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A Unique PDZ Ligand in PKCα Confers Induction of Cerebellar Long-Term Synaptic Depression

Report

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Summary

Induction of cerebellar long-term depression (LTD) requires a postsynaptic cascade involving activation of mGluR1 and protein kinase C (PKC). Our understanding of this process has been limited by the fact that PKC is a large family of molecules, many isoforms of which are expressed in the relevant postsynaptic compartment, the cerebellar Purkinje cell. Here, we report that LTD is absent in Purkinje cells in which the α isoform of PKC has been reduced by targeted RNA interference or in cells derived from PKCα null mice. In both of these cases, LTD could be rescued by expression of PKCα but not other PKC isoforms. The special role of PKCα in cerebellar LTD is likely to derive from its unique PDZ ligand (QSAV). When this motif is mutated, PKCα no longer supports LTD. Conversely, when this PDZ ligand is inserted in a nonpermissive isoform, PKCγ, it confers the capacity for LTD induction.

Introduction

Cerebellar LTD is a persistent attenuation of the parallel fiber-Purkinje cell synapse, which is induced by repeated coactivation of parallel fiber and climbing fiber synapses, as is suggested to occur in certain forms of motor learning, such as associative eyelid conditioning and adaptation of the vestibulo-ocular reflex (Bear and Linden, 2000; Hansel et al., 2001). Induction of cerebellar LTD is blocked by postsynaptic application of a wide range of PKC inhibitor drugs (Linden and Connor, 1991; Hartell, 1994; Freeman et al., 1998) or by postsynaptic expression of a PKC autoinhibitory domain peptide (De Zeeuw et al., 1998; Goossens et al., 2001) and is mimicked and occluded by the application of exogenous PKC activators (Crépel and Krupa, 1988; Linden and Connor, 1991; Chung et al., 2003). Cerebellar LTD is expressed by phosphorylation of ser-880 in the carboxy-terminal PDZ ligand motif of the AMPA receptor GluR2 (Chung et al., 2003), which follows PKC activation. This phosphorylation event promotes binding of the PDZ domain containing protein PICK1 (Matsuda et al., 1999; Chung et al., 2000; Xia et al., 2000; Perez et al., 2001) and ultimately results in clathrin-mediated internalization of postsynaptic AMPA receptors (Matsuda et al., 2000; Wang and Linden, 2000).

However, PKC comprises a family of 11 isoforms (Nishizuka, 2001), at least eight of which (α, βI, γ, δ, ϵ, η, ζ, and λ) have been reported to be expressed in cerebellar Purkinje cells (Shimohama et al., 1990; Wetsel et al., 1992; Bareggi et al., 1996; Garcia and Harlan, 1997; Barmack et al., 2000). Constructing a molecular model of cerebellar LTD has been limited by the inability to identify the particular isoform(s) of PKC involved in LTD induction: to date, the only attempt to address this point directly has involved the creation of a PKCγ null mouse that shows normal cerebellar LTD (Chen et al., 1995). Here we have used whole-cell patch-clamp recording from cultured cerebellar Purkinje cells together with both double-stranded RNA (dsRNA) knockdown and genetic deletion of various PKC isoforms to identify those isoforms that mediate cerebellar LTD.

Results

Because LTD induction requires activation of mGluR1 (Linden et al., 1991; Shigemoto et al., 1994) and this results in the production of 1,2-diacylglycerol and mobilization of Ca^{2+} from internal stores, we initially focused our attention on the "classical" PKC isoforms that are activated by Ca^{2+} and diacylglycerols: α, β, and γ. dsRNAs were constructed to target unique sequences in mouse mRNA for the three classical PKC isoforms. 21-base segments with dTdT overhangs were synthesized and coated onto gold particles for biolistic transfection together with a marker plasmid driving expression of enhanced green fluorescent protein (EGFP). After 20–54 hr, cultures of embryonic mouse cerebellum were fixed and stained with antibodies against PKCα, -β, and -γ. This revealed that dsRNA treatment was both potent and specific: for example, 8% of Purkinje cells transfected with PKCα dsRNA were immunoreactive for PKCα protein, while 95% of these same cells were PKCβ and 96% PKCγ immunoreactive ($p < 0.0001$ for each comparison by Student's t test). Similar potency and specificity of knockdown was seen for PKCβ and PKCγ dsRNAs (Figure 1A). As a control, the sequence of PKCα dsRNA was scrambled, and this produced no significant reduction of immunoreactivity for any of the three classical PKC isoforms.

With these tools in hand, we then asked whether cerebellar LTD was affected by these manipulations. Initially, this involved using a well-defined culture model system (Linden et al., 1991; Chung et al., 2003) in which parallel fiber input is replaced by iontophoretic pulses of glutamate and climbing fiber input is replaced by a 3 s long step depolarization to 0 mV. In this system, GABA-A

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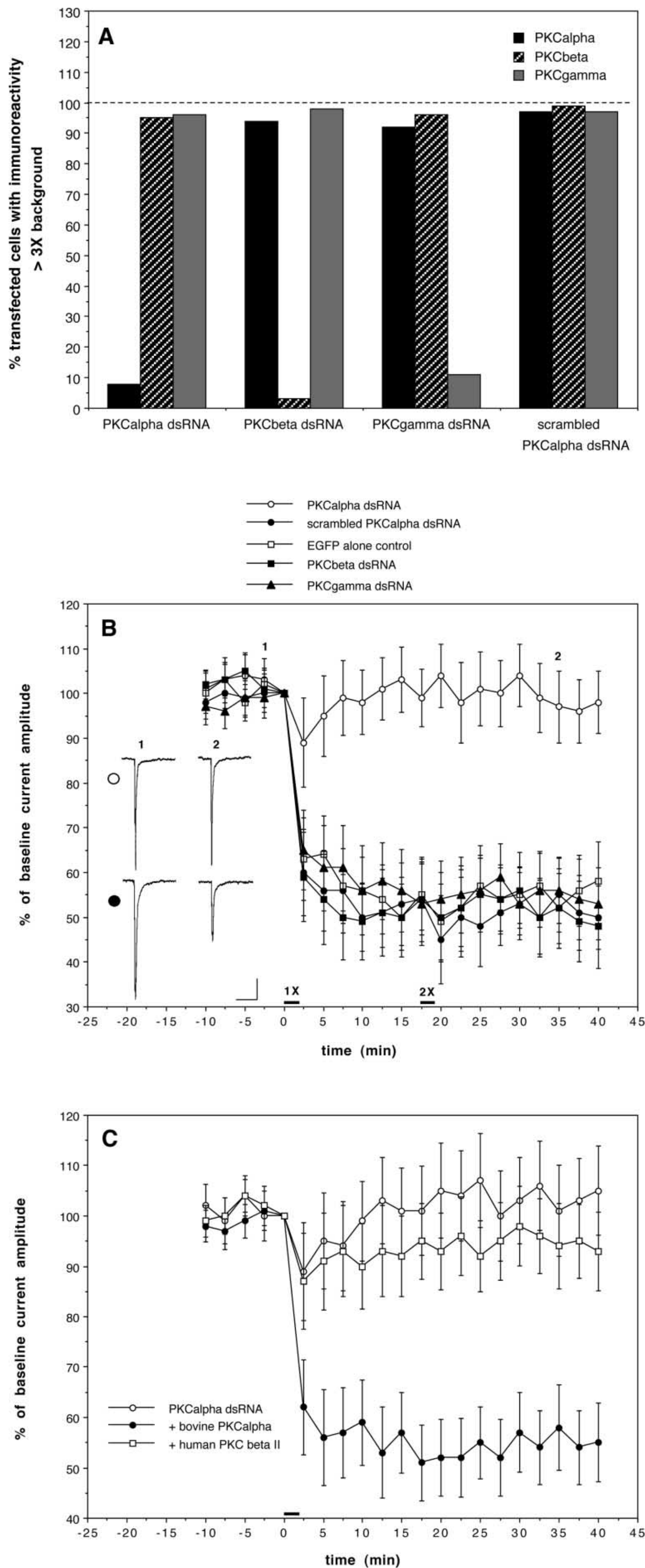


Figure 1. Knockdown of Mouse PKCα with dsRNA Produces a Complete Blockade of Cerebellar LTD Induction and LTD Is Rescued with Expression of Bovine PKCα

(A) Cerebellar cultures were transfected using gold particles coated with an EGFP reporter plasmid together with dsRNA directed against unique target sequences in mouse PKCα, PKCβ, and PKCγ as well as a scrambled PKCα control. EGFP-positive Purkinje cells were then scored for immunoreactivity for PKCα, PKCβ, and PKCγ to determine the potency and specificity of dsRNA knockdown.

(B) Following a period of baseline responses to glutamate test pulses, LTD was induced at $t = 0$ by six glutamate/depolarization pairing stimuli (1X) and test pulses were then resumed. At $t = 17.5$ min, an induction protocol consisting of 12 pairings (2X) was delivered. Representative current traces are taken from the points indicated on the time course graph. Scale bars, 1 s, 50 pA. Error bars indicate the SEM.

(C) Cotransfection of dsRNA directed against mouse PKCα together with expression plasmids encoding either bovine PKCα or human PKCβII was used to assess rescue of LTD. The horizontal bar at $t = 0$ min indicates six glutamate/depolarization pairing stimuli in this and all subsequent figures.

receptors are blocked with picrotoxin, and action potential generation is suppressed with tetrodotoxin added to the external saline. When Purkinje cells were held at −70 mV, test pulses of glutamate (30–110 ms long, delivered at 0.05 Hz) evoked an AMPA receptor-mediated inward current that was stable in the baseline recording period (Figure 1B). When control Purkinje cells, transfected with the EGFP marker alone, received pairing stimuli consisting of six 3 s long depolarizations to 0 mV, together with a pulse of glutamate, this produced an immediate and persistent depression in the response to subsequent test pulses (55% ± 7.9% of baseline, mean ± SEM, t = 17.5 min, n = 7). This LTD was maximal, as later pairing stimuli consisting of 12 pairings induced no further depression (58% ± 8.8% of baseline, t = 40 min). Purkinje cells transfected with dsRNA directed against mouse PKCβ (48% ± 9.4% of baseline at t = 40 min, n = 6) or mouse PKCγ (53% ± 8.0% of baseline, n = 5) or scrambled PKCα dsRNA (50% ± 7.1% of baseline, n = 6) showed similar, normal LTD. However, when dsRNA directed against mouse PKCα was used, LTD induction was completely eliminated (98% ± 6.9% of baseline, n = 8; $p < 0.005$ compared to EGFP marker control).

Knockdown of protein expression with dsRNA is highly intolerant of mismatches with the target mRNA. This can be exploited in a control experiment: because the sequence of bovine PKCα diverges from that of mouse PKCα at four bases in the target region, it is likely that bovine PKCα will not be degraded by mouse PKCα dsRNA. Indeed, when mouse PKCα dsRNA and a plasmid encoding bovine PKCα were coated together onto gold particles, transfected Purkinje cells displayed normal LTD (Figure 1C; 55% ± 8.9% of baseline at t = 40 min, n = 8). In contrast, when mouse PKCα dsRNA was cotransfected with a plasmid driving expression of human PKCβII, LTD remained almost completely blocked (93% ± 7.8% of baseline, n = 7; $p < 0.01$ compared with bovine PKCα transfection).

To confirm the requirement for PKCα in cerebellar LTD, we have also used a PKCα null mouse (Leitges et al., 2002). Cultured Purkinje cells derived from this mouse showed no LTD induction in a 40 min monitoring period following pairing stimulation (Figure 2A; 115% ± 8.1% of baseline at t = 40 min, n = 8). Transfection with a rescue plasmid driving expression of bovine PKCα restored LTD (56% ± 9.4% of baseline, n = 7; $p < 0.005$ compared with untransfected PKCα null) to nearly match the levels seen in wild-type Purkinje cells (47% ± 9.0% of baseline, n = 6). When rescue was attempted using human PKCβII or bovine/human chimeric PKCγ plasmids in the PKCα null background, this failed to restore LTD (99% ± 8.2% and 95% ± 9.3% of baseline, respectively, n = 7 for both groups). To confirm that the absence of LTD with PKCα deletion was not an artifact of cell culture conditions, the basic experiment was repeated in using acute cerebellar slices. Figure 2B shows data recorded in 200 μm thick acute parasagittal slices of P20–P30 mouse cerebellum using whole-cell patch-clamp recording, with a Cs-gluconate-based internal saline. Purkinje cells were held at −70 mV. A stimulation electrode was placed in the molecular layer and a paired pulse (100 ms interval) was delivered every 10 s to activate parallel fibers. The stimulation intensity was adjusted so that the first EPSC did not exceed 300 pA. After a period of baseline recording, pairing stimulation was given to induce LTD: parallel fibers were stimulated with a train of five stimuli at 100 Hz, and the Purkinje cell was depolarized to 0 mV for 100 ms. The depolarization preceded the onset of the parallel fiber train by 1 ms. The pairing stimulation was repeated 30 times at 2 s intervals. In this condition, LTD was completely abolished in the PKCα null as it also was using a stronger stimulation protocol with 15 parallel fiber stimuli/train delivered at t = 20 min (wt: 53% ± 7.6% of baseline, n = 6; PKCα null: 109% ± 7.5% of baseline, n = 7; t = 26 min; $p < 0.005$).

Taken together, these results suggest that PKCα has a central role in cerebellar LTD induction. However, the possibility remains that genetic deletion and knockdown of PKCα are blocking LTD induction through side effects on other processes. We have attempted to address this in several ways. First, measurements of mEPSC amplitude and kinetics were normal with these manipulations, suggesting that basal excitatory synaptic transmission is not altered (Supplemental Table S1 [http://www.neuron.org/cgi/content/full/44/4/585/DC1/]). Second, Ca^{2+} imaging was performed to measure dendritic transients evoked by depolarization and micropressure ejection of the mGluR1 agonist (S)-3,5-dihydroxyphenylglycine (DHPG). These values were also normal, indicating that two important signals for LTD induction, Ca^{2+} influx through voltage-gated channels and the sequelae of mGluR1 activation, are both intact (Supplemental Table S1). Third, an LTD-like phenomenon was evoked by bath application of the exogenous PKC activator phorbol-12,13-diacetate (PDA, 200 nM). This treatment, which evokes a form of depression that mutually occludes pairing-induced LTD (Linden and Connor, 1991), bypasses the initial signaling requirements for LTD induction. Similar to the results seen with pairing-induced LTD, treatment with PKCα dsRNA produced a complete blockade of depression evoked by a 10 min long bath application of PDA (Figure 3A; 92% ± 8.4% of baseline at t = 40 min, n = 7). Likewise, PDA-induced depression was normally evoked in Purkinje cells treated with PKCβ dsRNA (48% ± 8.5% of baseline, n = 6; $p < 0.005$ compared with PKCα dsRNA), PKCγ dsRNA (55% ± 9.2% of baseline, n = 6; $p < 0.01$ compared with PKCα dsRNA), or scrambled PKCα dsRNA (47% ± 8.4% of baseline, n = 6; $p < 0.005$ compared with PKCα dsRNA). Purkinje cells from PKCα null mice showed little depression in response to PDA (Figure 3B; 93% ± 8.1% of baseline, n = 7), but PDA-induced depression was conferred by rescue transfection with a PKCα expression plasmid (52% ± 8.5% of baseline, n = 7; $p < 0.01$ compared with untransfected PKCα null) and achieved levels of depression similar to that found in untreated wild-type Purkinje cells (57% ± 8.9% of baseline, n = 5). Thus, it is likely that the blockade of LTD seen with PKCα genetic deletion or dsRNA knockdown reflects the acute loss of PKCα and not side effects on upstream signals.

To this point, both induction and monitoring of LTD have used pulses of exogenous glutamate. This technique has the advantage that it only queries the status of the postsynaptic neuron but has the disadvantage that the pool of postsynaptic glutamate receptors acti-

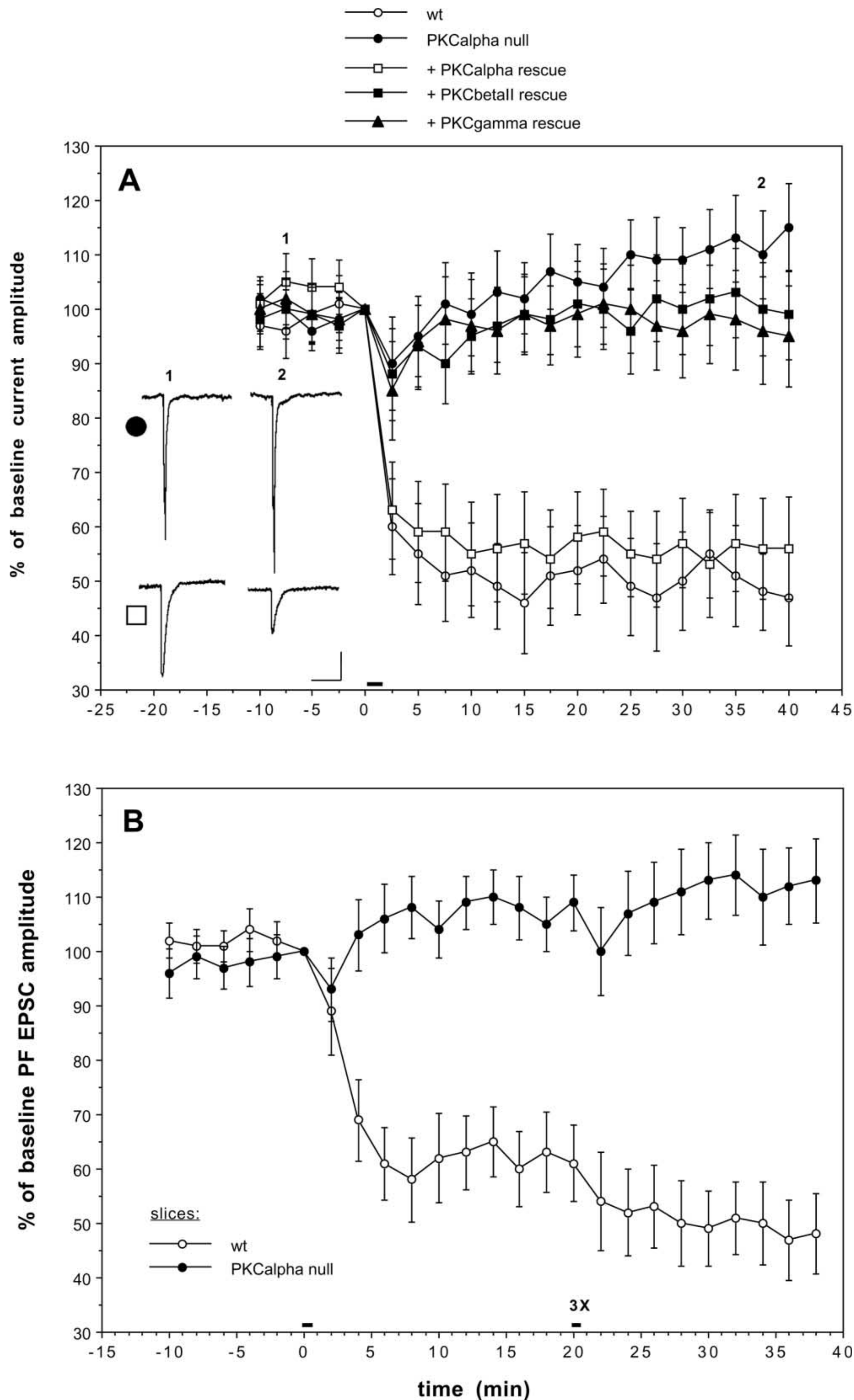


Figure 2. Cerebellar LTD Is Absent in Purkinje Cells Derived from PKCα Null Mice and Is Rescued with a PKCα Expression Construct

(A) Purkinje cells in cerebellar cultures derived from PKCα null mice were tested for LTD induction using glutamate depolarization pairing (at $t = 0$ min) either in the untransfected state or in cells transfected with expression plasmids encoding bovine PKCα, human PKCβII, or bovine/human chimeric PKCγ. Scale bars, 1 s, 60 pA.

(B) Parallel fiber-Purkinje cell EPSCs in acute parasagittal slices from wild-type (wt) or PKCa null mice were recorded, and LTD was induced by a parallel fiber burst/depolarization pairing protocol. The horizontal bar at $t = 0$ min indicates LTD induction using a protocol with five parallel fiber pulses/bursts, while that at $t = 20$ min indicates a stronger protocol with 15 pulses per burst (3X).

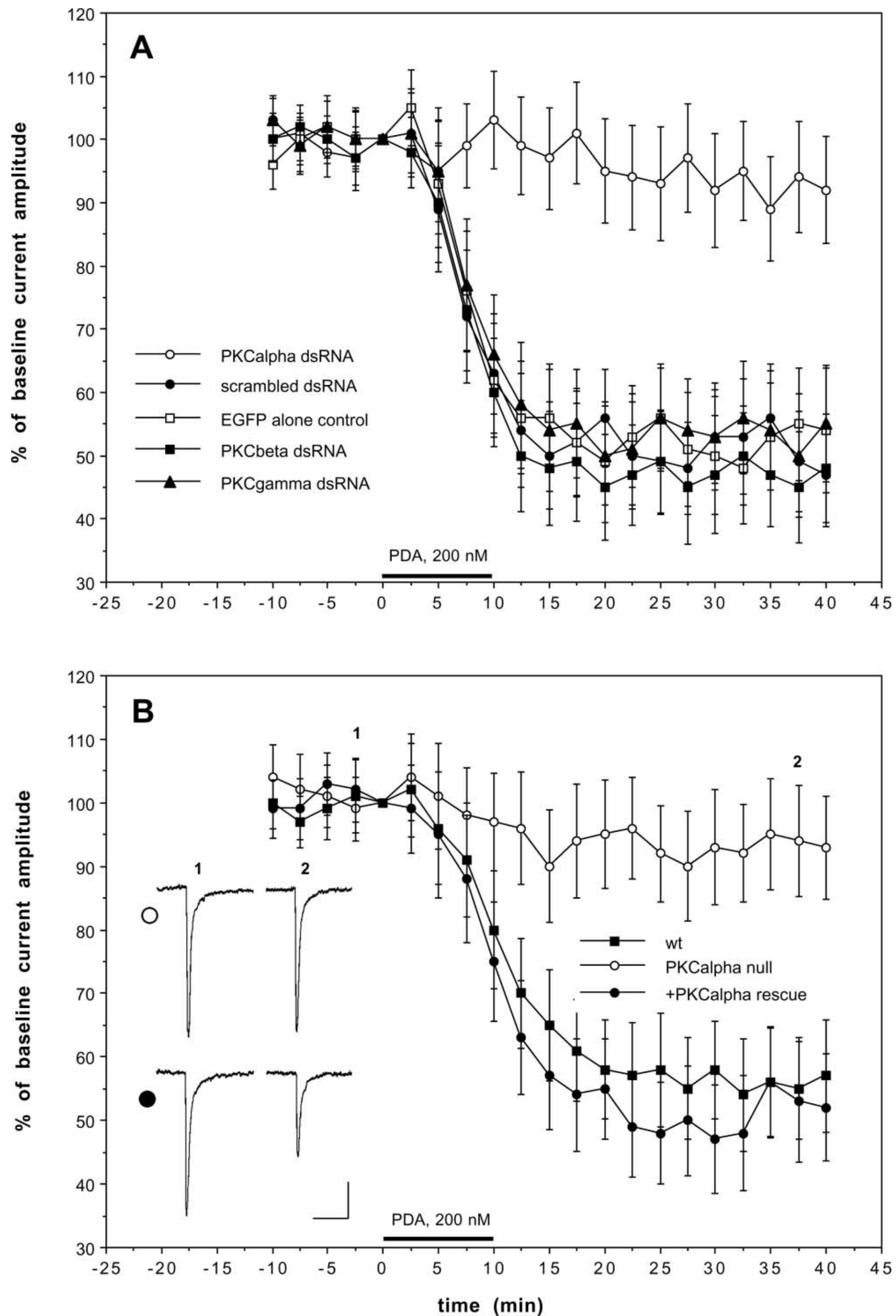
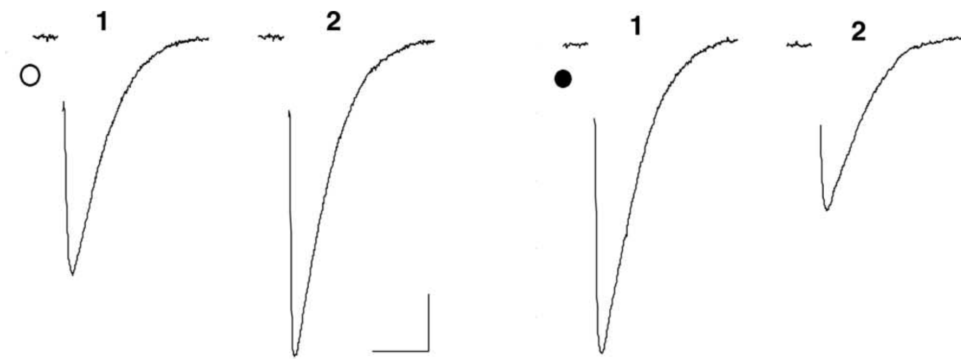


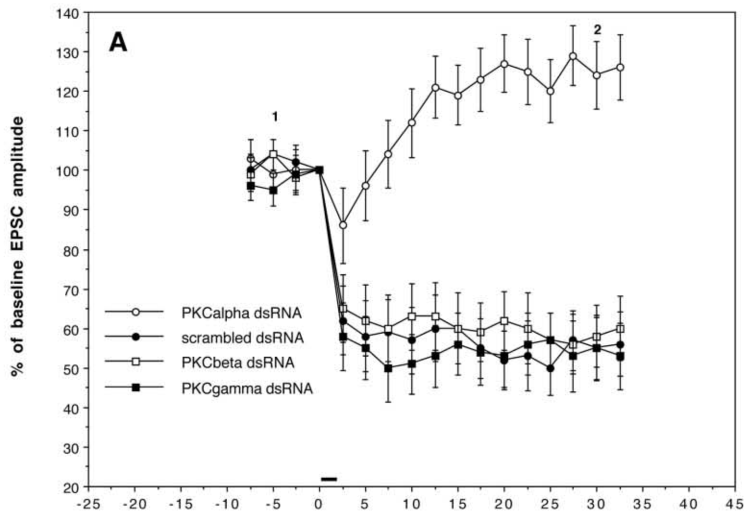
Figure 3. An LTD-like Phenomenon Produced by Bath Application of PKC-Activating Phorbol Ester Is Blocked by PKCα Knockdown or Genetic Deletion

(A) Bath application of the PKC-activating phorbol ester phorbol-12,13-diacetate (PDA), at t = 0–10 min, produces an LTD-like effect that is blocked with PKCα dsRNA treatment.

(B) The LTD-like effect produced by PDA is assessed in wild-type (wt) Purkinje cells, Purkinje cells derived from PKCα null mice, and in PKCα null Purkinje cells where PKCα expression is rescued with an expression plasmid. Scale bars, 1 s, 40 pA.

vated is not the same as those activated by synaptic stimulation (exogenous glutamate pulses activate a higher proportion of extrasynaptic receptors). To determine whether LTD of synaptic AMPA receptors requires PKCα, a set of experiments were conducted using synaptic activation in granule cell-Purkinje cell pairs in place of exogenous glutamate pulses (Figures 4A and 4B). Following pairing of granule cell-evoked EPSCs with





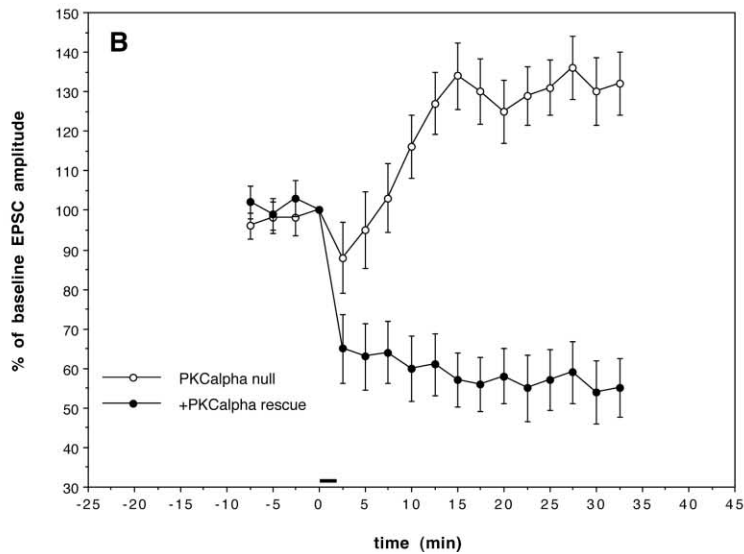


Figure 4. PKCα Knockdown or Genetic Deletion Blocks LTD in Cell Pairs

(A) These recordings were made using granule cell-Purkinje cell pairs together with dsRNA-mediated knockdown of PKCs. A mixture of EPSCs and synaptic failures were evoked in granule cell-Purkinje cell pairs. The mean EPSC amplitude, including failures, is plotted. LTD was induced by applying 60 pulses at 0.5 Hz at the test pulse stimulation strength to the granule cell, with each pulse paired with a 100 ms long depolarization of the Purkinje neuron to 0 mV (at $t = 0$ min). Current traces are the average of 12 consecutive responses. Scale bars, 5 ms, 10 pA.

(B) LTD in granule cell-Purkinje cell pairs assessed in cultures derived from PKCα null mice, with and without a PKCα rescue plasmid.

depolarizing pulses, normal LTD was induced in Purkinje cells transfected with PKCβ dsRNA (60% $\pm$ 8.1% of baseline at t = 32.5 min, n = 6), PKCγ dsRNA (53% $\pm$ 8.4% of baseline, n = 6), or scrambled PKCα dsRNA (56% $\pm$ 7.8% of baseline, n = 7). However, Purkinje cells transfected with PKCα dsRNA showed no LTD—in fact, a small degree of LTP was revealed (126% $\pm$ 8.2% of baseline, n = 7; $p < 0.001$ compared with scrambled PKCα dsRNA). A similar lack of LTD and induction of LTP was seen in cultures from PKCα null mice (Figure 4B; 132% $\pm$ 8.0%, n = 7). The LTP that was revealed from pairing stimuli in these two cases is likely to be presynaptic in origin (explaining why it was not seen with glutamate test pulses) as it is associated with decreases in the facilitation ratio of test pulse pairs delivered 100 ms apart (data not shown). A PKCα rescue plasmid was able to restore LTD in cell pairs derived from PKCα null mice (55% $\pm$ 7.4% of baseline, n = 6). However, a control construct consisting of a kinase dead mutant of PKCα (K368M, kindly provided by Peter J. Parker) failed in this regard (126% $\pm$ 9.3% of baseline, n = 6; Figure 4C; $p < 0.001$ compared with wild-type PKCα rescue). These experiments indicate that the requirement for PKCα in cerebellar LTD is not an artifact of measuring a pool containing extrasynaptic glutamate receptors.

What property of PKCα confers a special role for this isoform in cerebellar LTD induction? Immunohistochemical experiments have shown that cerebellar slices treated with high K and glutamate to produce a chemical form of LTD show cytosol to membrane translocation of PKCα and PKCβI (but not PKCγ; Hirono et al., 2001). Thus, it is unlikely that PKCα is uniquely activated by pairing stimulation. Rather, a clue to the special role of PKCα may come from protein-protein interactions involved in this phenomenon. When GluR2-containing AMPA receptors are phosphorylated at ser-880 in their carboxy-terminal PDZ ligand, this causes the PDZ domain-containing protein GRIP/ABP (Dong et al., 1997) to dissociate from GluR2 (Matsuda et al., 1999; Chung et al., 2000). GRIP/ABP unbinding allows another protein called PICK1 to bind the GluR2 PDZ ligand (Dev et al., 1999; Matsuda et al., 1999; Xia et al., 2000; Chung et al., 2000). PICK1 contains a single N-terminal PDZ domain and a BAR/coiled-coil domain, the latter of which allows homomultimerization and interaction with lipid bilayers. The PICK1 PDZ domain, in addition to binding GluR2, also binds a single isoform of PKC, which is PKCα (Staudinger et al., 1997). Previous work has shown that peptides and antibodies that interfere with PICK1 binding to GluR2 block cerebellar LTD (Xia et al., 2000). Based on these findings, we have sought to test the hypothesis that PKCα binding to PICK1 confers a special role upon PKCα in cerebellar LTD induction.

PKCα binds PICK1 through a PDZ binding motif (QSAV) located in the PKCα protein at the very carboxy-terminal end. Mutation of QSAV to AAAA prevents binding to PICK1 in a heterologous expression system (Staudinger et al., 1997). Guided by this study, we generated an expression plasmid coding for PKCα with the QSAV→AAAA mutation as well. When introduced into Purkinje cells derived from PKCα null mice, pairing stimulation in granule cell-Purkinje cell pairs fails to induce LTD (Figure 4C; 117% $\pm$ 8.3% of baseline at t = 32.5 min, n = 7; compare with wild-type PKCα rescue in Figure 2).

If the QSAV motif in PKCα does indeed confer LTD induction, then perhaps introducing a QSAV into the sequence of a nonpermissive PKC isoform would allow it to support LTD. We therefore generated a mutant PKCγ protein harboring a VPVM→QSAV mutation at the C terminus, which should transmit PICK1 binding capacity to the mutant protein. Using this mutant PKCγ protein in a coexpression system with PICK1, we were able to coimmunoprecipitate PICK1 together with mutant PKCγ, whereas wild-type PKCγ was not able to interact with PICK1 in this system (Figure 5, inset). This indicates that the QSAV motif is able to confer PICK1 binding on PKCγ. When this PKCγ with an inserted QSAV was introduced into PKCα null Purkinje cells, there was a significant, albeit submaximal, rescue of LTD (77% $\pm$ 8.3% of baseline, n = 8; $p < 0.02$ compared with PKCα null Purkinje cells transfected with wild-type PKCγ) and no significant side effects on basal synaptic transmission or Ca^{2+} dynamics (Supplemental Table S1 [http://www.neuron.org/cgi/content/full/44/4/585/DC1/]). It is likely that QSAV insertion alone is insufficient to completely reconstitute the PDZ ligand and that this accounts for the submaximal rescue of LTD.

Discussion

There are two main findings of this study. First, PKCα (but neither PKCβ nor PKCγ) is required for cerebellar LTD induction. Furthermore, the PKCα required for cerebellar LTD must be catalytically intact: a kinase-dead mutant in which the other structural domains of PKCα are expected to be preserved failed to support LTD. Second, PKCα is likely to have a special role in LTD based upon its unique type I PDZ ligand, the QSAV motif. This PDZ ligand is likely to confer the capacity for LTD induction on PKCα through binding with the multimeric GluR2 binding protein PICK1, although it cannot be ruled out that other PDZ domain-containing proteins may also bind PKCα to facilitate cerebellar LTD.

The sequence of molecular events involved in cerebellar LTD induction is becoming a fairly complete story, but there are some details that remain undefined. Biochemical studies have suggested that only PKCα in the activated state can bind PICK1 (Perez et al., 2001). One model is that PKCα, activated by Ca^{2+} and diacylglycerol from conjoint activation of voltage-gated Ca^{2+} channels and mGluR1/phospholipase C, binds PICK1. PICK1 bound PKCα then phosphorylates GluR2 ser-880, causing GRIP/ABP to dissociate. GRIP/ABP unbinding allows PICK1 to bind GluR2. PICK1 bound GluR2 is then primed for clathrin-mediated endocytosis (through mechanisms that have yet to be uncovered). Alternatively, once GluR2 is internalized by a constitutive process, it might be stabilized in an internal pool by PICK1 interactions. However, it should be cautioned that, while PKCα is required for LTD and phosphorylation of GluR2 ser-880 is also required, and the two are closely associated through PICK1, there is no direct evidence at present that proves that PKCα phosphorylates ser-880 as opposed to some other yet-to-be discovered critical residue. In this vein, it will be interesting to determine whether the transloca-

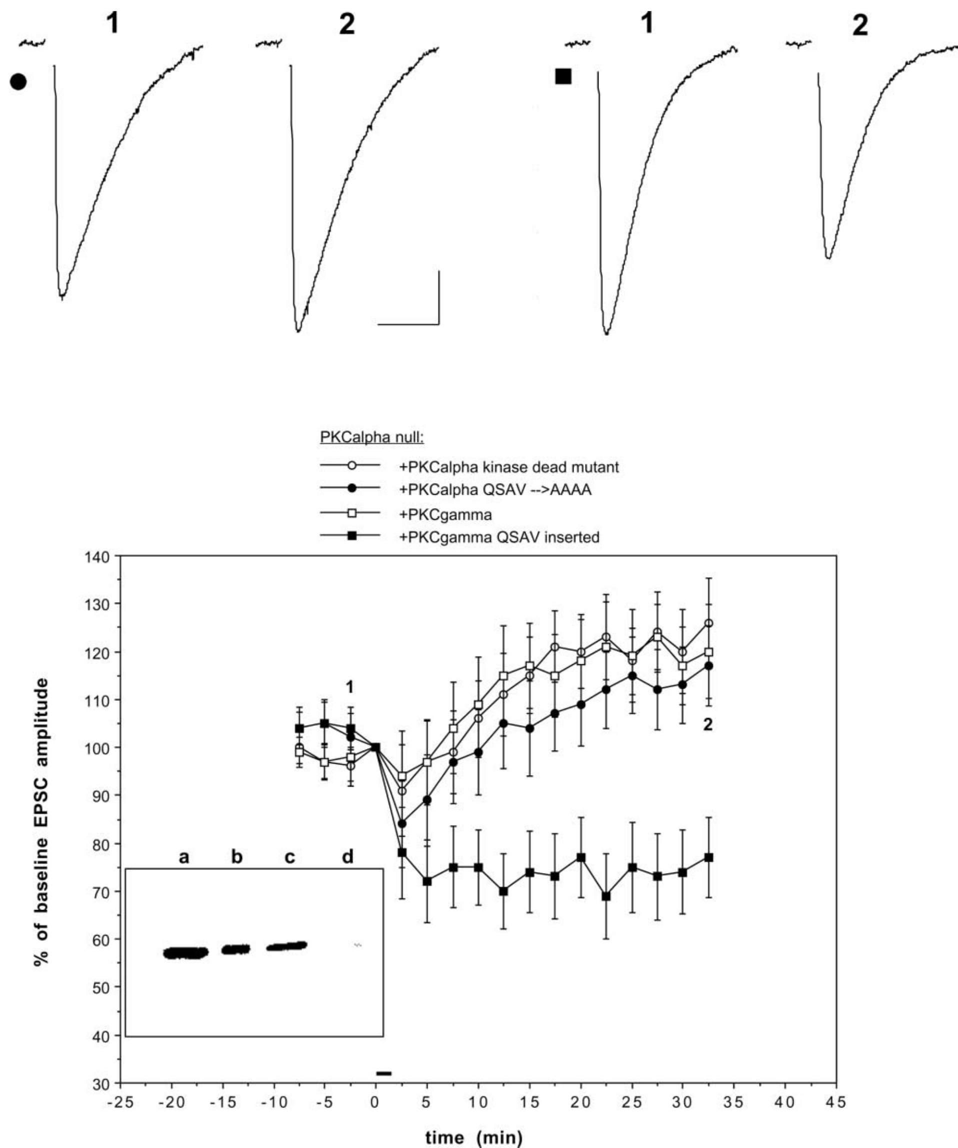


Figure 5. The PKCα QSAV Site, which Confers Binding to PICK1, Is Crucial for Cerebellar LTD

LTD in cell pairs from PKCα null mice with a variety of rescue constructs including the permissive isoform PKCα, PKCα with its PICK1 binding motif QSAV mutated to AAAA, the nonpermissive isoform PKCγ, and a mutant form of PKCγ in which QSAV was inserted to render it able to bind PICK1. Scale bars, 5 ms, 10 pA. (Inset) Coimmunoprecipitation of HA-tagged PICK1 and either QSAV-inserted PKCγ or wild-type PKCγ from cotransfected NIH 3T3 fibroblasts. Lanes: a, PKCγ QSAV-inserted cell lysate; b, IP: anti-PKCγ; c, wild-type PKCγ cell lysate; d, IP: anti-PKCγ.

tion of PKCα to the plasma membrane of Purkinje cells, which is produced by PKC-activating phorbol esters (Hirono et al., 2001), is blocked when either the QSAV domain of PKCα or the PDZ domain of PICK1 are mutated. Similarly, it would be interesting to determine whether introducing the QSAV motif into an LTD-nonpermissive isoform such as PKCγ would cause it to become translocated to the membrane upon activation.

While the present findings argue that PKCα is required for LTD and that PKCβ and PKCγ are not, it is not known whether PKCα activation is sufficient for LTD induction. It is formally possible that one or more of the nonclassical PKC isoforms expressed in Purkinje cells (δ, ϵ, η, ζ, and λ) may also function in cerebellar LTD.

Cerebellar LTD is thought to constitute a portion of the engram for certain forms of motor learning, including adaptation of the vestibulo-ocular reflex, adaptation of the horizontal optokinetic response, and associative eyelid conditioning (Bear and Linden, 2000). A transgenic mouse in which a peptide inhibitor of all PKC isoforms is expressed selectively in Purkinje cells to block cerebellar LTD shows strong deficits in both rapid adaptation of the vestibulo-ocular reflex (De Zeeuw et al., 1998; Goossens et al., 2001) and acquisition of associative eyelid conditioning (Koekkoek et al., 2003). A PKCγ null mouse, which had normal cerebellar LTD, was not impaired in associative eyelid conditioning (Chen et al., 1995). This mouse had only mild impairments in

adaptation of the horizontal optokinetic response, but adaptation was completely blocked by intracerebellar injection of a broad spectrum PKC inhibitor drug (in both PKCγ and wild-type mice; Shutoh et al., 2003). It will be useful to test the hypothesis that PKCα null mice will be profoundly impaired in these three motor learning tasks as a consequence of cerebellar LTD blockade.

Experimental Procedures

PKC Expression Vectors and Mutagenesis

All PKC cDNAs used in this study were cloned into the pMT2 backbone. cDNAs for PKCα, -βII, and -γ were originally described in Parker et al. (1986) and Coussens et al. (1986). The kinase-dead version of PKCα (K368M) was kindly provided by Peter J. Parker, UK Cancer Research, London. Mutagenesis has been performed according to manufacturer's protocol using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The following primer pairs have been used for the mutagenesis of PKCα: forward, 5′-G CAC CCC ATC CTG **GCG GCG GCG GCA** TGA GAC GCC TCG C-3′; and reverse, 5′-G CGA GGC GTC TCA TGC CGC CGC CGC CAG GAT GGG GTG C-3′. For the mutagenesis of PKCγ: forward, 5′-C AGC CCA ACG CCT **CAG TCA GCC GTG** TAA TCC CAC CTG-3′; and reverse, 5′-CAG GTG GGA TTA CAC GGC TGA CTG AGG CGT TGG GCT G-3′.

Coimmunoprecipitation Assay

NIH 3T3 fibroblasts plated on 6-well plates were transfected with the pMT2-PKCγ wild-type or pMT2-PKCγ/VPVM→QSAV mutant expression vectors in combination with a vector coding for HA-tagged PICK1 using FuGENE 6 (Roche) according to manufacturer's protocol. Twenty-four hours after transfection, cells were lysed by the addition of lysis buffer (50 mM Tris, pH 9.0, 1% deoxycholate) containing a protease inhibitor mix (Sigma) for 30 min on ice. Lysates were then incubated for 30 min at 37°C, and insoluble material was removed by centrifugation for 30 min at 10,000 × g. Resulting samples were dialyzed against 50 mM Tris/HCl, pH 7.4, and 0.1% Triton X-100 overnight at 4°C with agitation. For preclearing, the samples were incubated for 2 hr with protein A-agarose (Roche) and nonspecifically bound proteins removed by centrifugation at 1000 × g for 5 min. Precipitation was performed overnight with agitation by adding new protein A-agarose and 1 μg/sample rabbit polyclonal anti-PKCγ antiserum (clone C19, Santa Cruz) to the supernatants. The samples were washed extensively with PBS/0.1% Triton X-100 and eluted with double-concentrated SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE on a 10% gel and transferred to PVDF-membrane for subsequent immunodetection using a high-affinity anti-HA epitope antibody fused to peroxidase (clone 3F10, Roche).

Electrophysiology

Patch electrodes (2–4 MΩ) were filled with a solution containing CsCl (135 mM), HEPES (10 mM), EGTA (0.5 mM), Na_2-ATP (4 mM), and Na-GTP (0.4 mM), adjusted to pH 7.35 with CsOH. Cells were bathed in NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.8 mM), HEPES (10 mM), glucose (10 mM), D-AP5 (0.1 mM), tetrodotoxin (0.0005 mM), and picrotoxin (0.3 mM), adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. For experiments in granule cell-Purkinje cell pairs (Figures 4 and 5), TTX was omitted from the external saline, and granule cells were activated using 0.5 ms long constant-voltage pulses through a loose-patch electrode (5–6 MΩ) filled with external saline. Iontophoresis electrodes were filled with 10 mM glutamate (in 10 mM HEPES, pH 7.1) and were positioned ∼20 μm away from large-caliber dendrites. Test pulses were delivered using negative current pulses (600–900 nA, 30–110 ms duration). Membrane currents were digitized at 10 kHz and low-pass filtered at 2 kHz. Recordings in which R_{input} or R_{series} varied by more than 12% were excluded from the analysis.

Particle-Mediated Transfection

For gene delivery, a gold/glycerol mixture was first prepared as follows: 20 mg of 0.6 μm gold microcarriers were washed once in 70% ethanol, three times in sterile deionized, distilled water, and resuspended in 0.5 ml of sterile 50% glycerol. For each transfection into Purkinje cell cultures, 5 μg of plasmid cDNA, 5 μg of EGFP, 50 μl of 2.5 M $CaCl_2$, and 10 μl of 1 M spermidine were sequentially added to 50 μl of the gold/glycerol mixture. The preparation was gently vortexed for 15 min, after which the gold particles were centrifuged down and washed once with 70% ethanol and once with 100% ethanol. Washed gold particles were resuspended in 60 μl of 100% ethanol and pipetted onto macrocarrier disks (9 μL/disk). For RNA interference, dsRNA (Dharmacon) was diluted in RNase-free ddH_2O to yield a concentration of 20 μM. This solution (30 μl of) was mixed with 2 mg of 0.6 μm gold microcarriers (Bio-Rad) in a 50% glycerol solution and 6.5 μg of a DNA plasmid encoding EGFP. Following vortexing and an ethanol wash, microcarrier-containing solution was evaporated on the surface of six macrocarrier discs and delivered to the cerebellar cultures (at 6–7 DIV) using the Helios Gene Gun System (Bio-Rad) operating at a pressure of 450 psi and a vacuum of 20 in-Hg. Conditioned medium was aspirated from the surface of the cell culture immediately before transfection and was returned immediately thereafter. Cultures were then returned to the incubator for a minimum of 22 hr prior to electrophysiological recording. Transfected Purkinje neurons were identified by imaging GFP signals with 488 nm illumination.

dsRNA-Mediated Knockdown

Purified dsRNAs were obtained from Dharmacon and were chosen using their online sequence selection tool (Dharmacon siDESIGN Center). Mouse PKCα was targeted using the sequence AAGTCAT CAGCCCTTCAGA, mouse PKCβ using GTTACTAAGCCAGGAAGAG (a sequence common to both βI and βII splice forms), and mouse PKCγ using AGTTCACCGCTCGTTTCTT. Scrambled PKCα sequence was ATTGTATGCGATCGCAGAC. Protein knockdown was confirmed using immunohistochemistry. Antibodies from Santa Cruz Biotechnology were mouse monoclonal IgG dircted against human PKCα (catalog number sc-8393), rabbit polyclonal IgG directed against human PKBβI (sc-209), and rabbit polyclonal IgG directed against human PKCγ (sc-211). These were detected using Alexa Fluor 488-conjugated secondary antibodies raised in chickens (Molecular Probes) and standard visualization protocols with wide-field fluorescence microscopy.

Ca^{2+} Imaging

Bis-fura-2 ratio imaging of intracellular free Ca^{2+} was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system In these experiments, EGTA was removed from the internal saline and replaced with 100 μM bis-fura-2.

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