE. **D**, VAMP2 proteolysis by TeTx.

quantified using a confocal laser scan microscope (FV500; FluoView; Olympus, Tokyo, Japan). GluR2 internalization ratio in designated areas of PC dendrites was obtained as blue fluorescence intensity (internalized) divided by the total (blue plus red) fluorescence intensity.

Results

Blocking of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-dependent exocytosis

To examine whether the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent constitutive AMPAR trafficking is involved in AMPAR regulation at PF–PC synapses, we applied TeTx (150 nM), a blocker of VAMP, into PCs in cerebellar slices through a patch pipette. Within 6–8 min after establishing the whole-cell configuration, PF-EPSC amplitude started to decrease, and this rundown reached a steady state within 20–30 min ($60.0 \pm 2.2\%$; $n = 11$; $p < 0.01$) (Fig. 1A). This decrease in PF-EPSC amplitude was not accompanied by changes in the kinetics of EPSC (10–90% rising time, 1.94 ± 0.15 and 1.92 ± 0.16 ms; $p = 0.67$; and decay time constant, 12.3 ± 1.1 and 12.9 ± 1.4 ms; $p = 0.53$) or the PPFR (1.90 ± 0.06 and 2.05 ± 0.07 ; $p = 0.29$) of PF-EPSCs, suggesting that TeTx infusion decreased the number of synaptic AMPARs at PF–PC synapses; thus hereinafter this decrease in PF-EPSC amplitude is referred to as TeTx rundown. Heat-inactivated TeTx had no effect on PF-EPSC ($100.3 \pm 1.7\%$; $n = 8$) (Fig. 1A). To examine whether TeTx rundown is activity dependent or constitutive, PF stimulation was interrupted during the 5–20 min period after the onset of recording. This interruption of PF stimulation did not alter the extent of TeTx rundown ($60.2 \pm 3.3\%$; $n = 8$; $p = 0.94$) (Fig. 1A), indicating that TeTx rundown was caused by the constitutive elimination of synaptic AMPARs. TeTx at a low concentration (50 nM) did not cause a significant decrease in PF-EPSC amplitude ($90.3 \pm 2.8\%$; $n = 5$) (Fig. 1B), but TeTx at 150 and 300 nM

induced the maximum EPSC rundown ($63.5 \pm 1.5\%$; $n = 8$; 300 nM TeTx) (Fig. 1B), indicating that TeTx in 150 nM caused saturated reduction in PF-EPSC amplitude. To examine the possibility that TeTx-insensitive VAMP contributes to AMPAR trafficking at the steady state after TeTx rundown, we examined the effect of botulinum toxin C (BoTx C) ($1 \mu\text{M}$), a blocker of syntaxin. BoTx C infusion caused a similar rundown of PF-EPSC amplitude at the same steady state ($64.1 \pm 1.6\%$; $n = 6$) (Fig. 1C), suggesting that the contribution of TeTx-insensitive VAMP to AMPAR trafficking at the steady state after TeTx rundown was unlikely. To assess the concentration–activity relationship of TeTx, VAMP2 proteolysis by TeTx was examined *in vitro*. The proteolytic activity of TeTx was saturated at 150 nM and completely inactivated by boiling (Fig. 1D).

To test a hypothesis that AMPARs at the steady-state level after TeTx rundown are stabilized at PF–PC synapses, GluR2 internalization ratio was measured by immunocytochemical analysis using dissociated PCs in culture. After the 30 min recording of GC–PC EPSC through a patch pipette filled with or without TeTx, the GluR2 proteins were stained immunocytochemically using the monoclonal antibody against its N terminus (Man et al., 2000). In dissociated PCs in culture, TeTx infusion caused the rundown of GC–PC EPSC ($38.2 \pm 5.5\%$; $n = 6$) (Fig. 2B). GluR2 subunits were constitutively internalized in control PCs (Fig. 2A, top), whereas few GluR2 subunits were internalized in TeTx-infused PCs (Fig. 2A, bottom). The GluR2 internalization ratio was smaller in TeTx-infused PCs (0.25 ± 0.03 ; $n = 7$; $p < 0.01$) than that in control PCs (0.49 ± 0.02 ; $n = 7$), indicating that AMPARs remaining at the steady state after TeTx rundown are relatively stable at GC–PC synapses.

Involvement of PKC and mitogen-activated protein kinase kinase–ERK1/2 in constitutive endocytotic AMPAR elimination

Cerebellar LTD requires specific isoforms of conventional PKC (cPKC), namely, PKC α (Leitges et al., 2004); however, whether cPKC regulates the constitutive AMPAR trafficking at PF–PC synapses remains unclarified. Thus, we examined the effect of Gö6976, a selective inhibitor of cPKC isoforms, on TeTx rundown. Gö6976 (100 nM) significantly suppressed this rundown when applied through a patch pipette ($77.7 \pm 2.1\%$; $n = 14$; $p < 0.01$ compared with the control DMSO) (Fig. 3A,E). DMSO (0.03%) did not change the TeTx rundown ($62.5 \pm 1.0\%$; $n = 7$; $p = 0.34$) (Fig. 3A,E). However, the activities of mitogen-activated protein kinase kinase (MEK)–ERK1/2 are required for cerebellar LTD induction (Kawasaki et al., 1999; Endo and Launey, 2003), the effects of MEK–ERK1/2 activities on the constitutive AMPAR elimination from PF–PC synapses are unknown. Thus, we examined the effect of PD98059, a specific MEK inhibitor, on TeTx rundown. PD98059 (10 μM) significantly minimized TeTx rundown ($80.7 \pm 0.4\%$; $n = 10$; $p < 0.01$ compared with the control DMSO) (Fig. 3B,E), indicating the involvement of MEK–ERK1/2 activities in the constitutive elimination of synaptic AMPARs. To examine whether the effects of cPKC and MEK–ERK1/2 are additive, both Gö6976 and PD98059 were applied simultaneously. Because simultaneous application of both inhibitors caused the complete inhibition of TeTx rundown ($96.5 \pm 3.9\%$; $n = 10$; $p < 0.01$ compared with the control DMSO) (Fig. 3B,E), cPKC and MEK–ERK1/2 were shown to act on TeTx rundown additively.

PKC activity enhancement induced the LTD of PF-EPSC in cultured PCs (Linden et al., 1992), but whether PKC activity enhancement increases TeTx rundown is unknown. We then ex-

Figure 2. GluR2 internalization of GC–PC synapses. **A**, LY (fluorescent image of Lucifer yellow) infused into PCs through the pipette with or without TeTx. Surface GluR2 (red) under nonpermeant condition (Non-perm) and internalized GluR2 (blue) under permeant condition (Perm) were visualized using the antibody against the N terminus of GluR2. Scale bar, 5 μm . **B**, Effects of TeTx on GC–EPSC recorded from dissociated PCs. Error bars indicate SE.

amined the effect of PKC activators on TeTx rundown. Neither TPA (100 nM; $59.9 \pm 3.7\%$; $n = 7$; $p = 0.88$) nor (–)-indolactamV (1 μM ; $59.8 \pm 4.9\%$; $n = 8$; $p = 0.30$) enhanced TeTx rundown (Fig. 3C,E), suggesting that basal PKC activity was sufficient for TeTx rundown.

The suppression of the basal activities of protein phosphatases (PPs) elicited cerebellar LTD (Eto et al., 2002; Launey et al., 2004). Okadaic acid (OA) (100 nM), an inhibitor of PP1/2A, enhanced TeTx rundown only slightly (53.2 ± 1.6 ; $n = 8$; $p < 0.05$) (Fig. 3D,E), suggesting that the basal activities of PP1/2A affecting TeTx rundown were weak. We also tested the effect of PP2B inhibitor on TeTx rundown. Cyclosporin A (100 nM) did not affect TeTx rundown when applied through a patch pipette ($64.1 \pm 3.6\%$; $n = 8$) (Fig. 3D,E). The simultaneous infusion of OA and TPA enhanced TeTx rundown maximally ($49.3 \pm 2.9\%$; $n = 9$; $p < 0.01$) (Fig. 3D,E), but almost one-half of AMPARs remained at PF–PC synapses. The simultaneous infusion of PD98059 and Gö6976 without TeTx did not affect PF-EPSC amplitude ($127.4 \pm 9.9\%$; $n = 6$; data not shown). None of these inhibitors or activators affected TeTx proteolytic activity *in vitro* (Fig. 3F).

Actin polymerization level and TeTx-resistant AMPARs

The balance between actin polymerization and depolymerization has been shown to regulate synaptic plasticity in hippocampal pyramidal neurons (Okamoto et al., 2004). LatrunculinA (10 μM), an inhibitor of actin polymerization, significantly decreased

Figure 3. Protein phosphorylation and TeTx rundown of PF-EPSC. **A**, Effects of PKC α -selective inhibitor, Gö6976 (100 nM; $n = 11$) or vehicle (0.03% DMSO; $n = 8$) on TeTx rundown. **B**, Effects of MEK inhibitor PD98059 (10 μ M; $n = 10$) or simultaneously applied Gö6976 and PD98059 ($n = 8$) on TeTx rundown. **C**, Effects of TPA (100 nM; $n = 8$) or indolactamV (1 μ M; $n = 7$) on TeTx rundown. **D**, Effects of PP1/2A inhibitor OA (100 nM; $n = 8$), PP2B inhibitor cyclosporin A (100 nM; $n = 8$), or simultaneously applied OA and TPA ($n = 8$) on TeTx rundown. **E**, PF-EPSC amplitude (26–30 min) normalized by amplitude of baseline response (3–5 min). * $p < 0.05$; ** $p < 0.01$. Error bars indicate SE. **F**, Effects of chemicals on VAMP2 proteolysis by TeTx conc., Concentration; ctrl., control; Cyclo, cyclosporin A; Go, Gö6976; Indo, (–)-indolactamV; PD, PD98059.

the steady-state level of TeTx rundown ($51.0 \pm 2.0\%$; $n = 9$; $p < 0.05$) (Fig. 4A), whereas jasplakinolide (1 μ M), an inhibitor of actin depolymerization, significantly increased this level ($77.9 \pm 3.3\%$; $n = 9$; $p < 0.01$) (Fig. 4A) with no change in TeTx proteolytic activity (Fig. 4B). The effect of these inhibitors on GluR2 internalization ratio was slightly enhanced by latrunculinA treatment (0.54 ± 0.01 ; $n = 9$; $p < 0.05$) or suppressed by jasplakinolide treatment (0.41 ± 0.01 ; $n = 10$; $p < 0.01$), compared with the control DMSO (0.49 ± 0.01 ; $n = 10$) (Fig. 4C,D).

To examine whether the effects of phosphorylation-dominant condition (OA/TPA treatment) and latrunculinA on the steady state of TeTx rundown are additive, these three chemicals were applied simultaneously. The effects of these chemicals on the steady state of TeTx rundown were occluded ($50.9 \pm 2.2\%$; $n = 8$; $p = 0.63$) (Fig. 4A), indicating that almost one-half of AMPARs

at the PF–PC synapse were resistant to simultaneous treatment with TeTx, OA/TPA, and latrunculinA.

Discussion

TeTx rundown of PF-EPSCs as a measure of constitutive AMPAR elimination from PF–PC synapses

The present results demonstrate that the SNARE-dependent exocytosis is involved in constitutive AMPAR incorporation into PF–PC synapses and that TeTx rundown reflects a counterbalanced constitutive AMPAR elimination from PF–PC synapses. We conclude that TeTx rundown in PF-EPSC amplitude is a measure of AMPAR elimination through constitutive endocytosis for the following reasons: First, TeTx infusion into postsynaptic PCs caused no change in PPFR during TeTx rundown, indicating that a decrease in presynaptic release probability by a retrograde transmitter, such as endocannabinoid (Maejima et al., 2001), is unlikely. Second, no change was detected in the rising and decaying kinetics of PF-EPSC amplitude throughout the TeTx rundown, indicating that the decrease of EPSC amplitude is not attributable to changes in the kinetic properties of AMPARs. Third, the immunocytochemically measured GluR2 internalization ratio decreased after the TeTx rundown of GC–PC EPSC in dissociated PCs in culture (Fig. 2). Furthermore, the TeTx rundown of PF-EPSC is not activity dependent (Fig. 1). Thus, the TeTx rundown of PF-EPSC is concluded to reflect AMPAR elimination from PF–PC synapses through constitutive endocytosis. In hippocampal pyramidal neurons, the constitutive exocytotic incorporation and endocytotic elimination of AMPARs are balanced under normal conditions (Lüscher et al., 1999; Brecht and Nicoll, 2003). TeTx infusion into PCs blocked the exocytosis and AMPAR incorporation into PF–PC synapses, and revealed a hidden constitutive elimination of synaptic AMPARs through endocytosis.

TeTx rundown of PF-EPSC is dependent on background activities of protein kinases

Present results indicated that TeTx rundown, representing a constitutive internalization of AMPARs from PF–PC synapses, required the background activities of cPKC and ERK1/2, as confirmed by the complete suppression of TeTx rundown of PF-EPSC by the inhibition of cPKC and ERK1/2 (Fig. 3B). These inhibitors had no effects on the TeTx proteolytic activity *in vitro* (Fig. 3F). Cerebellar LTD induction requires the activation of PKC α (Perez et al., 2001; Leitges et al., 2004) or ERK1/2 (Kawasaki et al., 1999) (for review, see Ito, 2001), whereas the background PKC activity was sufficient for the constitutive endocytotic AMPAR elimination, because the PKC activator had no effect on TeTx rundown (Fig. 3C). This is in sharp contrast to cerebellar LTD induction.

Cerebellar LTD is induced by a PP2A inhibitor (Launey et al., 2004), whereas OA, a PP1/2A inhibitor, enhanced TeTx rundown only to a slight extent (Fig. 3D), suggesting that the basal activity of PP1/2A did not affect the constitutive internalization of synaptic AMPARs. Similarly, cyclosporin A, a PP2B inhibitor, did not affect the constitutive internalization of AMPARs, indicating that PP2B was not active under the basal condition and/or not involved in constitutive AMPAR internalization. This observation would be consistent with the previous finding that the application of cyclosporin A had no effect on the basal EPSC amplitudes (Belmuguenai and Hansel, 2005).

The phosphorylation of Ser880 of GluR2 by PKC causes the dissociation of GluR2 from GRIP (glutamate receptor interacting protein), and GluR2 is released from PSD (postsynaptic density),

and indeed the phosphorylation of Ser880 of GluR2 is required for cerebellar LTD induction (Xia et al., 2000). Although simultaneous infusion of OA and TPA maximized TeTx rundown (Fig. 3D), still about one-half of AMPARs remained at PF–PC synapses, suggesting that PKC activation and PP1/2A suppression are not sufficient to destabilize AMPARs in the “stable pool” at PF–PC synapses.

In both cerebellar LTD and TeTx rundown, the surface expression of AMPARs at PF–PC synapses is downregulated. However, the signal transduction mechanism differs between these two processes in the following aspects: (1) AMPAR activation is required for cerebellar LTD (Linden et al., 1991), but not for TeTx rundown (Fig. 1A); (2) a large increase in intracellular Ca^{2+} concentration (Konnerth et al., 1992) is essential for cerebellar LTD, but basal intracellular Ca^{2+} concentration is sufficient for TeTx rundown (Fig. 1A); (3) transient PKC activation is required for cerebellar LTD (Xia et al., 2000; Chung et al., 2003), but TeTx rundown requires only the background activities of PKC and ERK1/2. These differences in TeTx rundown and cerebellar LTD induction may be explicable if we assume that the constitutive endocytosis of AMPARs from PF–PC synapses is “the final common pathway” for both processes and that a large Ca^{2+} concentration increase and AMPAR activation are required only for cerebellar LTD induction.

Balance between actin polymerization and depolymerization modulates “stable synaptic pool” size of AMPARs

It should be noted that one-half of synaptic AMPARs remain as a TeTx-resistant pool, which is resistant to endocytotic elimination, even under the phosphorylation-dominant condition that might provide the maximum facilitation of rapid recycling (Fig. 3D). The steady-state level (50–60%) of TeTx rundown could indicate the size of the TeTx-resistant “stable pool” of AMPARs, whose recycling rate is slow. Indeed, GluR2 internalization ratio in TeTx-infused PCs in culture significantly decreased (Fig. 2A,C). Thus, we hypothesized that functional AMPARs at PF–PC synapses could be classified into two pools: the “constitutively recycling pool” and “stable synaptic pool.” Recently, actin cytoskeleton has been indicated to be involved in synaptic plasticity in hippocampal neurons (Okamoto et al., 2004; Zhou et al., 2004). Moreover, in the present study, the inhibition of actin depolymerization increased the size of the “stable synaptic pool” of AMPARs (Fig. 4A) and decreased the GluR2 internalization ratio (Fig. 4C,D). In contrast, the inhibition of actin polymerization decreased the size of the “stable synaptic pool” of AMPARs (Fig. 4A) and increased the GluR2 internalization ratio (Fig. 4C,D). Thus, actin polymerization is suggested to be involved in the regulation of the stable pool size of AMPARs at PF–PC synapses, in addition to regulation by AMPAR phosphorylation. However, the involvement of other factor(s) was suggested in the

Figure 4. Actin polymerization and TeTx rundown of PF-EPSC. **A**, Effects of depolymerization inhibitor jasplakinolide ($1 \mu M$; $n = 9$), latrunculinA ($10 \mu M$; $n = 9$), or simultaneously applied latrunculinA and OA/TPA on TeTx rundown. **B**, Effects of jasplakinolide or latrunculinA on VAMP2-cleaving activity of TeTx. **C**, Immunocytochemically detected surface GluR2 (red; Non-perm), internalized GluR2 (blue; Perm), and calbindin (green; Perm + Calb). DMSO (0.1%), latrunculinA ($100 \mu M$), and jasplakinolide ($10 \mu M$) were incubated (1 h) after antibody binding. Scale bar, $5 \mu m$. **D**, Actin polymerization and GluR2 internalization ratio in PCs. Error bars indicate SE. $*p < 0.05$; $**p < 0.01$. conc., Concentration; Jas, jasplakinolide; Lat A, latrunculinA.

regulation of the stable pool size of AMPARs, because about one-half of AMPARs at PF–PC synapses remained stable after the simultaneous infusion of OA/TPA and latrunculinA (Fig. 4A). AMPAR activation and a large Ca^{2+} concentration increase, required for cerebellar LTD, possibly destabilize AMPARs in the stable pool at PF–PC synapses, and destabilized AMPARs might be eliminated from PF–PC synapses by background PKC and MEK–ERK1/2 activity-dependent constitutive endocytosis.

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