Brief Communications

Transient Receptor Potential Canonical Channels Regulate the Induction of Cerebellar Long-Term Depression

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In the cerebellum, synaptic strength at the synapses between parallel fibers and Purkinje cells is best known to be modulated via metabotropic glutamate receptor 1 (mGluR1)-dependent cerebellar long-term depression (LTD). An increase in intracellular calcium levels plays an important role in inducing mGluR1-dependent cerebellar LTD. Downstream of mGluR1, there are two major sources of calcium: transient receptor potential canonical (TRPC) channels and inositol trisphosphate receptors (IP_3R). IP_3R triggers a calcium release from the intracellular calcium store. Here, we show that TRPC channels mediate mGluR1-evoked slow currents to regulate cerebellar LTD in Sprague Dawley rats. We found that the inhibition of TRPC channels blocks the induction of cerebellar LTD. Moreover, we show that processes known to underlie cerebellar LTD induction, such as increases in intracellular calcium concentration, the activation of protein kinase C, and the internalization of GluR2, are also hindered by blocking TRPC. These results suggest that the mGluR1-evoked activation of TRPC channels is required for the induction of cerebellar LTD.

Introduction

Cerebellar long-term depression (LTD) can be induced *in vitro* in brain slices by repetitive excitatory synaptic inputs from both parallel fibers (PFs) and climbing fibers (CFs) at low frequencies (Ito et al., 1982). A postsynaptic calcium increase in the Purkinje cell plays a central role in inducing LTD (Ito, 1989; Linden and Connor, 1995; Daniel et al., 1998). In dendritic spines of Purkinje cells, the activation of CF exhibits a distinct calcium increase that spans the entire dendritic branch via various voltage-gated calcium channels (VGCCs) (Schmolesky et al., 2002). Glutamate spillover from bursts of PF signaling activates metabotropic glutamate receptor type 1 (mGluR1) and increases calcium levels through two parallel pathways: extracellular calcium influx from a slow EPSC (sEPSC) (Canepari et al., 2004) and a release of calcium from internal calcium stores (Finch and Augustine, 1998). While calcium release from the internal stores is known to be induced via phospholipase Cβ activation and subsequent inositol trisphosphate production, the mechanism of calcium influx from the extracellular environment is not well understood (Okubo et al., 2004; Doi et al., 2005).

Transient receptor potential canonical (TRPC) channels in the dendritic spine of Purkinje cells are accountable for sEPSC (Kim et al., 2003). In TRPC3 knock-out mice, sEPSC was absent, and these mice showed behavior deficits (Hartmann et al., 2008). Whether the activation of TRPCs by mGluR1 is required to induce long-term changes of synaptic strength is unknown. We thus sought to elucidate the contribution of TRPCs in cerebellar LTD by blocking TRPC channels and monitoring changes in sEPSCs, LTD induction, calcium transients, and downstream signaling molecules.

Materials and Methods

Slice preparation. Parasagittal cerebellar brain slices (250 μm thick) were cut from 18- to 21-d-old Sprague Dawley rats of either sex at 2–3°C using a vibrating tissue slicer. Brain slices were incubated for 30 min at 35°C and then kept at room temperature before recording. Artificial CSF (ACSF) used for cutting contained the following (mM): 110 choline chloride, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 2.4 sodium-pyruvate, 1.3 sodium-ascorbate, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 20 D-glucose. ACSF used for incubating contained the following (mM): 124 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 2.5 NaH_2PO_4, 26.2 $NaHCO_3$, and 20 D-glucose.

Patch-clamp recordings. Brain slices were placed on an Olympus BX-50WI upright light microscope and perfused with standard ACSF containing picrotoxin (100 μM) to block the $GABA_A$ receptor. Whole-cell patch-clamp recordings were obtained from lobules 3–6 in Purkinje cells using an EPC-9 amplifier (HEKA Elektronik) at 30–32°C. A patch electrode (2.5–3.0 MΩ) was filled with the following (in mM): 135 CsMS, 10 CsCl, 10 HEPES, 0.2 EGTA, 4 Na_2-ATP, and 0.4 Na_3-GTP for sEPSC experiments; and 130 K-gluconate, 10 NaCl, 10 HEPES, 0.3 EGTA, 2 $MgCl_2$, 4 Na_2-ATP, 0.4 Na_3-GTP, and 10 Tri-phosphocreatine for the other experiments. The holding potential was −70 mV in voltage-clamp mode. Recordings were excluded if the series resistance varied by >15% and the injection current for the holding potential exceeded 600 pA. PF inputs were activated by an ACSF-filled electrode in the molecular layer and CF inputs in the granule cell layer. LTD was induced by 30 repeats of a pairing stimulation for 5 min in the current-clamp mode. Pairing stim-

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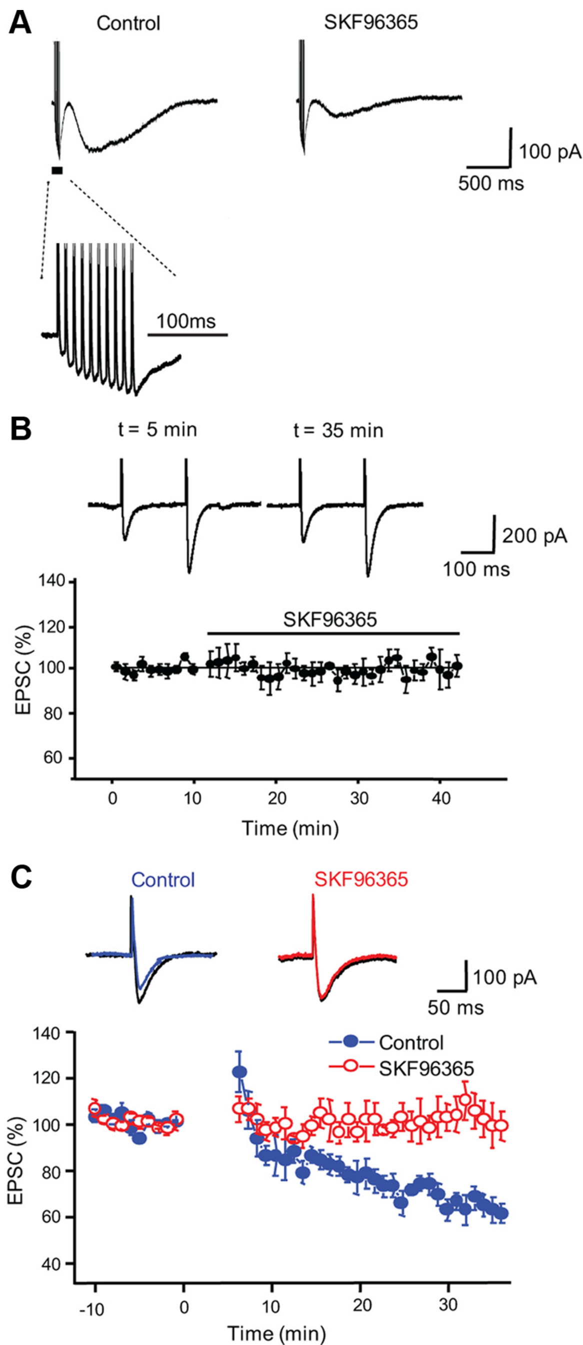

Figure 1. The TRPC blocker SKF96365 affected sEPSC and cerebellar LTD. ***A***, sEPSC was reduced by SKF96365. Shown is an sEPSC trace before and after treatment with SKF96365 (10 μM). ***B***, SKF96365 did not impair presynaptic neurotransmission. A paired pulse of PF stimulus was monitored throughout a 10 min baseline and 30 min application of SKF96365 (10 μM). Time graph showing the amplitude of EPSC ($n = 5$). ***C***, Cerebellar LTD was blocked by SKF96365. LTD was induced by 30 pairing stimulations for 5 min. Representative current trace before (black) and after the induction protocol with (red) or without (blue) SKF96365. Time graph showing the amplitude of EPSC ($n = 5$).

ulation consisted of 7 PF stimuli at 100 Hz with 1 CF stimulus delayed by 150 ms. In slow EPSC experiments, test stimulations were evoked by a 10 PF stimuli at 100 Hz every 1 min, and the ASCF contained NBQX (2.5 μM) to block the AMPA receptor.

Calcium imaging. A confocal laser-scanning fluorescent microscope (Fluoview 300, Olympus) was used to acquire calcium images during the whole-cell recording. To measure dendritic calcium transients, we replaced EGTA in the internal solution with Fluo-5F (Invitrogen). Calcium imaging was performed at least 20 min after the whole-cell configuration

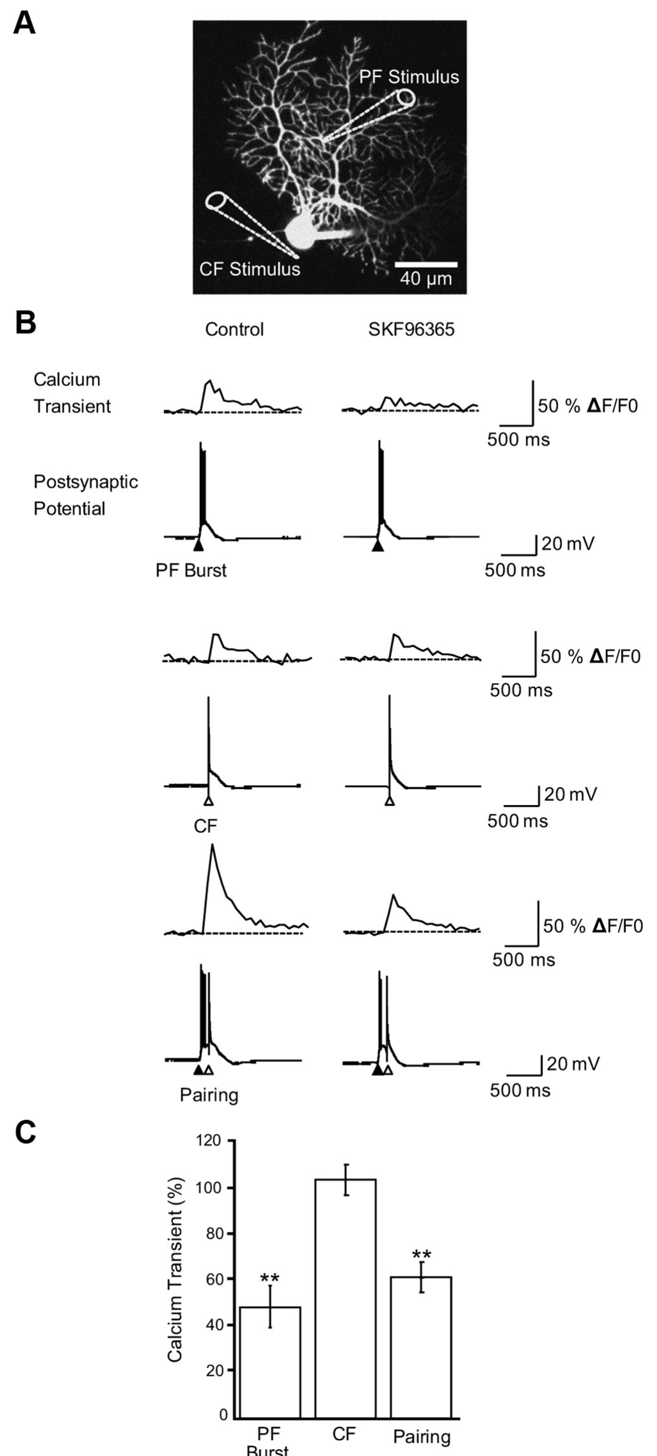

Figure 2. SKF96365 reduced dendritic calcium transients evoked by a burst of PF and pairing stimulations. ***A***, Purkinje cells were loaded with a calcium indicator (Fluo-5F, 200 μM) to measure calcium transients and postsynaptic potential simultaneously. Stimulating electrodes were placed on the molecular layer surface (to evoke PF bursts) and on the granule cell layer (for CF stimulation), as shown. ***B***, Representative calcium transient (top trace) and postsynaptic potential traces (bottom trace) are shown before and after the SKF96365 application for each stimulus applied. PF burst (closed triangle), single CF (open triangle), and pairing of PF burst and CF (closed and open triangle) test pulses produced postsynaptic potential and an accompanying calcium transient at −70 mV with current injection. ***C***, Summary bar graph shows the change in calcium transients after 15–20 min bath application of SKF96365 (10 μM; $**p < 0.01$).

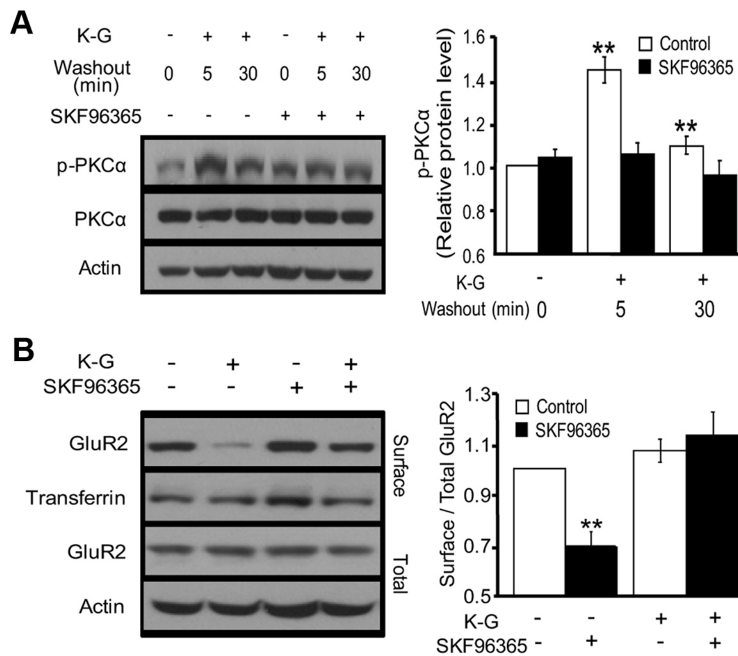

Figure 3. Activation of PKCα and GluR2 internalization via chemically induced LTD were blocked by SKF96365. ***A***, Phosphorylation of PKCα was blocked during chemically induced LTD in cerebellar slices. Lysates of cerebellar slices were immunoblotted with antibodies against p-PKCα, total PKCα, and actin. Stimulation of slices with 50 mM K^+ and 10 μM glutamate treatment led to an expression of p-PKCα that was reduced by SKF96365. The relative level of p-PKCα was quantified by densitometry (**$p < 0.01$, $n = 5$). ***B***, Internalization of AMPA receptor was blocked during chemically induced LTD in cerebellar slices. Surface level of the GluR2 was determined by slice biotinylation assay. Slices were treated with or without 10 μM SKF96365 for 1 h before K-G treatment. K-G treatment led to an internalization of GluR2 that was blocked by SKF96365. The relative ratio of surface to total GluR2 expression was calculated as (pixel intensity of surface GluR2)/(pixel intensity of total GluR2) (**$p < 0.01$, $n = 5$).

to allow the dendritic perfusion of Fluo-5F. The pairing stimulus for LTD induction was used. Images were analyzed in Fluoview (Olympus).

Immunoblotting analysis. Lysates were denatured at 90°C and loaded by 5% SDS-PAGE gel. Proteins were transferred to PVDF; blocked with PBS containing 0.03% Tween 20 and 5% skim milk for 1 h; and incubated using mouse anti-GluR2 (BD Bioscience), mouse anti-protein kinase Cα (PKCα) (Chemichon), rabbit anti-phosphorylated PKCα (Chemichon), mouse anti-transferrin (Zymed), or rabbit anti-actin (Abchem). A horseradish-peroxidase-conjugated goat IgG as secondary antibody (Santa Cruz Biotechnology) was used. The bands were developed with the SuperSignal chemiluminescent detection kit (Pierce). For the analysis, bands were quantified using ImageJ (NIH). At least five independent experiments were conducted.

Slice biotinylation assay. Cerebellar slices (200 μm) were kept in standard ACSF containing 0.5 mg/ml sulfo-NHS-LC-Biotin (Pierce) for 1 h on ice, and bubbled with 95% O_2 and 5% CO_2. Unreacted biotinylation reagent was washed once with ice-cold ACSF, followed by two washes in ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl). Slices were briefly sonicated in homogenization buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, and protease inhibitor cocktail, Sigma-Aldrich). After sonication, the samples were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was obtained. The protein concentration in the soluble fraction was then measured by Bradford assay. Biotinylated protein (400 μg) in the supernatant was precipitated with 50 μl of 50% Neutravidin agarose (Pierce) for 16 h at 4°C and washed four times with homogenization buffer. Bound protein was eluted with SDS sample buffer by boiling it for 10 min. To quantify the total receptor expression, aliquots of the lysates were saved for immunoblotting.

Drugs and statistics. All drugs were purchased from Sigma except for NBQX, (s)-DHPG (Tocris Cookson). Anti-TRPC antibodies were obtained from Alomone Labs (defined as TRPC antibody), and preincubated antibodies were prepared at room temperature by incubating them with the control peptide for at least 2 h. All group data are shown as the mean ± SEM. For statistical analysis, we used a Student's paired *t* test.

Results

Whole-cell voltage-clamp recordings on cerebellar Purkinje cells were performed in sagittal brain slices. ACSF was complemented with a GABA receptor antagonist, picrotoxin (100 μM). To readily evoke sEPSCs with mild stimulating intensity, the slice was perfused with the AMPA receptor antagonist NBQX (2.5 μM) to block ~90% of the AMPA receptor responses (Fig. 1*A*). To identify the functional role of TRPC on an mGluR1-evoked sEPSC, we applied the TRPC channel blocker SKF96365 (10 μM). With SKF96365, a significant reduction of sEPSC was observed, leaving the fast component unaltered [Fig. 1*A*; fast EPSC (fEPSC) 96.3 ± 2.2%; sEPSC, 43.8 ± 8.3%, $n = 5$]. Without NBQX in the bath solution, SKF96365 had no effect on the amplitude of the EPSC of the AMPA receptors (Fig. 1*B*; 100.3 ± 1.5%, $n = 5$) or the paired-pulse ratio (PPR; 101.5 ± 1.7%, $n = 5$), indicating that presynaptic glutamate transmission was not altered by SKF96365.

Next, we electrically induced LTD to test whether TRPC channels are involved in the induction of cerebellar LTD. Cerebellar slices were preincubated for at least 30 min with SKF96365 (10 μM) before whole-cell configuration. LTD was induced in the current-clamp mode for 5 min with the protocol of 30 repeated parallel fiber bursts and single climbing fiber pairing every 10 s (Safo and Regehr, 2008). In the presence of SKF96365, LTD was blocked (Fig. 1*C*; control, 63.8 ± 1.2%, $n = 5$; SKF96365, 101.0 ± 2.1%, $n = 5$), while PPR was not changed in either the control condition or with SKF96365 (Fig. 1*C*; control, 102.1 ± 2.0%, $n = 5$; SKF96365, 99.8 ± 1.5%, $n = 5$). In Purkinje cells, a high concentration of SKF96365 (30 μM) was reported to partially affect low voltage-activated T-type calcium channels (Singh et al., 2010). Although we used a lower concentration of SKF96365 (10 μM), a partial blocking of the T-type calcium channel by SKF96365 may have inhibited the induction of LTD. We therefore tested the effect of NNC55–0396 (10 μM), a specific T-type calcium channel blocker, on the induction of cerebellar LTD. LTD, however, was not affected by NNC55–0396, suggesting that the blocking of cerebellar LTD by SKF96365 was not mediated by the inhibition of T-type calcium channels (data not shown).

To test whether SKF96365 affects the calcium transients during LTD induction, we performed dendritic calcium imaging (Fig. 2). EGTA was replaced by the calcium indicator (Fluo-5F) in the internal solution. Twenty minutes after whole-cell configuration, to allow the dendritic perfusion of Fluo-5F, calcium transients were measured before and after SKF96365 application. As test stimuli, a pairing stimulus used in LTD induction and each of the components of pairing, a burst of PF stimulus or a single CF stimulus, was applied. We observed that calcium transients resulting from the brief burst of PF stimulus and pairing stimulation were reduced by SKF96365, but the calcium transients resulting from a single CF stimulus through VGCC was not affected by SKF96365 (Fig. 2; PF burst, 48.2 ± 9.2%; CF, 103.52 ± 6.68%; pairing, 61.3 ± 6.4%, $n = 5$).

To further verify that the TRPC channel is involved in cerebellar LTD, the internalization of AMPA receptors was monitored. In cerebellar LTD, the activation of PKC is dependent on calcium, and activated PKC phosphorylates AMPA-type glutamate receptors (Hirono et al., 2001; Chung et al., 2003). Biochemical assays were used to determine the activated PKC during the LTD induction period. LTD was chemically induced by a combined application of 50 mM K^+ and 10 μM glutamate for 5 min to cerebellar slices (K-G treatment) (Tanaka and Augustine, 2008). The K-G treatment increased phospho-PKCα (p-PKCα) in the lysates of the cerebellar slices (Fig. 3*A*; 1.4 ± 0.1 at 5 min, 1.1 ± 0.1 at 30 min, $p < 0.01$, $n = 5$). This increase in p-PKCα was reduced by the application of SKF96365, indicating that PKCα activation by chemically induced LTD requires an activation of TRPC channels. Next, a slice biotinylation assay was performed to investigate the internalization of the AMPA receptors. The surface level of GluR2 decreased after the K-G treatment in the slices, without any change in the total protein level of GluR2 (Fig. 3*B*; 0.7 ± 0.1, $p < 0.01$, $n = 5$). This decrease in the surface expression of GluR2 was blocked by SKF96365, indicating that the internalization of AMPA receptors under the chemically induced LTD requires the activation of the TRPC channels.

To selectively block a certain type of TRPC channel in cerebellar LTD induction, Purkinje cells were loaded with specific antibodies directed against a cytoplasmic epitope of TRPC3 in the internal solution (0.5 μg/ml) (Alomone Labs) (Albert et al., 2006; Alvarez et al., 2008). TRPC3 was selected among various subtypes of the TRPC family since the sEPSC in Purkinje cells is absent in TRPC3 knock-out mice (Hartmann et al., 2008). We observed that mGluR1-evoked sEPSC gradually reduced for up to 30 min after achieving a whole-cell configuration while leaving the fast component unaltered (Fig. 4*A*; fEPSC, 93.3 ± 9.4%, $n = 6$). sEPSC were significantly reduced to 16.8 ± 4.1% ($n = 6$) of the initial response in the TRPC3 antibody-loaded cells. Preincubated antibodies had no effect on the amplitude of fEPSC or sEPSC (Fig. 4*A*; fEPSC, 101.0 ± 8.2%; sEPSC, 98.9 ± 4.9%; $n = 6$). We next hypothesized that TRPC3 antibodies, which showed a significant reduction of sEPSC, would obstruct the induction of the cerebellar LTD. Considering the delay time of antibodies to diffuse into the dendrites (30 min after whole-cell configuration), LTD was completely blocked in the presence of TRPC3 antibodies (Fig. 4*B*; control, 59.1 ± 2.3%, $n = 9$; TRPC3, 101.4 ± 5.7%, $n = 12$), and PPR was not affected by either preincubated TRPC3 antibody or TRPC3 antibody (Fig. 4*B*; control, 98.5 ± 4.0%; TRPC3, 93.3 ± 6.2%). These results demonstrate that the TRPC3 channels are necessary for the induction of cerebellar LTD.

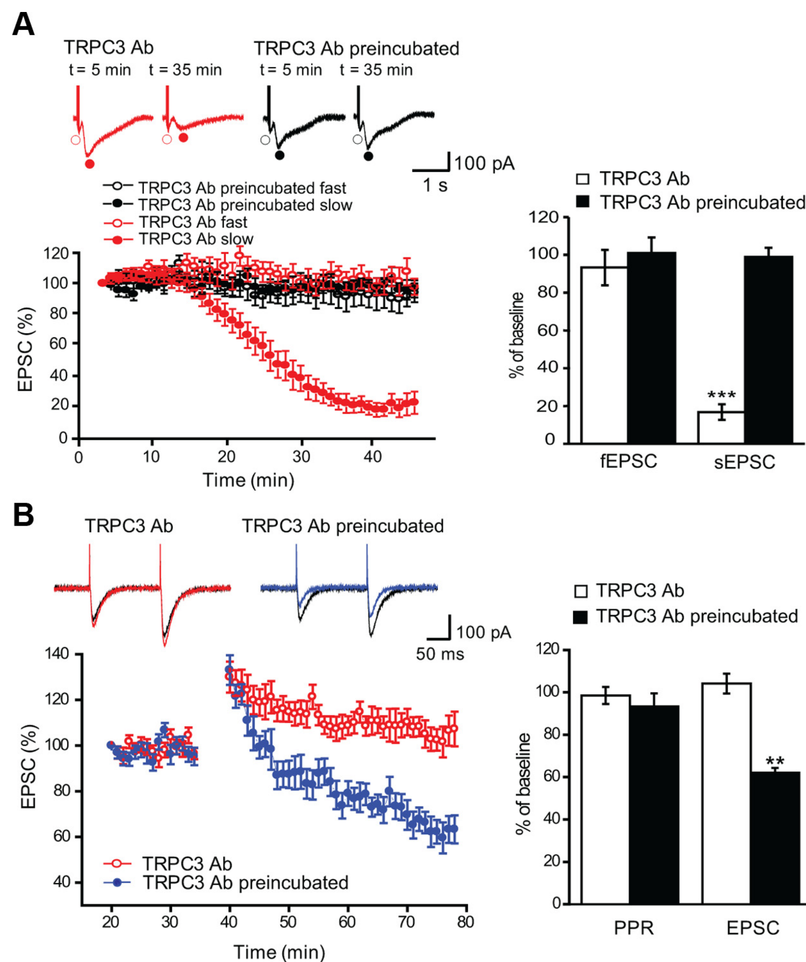

Figure 4. TRPC3 antibody reduced mGluR1-mediated slow EPSC and blocked cerebellar LTD. ***A***, Application of TRPC3 antibody reduced sEPSC. Representative current traces showing parallel fiber burst-evoked slow EPSC with TRPC3 antibody ($n = 6$) and preincubated TRPC3 antibody ($n = 10$). Time course graph showing sEPSC amplitude reduced over time, which indicates the diffusion time course from the patch pipette to the dendrite spine. Summary bar graph displays a significant decrease of sEPSC in the presence of TRPC3 antibody (***$p < 0.001$). ***B***, Cerebellar LTD was impaired in the presence of TRPC3 antibody. Representative current traces of EPSC before induction (black) and after treatment with TRPC3 antibody (red, $n = 10$) or preincubated TRPC3 antibody (blue, $n = 11$) in the internal solution (0.5 μg/ml). Time course graph indicates the percentage change of EPSC amplitude before and after LTD induction. LTD was completely blocked in the TRPC3 antibody-applied group. Summary bar graph shows the numerical value of the change in EPSC and the paired-pulse ratio in cells applied with the set of antibodies (**$p < 0.01$).

Discussion

We used SKF96365 and TRPC-specific antibodies to examine the role of TRPC channels in the induction of cerebellar LTD. Our observations indicate that the TRPC channel is required for the mGluR1-evoked sEPSC and the consequent cerebellar LTD. The underlying mechanisms of cerebellar LTD, such as an increase in calcium transients, the consequent activation of PKC, and the internalization of AMPA receptors, were all blocked by SKF96365.

While SKF96365 has been used extensively as a tool to define the functional roles of TRPC channels in various cell and tissue types (Bengtson et al., 2004; Fowler et al., 2007), previous studies reported notable overlapping effects on numerous channels in various organs (Merritt et al., 1990; Hong and Chang, 1994; Schwarz et al., 1994; Juvin et al., 2007; Liu et al., 2007). A high concentration of SKF96365 (30 μM) partly affected low voltage-activated T-type calcium channels in Purkinje cells (Singh et al., 2010). This T-type calcium channel is responsible for shaping complex spikes by CF stimulation and subsequent dendritic cal-

cium transients (Cavelier et al., 2008). In this study, we used a lower concentration of SKF96365 (10 μM) and did not observe a significant difference in dendritic calcium transients in response to CF stimulus, suggesting that the inhibition of T-type calcium channels at this concentration was limited. We also tested cerebellar LTD with a specific T-type blocker using the same concentration that was used to study T-type VGCC. NNC 55–0396 did not affect the induction of cerebellar LTD. Further investigations need to be conducted to fully assess the effect of SKF96365.

Intracellular calcium potentiates the G-protein-coupled receptor-evoked activation of TRPC channels (Blair et al., 2009). In Purkinje cells, during the pairing stimulation of PF and CF for LTD induction, calcium influx from VGCC by CF activation may potentiate the mGluR1-evoked activation of the TRPC. This potentiated activation of calcium-permeable TRPC channels may contribute to a supralinear calcium signal, a characteristic of greater calcium influx than the sum of each PF burst and CF inputs when both stimuli are paired (Wang et al., 2000). Since supralinear calcium signal is crucial in cerebellar LTD (Wang et al., 2000; Tanaka et al., 2007), a TRPC-mediated supralinear calcium signal may be necessary for LTD induction. While the present study suggests that TRPC channels participate in calcium signaling during PF burst, a near-to-normal dendritic calcium signal during PF burst was observed in TRPC3 KO mice (Hartmann et al., 2008). This unaffected calcium signaling may result from a compensatory increase in calcium release from an intracellular calcium store in the TRPC3 KO mouse. Alternatively, this observation may also be explained by the difference in experimental conditions. For instance, we used a low-affinity calcium indicator, Fluo-5F, to minimize exogenous calcium buffering that might distort the kinetics of calcium transients and detection. The pharmacological inhibition of both mGluR1-evoked EPSC and its associated dendritic calcium signal is consistent with our data (Canepari et al., 2004). Further studies are needed to fully assess the detailed calcium profiles of LTD and the contributions of TRPC3 in this process.

Our data have shown that TRPC3 is required for the induction of cerebellar LTD, which has long been considered as the molecular basis of motor coordination and cerebellar motor learning (Aiba et al., 1994; Kim and Thompson, 1997; De Zeeuw et al., 1998; Feil et al., 2003; Boyden et al., 2006; Hansel et al., 2006). Specifically in TRPC3 knock-out mice, the walking behavior and sEPSC induced through the brief burst of a PF stimulus are impaired (Hartmann et al., 2008). It might thus be useful to study the role of TRPC3 in cerebellar Purkinje cells to obtain a better understanding of plasticity occurring at the PF–Purkinje cell synapse.

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