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Activation of Presynaptic cAMP-Dependent Protein Kinase Is Required for Induction of Cerebellar Long-Term Potentiation

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Cerebellar long-term potentiation (LTP) is a persistent increase in the strength of the granule cell–Purkinje neuron synapse that occurs after brief stimulation of granule cell axons at 2–8 Hz. Previous work has indicated that cerebellar LTP induction requires presynaptic Ca influx, stimulation of Ca-sensitive adenylyl cyclase, and activation of PKA. The evidence implicating PKA has come from bath application of drugs during LTP induction, an approach that does not distinguish between PKA activation in the presynaptic or postsynaptic cell. Although bath application of PKA inhibitor drugs (KT5720, Rp-8CPT-cAMP-S) blocked LTP induction in granule cell–Purkinje neuron pairs in culture, selective application to granule cell or Purkinje neuron somata via patch pipettes did not. We hypothesized that presynaptic PKA activation is required for LTP induction but that drugs applied to the granule cell soma cannot diffuse to the terminal within this timescale. To test this hypothesis, we transfected cerebellar cultures with an expression vector encoding a peptide inhibitor of PKA [Rous sarcoma virus (RSV)-protein kinase A inhibitor (PKI)]. Transfection of RSV-PKI into presynaptic granule cells, but not postsynaptic Purkinje neurons or glial cells, blocked LTP induction produced by either synaptic stimulation or an exogenous cAMP analog. An expression vector encoding a control peptide with no PKA inhibitory activity was ineffective. These results show that induction of cerebellar LTP requires a presynaptic signaling cascade, including Ca influx, stimulation of Ca-sensitive adenylyl cyclase, and activation of PKA, and argue against a requirement for postsynaptic Ca signals or their sequelae.

Key words: granule cell; Purkinje neuron; glia; particle-mediated gene transfer; synaptic transmission; motor learning.

The cerebellar cortex has been suggested to include an essential circuit for certain forms of motor learning, including associative eyeblink conditioning and adaptation of the vestibulo-ocular reflex. One cellular model system thought to contribute to learning in this structure is cerebellar long-term depression in which coactivation of inferior olive (climbing fiber) and granule cell (parallel fiber) axons to a Purkinje neuron induces a persistent, input-specific depression of the parallel fiber–Purkinje neuron synapse (Daniel et al., 1998). The converse phenomenon, cerebellar long-term potentiation (LTP), has also been described in which granule cell–Purkinje neuron synapses are strengthened by repetitive parallel fiber stimulation at low (2–8 Hz) frequencies (Sakurai, 1987, 1990; Hirano, 1990, 1991; Crepel and Jaillard, 1991; Shibuki and Okada, 1992; Salin et al., 1996; Linden, 1997, 1998; Kimura et al., 1998; Storm et al., 1998). Recently, a molecular description of cerebellar LTP induction has begun to emerge. Several converging lines of evidence have suggested that the initial trigger for cerebellar LTP induction is presynaptic Ca influx. Neither application of glutamate receptor antagonists during the tetanic stimulation (Salin et al., 1996; Linden, 1997, 1998) nor loading of the Purkinje neuron with a Ca chelator (Sakurai, 1990; Shibuki and Okada, 1992; Salin et al., 1996; Linden, 1997, 1998; Storm et al., 1998) is effective in blocking cerebellar LTP induction. However, LTP is blocked when external Ca is removed during tetanic stimulation (Salin et al., 1996; Linden, 1997, 1998). One potential mechanism by which an increase in presynaptic Ca could be linked to LTP induction is the activation of a Ca-sensitive adenylyl cyclase and consequent production of cAMP. It has been shown that an LTP-like effect may be produced by bath application of the adenylyl cyclase activator forskolin or membrane-permeable cAMP analogs (Salin et al., 1996; Chavis et al., 1998; Kimura et al., 1998; Storm et al., 1998). In addition, cerebellar LTP induced by granule cell stimulation (but not an exogenous cAMP analog) is attenuated in cell cultures from a type I Ca-sensitive adenylyl cyclase knock-out mouse (Storm et al., 1998). Production of cAMP could induce LTP via activation of PKA. PKA inhibitors have been shown to block induction of LTP produced by tetanic stimulation (Salin et al., 1996; Kimura et al., 1998) or an LTP-like effect produced by exogenous cAMP analogs (Chavis et al., 1998; Storm et al., 1998).

Is the PKA activation required for cerebellar LTP induction occurring in the presynaptic cell, the postsynaptic cell, or some other compartment, such as glial cells? All of the previous studies using PKA activators and inhibitors have relied on bath application, which, of course, cannot address this issue. In the present study, we have performed recordings from granule cell–Purkinje neuron pairs in culture. We have attempted to apply PKA inhibitors selectively to presynaptic and postsynaptic cells using two different techniques, via patch pipettes attached to the neuronal somata and via particle-mediated gene transfer of a specific PKA inhibitor peptide.

MATERIALS AND METHODS

Neurons and glia from embryonic mouse cerebellum were prepared and cultured as described previously (Linden et al., 1991). At 4 d *in vitro* (DIV), a fraction of gold particles (0.6 μm in diameter, 25 mg) that were coated with 50 μg of enhanced green fluorescent protein (GFP) plasmid

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(Clontech, Palo Alto, CA), and 50 μg of either Rous sarcoma virus-protein kinase A inhibitor (RSV-PKI) or RSV-PKImut expression vectors were delivered by particle-mediated gene transfer using the Helios Gene Gun System (Bio-Rad, Hercules, CA) as described previously (Qian et al., 1998). Cultures were then returned to the incubator and maintained for a total of 7–8 d *in vitro* at the time of use in recording experiments. Transfected Purkinje neurons, granule cells, and glia were identified by imaging GFP signals with 488 nm illumination. The criterion for transfection was that the peak luminance of the 488 nm GFP signal in the soma of the transfected neuron had to be more than fourfold higher than that of the background. This was typically assessed using a 200-msec-long exposure. Identification of Purkinje neurons at this early stage is much easier in GFP-filled (as opposed to nontransfected) cells because this allows for a clear view of the dendrites (see Fig. 4*A*). In general, Purkinje neurons appeared as large (>20 μm) multipolar cells with thick, elaborate dendrites. This morphological identification was confirmed by a unique electrophysiological signature (BK channel openings observed in whole-cell mode). Granule cells were identified as small (<7 μm) round clustering cells with short dendrites that evoked an EPSC in neighboring Purkinje neurons that showed paired-pulse facilitation when stimulated at an interval of 50 msec.

Whole-cell recordings were made from neurons and glial cells (7–12 DIV) as described previously (Linden, 1997, 1998; Storm et al., 1998). Cultures were bathed in a solution that contained (in mM) NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, and picrotoxin 0.2, adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. The electrode for Purkinje neuron recording typically contained (in mM): CsCl 120, HEPES 10, and Cs_4-BAPTA 10, adjusted to pH 7.35 with CsOH. In one set of experiments, illustrated in Figure 1, an internal saline was used containing (in mM): CsCl 50, HEPES 10, and Cs_4-BAPTA 35, adjusted to pH 7.35 with CsOH. The electrode for granule cell stimulation (and recording) contained (in mM): CsCl 135, HEPES 10, EGTA 1, Na2-ATP 4, and Na-GTP 0.4, adjusted to pH 7.35 with CsOH. The electrode for glial cell recording contained (in mM): CsCl 110, TEA-Cl 10, HEPES 10, and Cs_4-BAPTA 10, adjusted to pH 7.35 with CsOH. KT5720 and KT5823 were purchased from Calbiochem (La Jolla, CA), Rp-8CPT-cAMP-S and Sp-8CPT-cAMP-S from Biolog (Hayward, CA), tetrodotoxin from Alexis Biochemicals (San Diego, CA), QX-314-Br from Alomone Labs (Jerusalem, Israel), Cs_4-BAPTA from Molecular Probes (Eugene, OR), and all other compounds from Sigma (St. Louis, MO). Patch electrodes were pulled from N51A glass and yielded a resistance of 3–5 MΩ. For stimulation–recording of granule cells, slightly smaller electrodes (5–6 MΩ) were fabricated (except for an experiment shown in Fig. 2*C* in which 3 MΩ electrodes were used). Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in resistive voltage-clamp mode, filtered at 2 kHz, and digitized at 5 kHz. R_{series} was uncompensated. Experiments were conducted at room temperature. Cell pairs in which R_{input} or R_{series} varied by >15% were excluded from the analysis.

RESULTS

Whole cell voltage-clamp recordings were made from granule cell–Purkinje neuron pairs in dispersed cultures of embryonic mouse cerebellum. The process of identifying synaptically connected pairs of cells was as described previously (Linden, 1997, 1998) and was used herein with one exception. Whereas previous studies from this laboratory used a loose patch configuration for stimulating the presynaptic granule cell, the present study used the whole-cell configuration to allow for presynaptic perfusion. Both cells were held at −80 mV. To evoke an action potential, the presynaptic cell was stepped to a command potential of +10 mV for 1 msec. When this stimulation was repeated at 0.1 Hz, it resulted in a mixture of successful and failed synaptic events, which were averaged to produce a measure of mean EPSC amplitude. Failure of the presynaptic cell to fire an action potential was <1% in all experiments and was not altered by any treatment (data not shown).

Previous work had shown that LTP could be induced when 10 mM BAPTA was included in the patch pipette of the postsynaptic cell in either granule cell–Purkinje neuron or granule cell–glial cell pairs (Linden, 1997, 1998; Storm et al., 1998). This finding

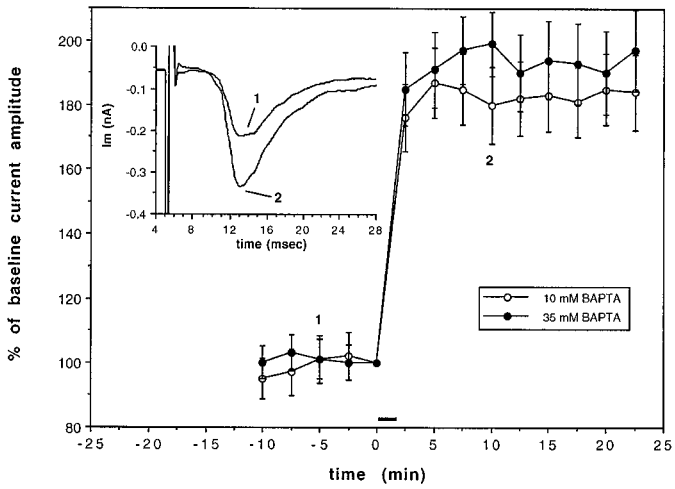

Figure 1. High concentrations of postsynaptic Ca chelator fail to block cerebellar LTP induction in granule cell–Purkinje neuron pairs. Purkinje neurons were loaded with a Cs-based patch pipette saline that contained either 10 or 35 mM BAPTA. LTP was induced by 4 Hz stimulation for 100 pulses, as indicated by the *thick horizontal bar* at $t = 0$ min. *Inset* illustrates current traces representing the average of 10 consecutive responses (including failures) recorded from a granule cell–Purkinje neuron pair loaded with 10 mM BAPTA. Traces are from the time points indicated on the graph.

suggested to us that postsynaptic Ca transients are not required for this process. However, a recent report examining LTP of the mossy fiber–CA3 synapse in the hippocampal slice has demonstrated that inclusion of 10 mM BAPTA in the postsynaptic patch pipette saline failed to completely block dendritic Ca transients associated with high-frequency synaptic stimulation and, consequently, LTP induction remained. However, concentrations of BAPTA >30 mM were effective in blocking these processes (Yeckel et al., 1999). Thus, to provide a more stringent test of the requirement for postsynaptic Ca transients, we have repeated cerebellar LTP experiments using both 10 and 35 mM BAPTA (Fig. 1). After a 10 min baseline recording period, LTP was induced by applying presynaptic stimulation at 4 Hz for 100 pulses. In Purkinje neurons filled with 10 mM BAPTA, this resulted in a potentiated response that persisted for the duration of the recording period (184 ± 11.6% of baseline at $t = 22.5$ min, mean ± SEM, $n = 7$ cells) and that was associated with a decrease in the rate of synaptic failures (38 ± 7% at $t = -5$ min, before LTP induction, compared with 16 ± 6% at $t = 20$ min). This is quite similar to LTP reported previously using this protocol when the presynaptic cell was loose-patched (Linden, 1997, 1998; Storm et al., 1998). When 35 mM BAPTA was used, LTP of similar amplitude and duration was produced (197 ± 12.5% of baseline at $t = 22.5$ min, $n = 5$ cells), indicating that postsynaptic Ca transients are not required for induction of cerebellar LTP in culture. No Ca transients were seen in either dendritic spines or shafts of fura-2 filled Purkinje neurons coloaded with either 10 or 35 mM BAPTA and stimulated with granule cell activation at 4 Hz for 100 pulses (data not shown).

When two different membrane-permeant PKA inhibitors were bath applied throughout the recording, both produced a near complete blockade of LTP (KT5720, 10 μM, 100 ± 8.9% of baseline at $t = 22.5$ min, $n = 6$; Rp-8CPT-cAMP-S, 100 μM, 105 ± 9.8% at $t = 22.5$ min, $n = 4$) (Fig. 2*A*). An inhibitor of cGMP-dependent protein kinase was ineffective in blocking LTP

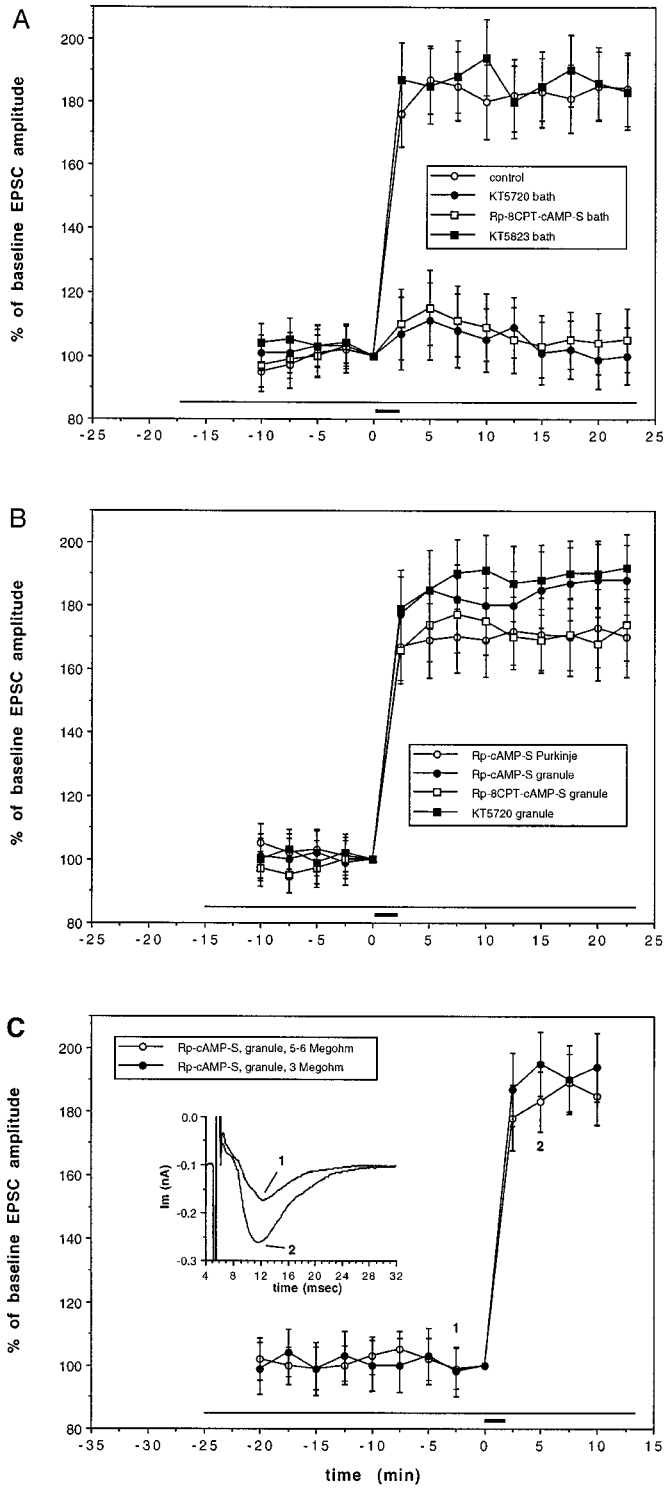

Figure 2. PKA inhibitor drugs block cerebellar LTP induction in granule cell–Purkinje neuron pairs when applied in the bath but not in patch pipettes. *A*, PKA inhibitors KT5720 (10 μM) and Rp-8CPT-cAMP-S (100 μM) and the PKG inhibitor KT5823 (10 μM) were applied in the bath starting at $t = -17.5$ min, as indicated by the *thin horizontal bar*. The *control* group in this graph is the same as the 10 mM BAPTA group from Figure 1 and is reproduced here for comparison. *B*, PKA inhibitors Rp-cAMP-S, Rp-8CPT-cAMP-S, and KT5720 (all 1 mM) were included in the patch pipette saline of either the granule cell or Purkinje neuron. The time of initiating whole-cell recording was at $t = 15$ min (or slightly earlier), as indicated by the *thin horizontal bar*. *C*, Rp-cAMP-S (2 mM) was included in the granule cell patch pipette saline with an extended baseline recording to allow for maximal presynaptic perfusion. Recordings were made using either our standard electrode for granule cell recording (5–6 MΩ) or a somewhat larger electrode (3 MΩ) to further maximize perfusion. The *inset* illustrates current traces representing the average of 10 consecutive responses (including failures) recorded from a granule cell–Purkinje neuron pair recorded with a 5–6 MΩ electrode.

induction (KT5823, 10 μM, 182 ± 12.0% at $t = 22.5$ min, $n = 5$). To test the hypothesis that activation of PKA in the granule cell axon terminal is necessary for LTP induction, Rp-cAMP-S (1 mM) was included in the pipette saline of either the presynaptic or postsynaptic electrode (Fig. 2*B*). This drug was chosen initially because it is somewhat less membrane-permeant than Rp-8CPT-cAMP-S used previously in bath application experiments. Whole-cell mode was achieved at least 5 min before the onset of baseline recording, so the cells in these experiments were perfused for at least 15 min before LTP induction. When Rp-cAMP-S was included in the Purkinje neuron recording pipette, no effect was seen upon either the initial amplitude (167 ± 10.5% at $t = 2.5$ min, $n = 5$) or the time course of LTP (170 ± 12.3% at $t = 22.5$ min). However, when this drug was included in the granule cell recording pipette, much to our consternation, LTP was similarly unaffected (188 ± 11.0% at $t = 22.5$ min, $n = 6$). LTP remained unaffected when the experiment was repeated using different PKA inhibitors (Rp-8CPT-cAMP-S, 1 mM, 174 ± 11.2% at $t = 22.5$ min, $n = 5$; KT5720, 1 mM, 192 ± 10.7% at $t = 22.5$ min, $n = 6$).

In an attempt to maximize perfusion of the granule cell axon, experiments were performed in which the dose of Rp-cAMP-S was increased (2 mM), as was the baseline recording time (at least 25 min of whole-cell mode before LTP induction) (Fig. 2*C*). Because the recording time of these experiments is quite limited as a result of the difficulties inherent in cell pair recording, this is the maximum perfusion time that could be reliably obtained. Unfortunately, this design still failed to block LTP induction (185 ± 9.4% at $t = 10$ min, $n = 7$). The 5–6 MΩ pipettes used for granule cell stimulation yielded R_{series} of 11.5 ± 3.0 MΩ, measured at $t = 0$ min). To further maximize axonal perfusion, this protocol was then repeated using a larger (3 MΩ) granule cell pipette (R_{series} = 7.9 ± 2.6 MΩ), which also failed to block LTP (194 ± 10.9% at $t = 10$ min, $n = 5$).

There are several potential explanations for the failure of presynaptic PKA inhibitors to block LTP induction when included in the internal saline of the granule cell electrode. First, the PKA activation necessary for LTP induction is not in either the granule cell or the Purkinje neuron but rather is in some other cellular compartment such as nearby inhibitory interneurons or glial cells. Second, LTP induction requires PKA activation in both the granule cell and the Purkinje neuron, such that PKA inhibition in either compartment alone is ineffective. Third, PKA activation in the granule cell is required for LTP induction, but somatic application of PKA inhibitors is insufficient to inhibit PKA in the granule cell presynaptic terminals because of limited perfusion.

As an initial test of this last hypothesis, we applied a compound to the soma for which there is an independent assay for its arrival at the terminals. QX-314 is a drug that blocks voltage-gated Na channels from the cytoplasmic side in a use-dependent manner. QX-314 (1–5 mM) was included in the granule cell pipette saline and was allowed to perfuse the granule cell while retrograde spikes were evoked by direct stimulation of the granule cell

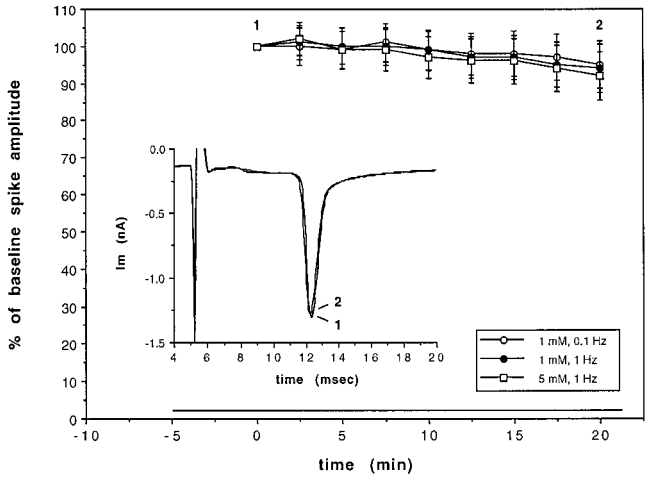

Figure 3. The Na channel blocker QX-314 fails to block axonally evoked retrograde spike current when applied in the granule cell patch pipette. QX-314 was included in granule cell patch pipette at a concentration of either 1 or 5 mM and was delivered beginning with the initiation of whole-cell recording (*thin horizontal bar*). Retrograde spike current was evoked by extracellular stimulation of the granule cell terminals–axon, and the effect of QX-314 perfusion was assessed with test pulses applied at either 0.1 or 1 Hz. *Inset* illustrates single current traces from a cell perfused with 5 mM QX-314 and stimulated at 1 Hz, taken at the time points indicated on the graph.

terminals. These spikes resulted in a local voltage-clamp failure, and the resultant short-latency inward current was propagated to the somatic recording electrode (Fig. 3). If QX-314 diffuses to the axonal stimulation site in sufficient concentration to block voltage-gated Na channels, then the retrograde spike current recorded in the soma should be blocked. Axons were stimulated at either 0.1 or 1 Hz, the latter to promote use-dependent blockade of Na channels. However, after 20 min of recording (≥25 min of perfusion), no significant blockade of the retrograde spike current was observed, even at the higher stimulation frequency and QX-314 concentration (5 mM, 1 Hz: 92 ± 6.5% at $t = 20$ min, $n = 5$). Na current evoked by somatic depolarizing steps to −50 mV was completely blocked by both doses of QX-314 within 10 test pulses, indicating that the QX-314 was effective (data not shown). These results are consistent with the hypothesis that granule cell terminals are not effectively perfused in these experiments.

As a further test, we used particle-mediated gene transfer to transfect cerebellar cultures with an expression vector in which a Rous sarcoma virus promoter drives expression of PKI peptide (RSV-PKI) (Day et al., 1989; Ginty et al., 1991), which inhibits PKA types I and II (Glass et al., 1989). The gold particles were also coated with another plasmid designed to drive expression of enhanced GFP. After a waiting period of 3–4 d to allow for the synthesis of the peptide and its transport throughout the cell, cell pairs were chosen (using GFP fluorescence as a marker) in which granule cells or Purkinje neurons had been selectively transfected. Figure 4*A* illustrates a cell pair in which both the granule cell and the Purkinje neuron have been transfected and imaged with 488 nm illumination and a cooled slow-scan CCD camera.

Cell pairs in which the granule cell was selectively transfected had a nearly complete blockade of LTP (101 ± 9.0% at $t = 22.5$ min, $n = 6$), as did pairs in which both granule cells and Purkinje neurons were transfected (102 ± 12.9% at $t = 22.5$ min, $n = 3$). However, selective transfection of the Purkinje neuron was ineffective in blocking LTP induction (178 ± 11.5% at $t = 22.5$ min, $n = 5$). Likewise, transfection of granule cells with a mutant form of the peptide that does not inhibit PKA (RSV-PKImut) (Day et al., 1989; Ginty et al., 1991) resulted in normal cerebellar LTP (179 ± 10.2% at $t = 22.5$ min, $n = 5$).

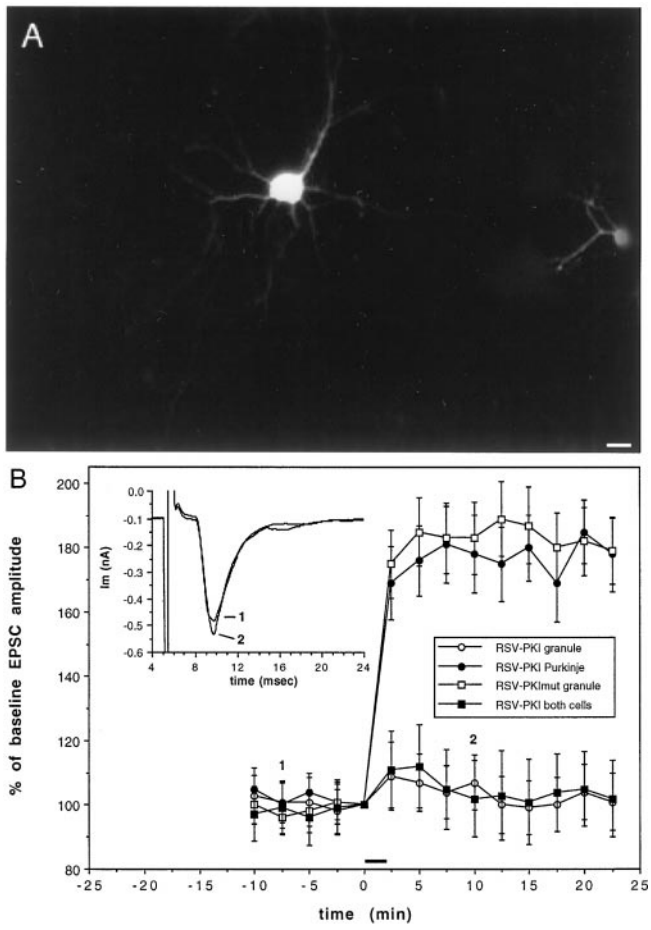

Figure 4. Transfection of the granule cell with a PKA inhibitor construct in a granule cell–Purkinje neuron pair blocks LTP induction. Granule cells or Purkinje neurons were selectively transfected with either a PKA inhibitor peptide expression vector (RSV-PKI) or a mutant peptide expression vector that is inactive with respect to PKA (RSV-PKImut), 3–4 d before recordings. Localization was determined by cotransfection with GFP and subsequent illumination at 488 nm. *A*, Photomicrograph illustrating a GFP-transfected granule cell (*far right*) and Purkinje neuron (*center*) illuminated with 488 nm light. Scale bar, 10 μm. *B*, LTP experiments. *Inset* illustrates current traces representing the average of 10 consecutive responses recorded from a granule cell–Purkinje neuron pair in which the granule cell was transfected with the RSV-PKI construct.

Is the blockade of cerebellar LTP by PKA inhibitors a relatively specific effect or is it a consequence of a large alteration in basal synaptic parameters? To address this issue, we measured a number of basal parameters of Purkinje neurons and granule cell–Purkinje neuron pairs [R_{input}, miniature EPSC (mEPSC) frequency, mEPSC amplitude, failures, evoked EPSC amplitude, and paired-pulse facilitation] together with PKA inhibitor application (Table 1). None of these parameters showed a statistically significant difference from the control group ($p > 0.05$, Student's t test) with treatments that blocked LTP (either bath-applied or granule cell-transfected PKA inhibitors). However, it should be noted that the PKA inhibitor treatments did produce a cluster of small effects, that, although not statistically significant for this

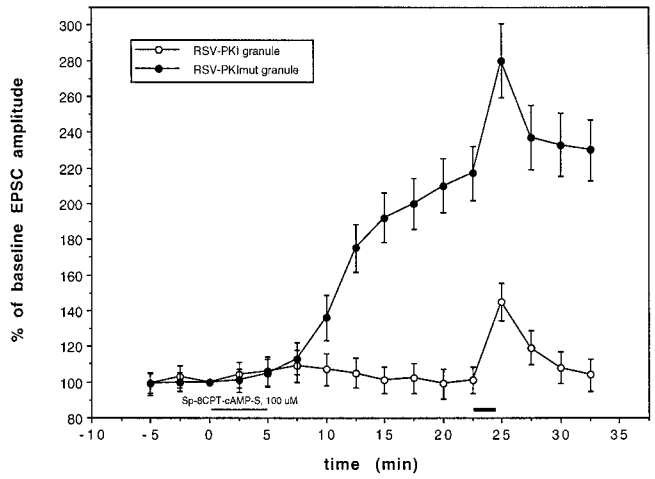

Figure 5. Transfection of the granule cell with a PKA inhibitor construct in a granule cell–Purkinje neuron pair blocks synaptic potentiation induced by an exogenous cAMP analog. Recordings were made from cell pairs in which the granule cell was transfected with either the PKA inhibitor construct RSV-PKI or the control construct RSV-PKImut. The cAMP analog Sp-8CPT-cAMP-S was applied in the bath from $t = 0$–5 min (as indicated by the *thin horizontal bar*). At $t = 22.5$ min, 4 Hz stimulation for 100 pulses was applied to the granule cell (as indicated by the *thick horizontal bar*).

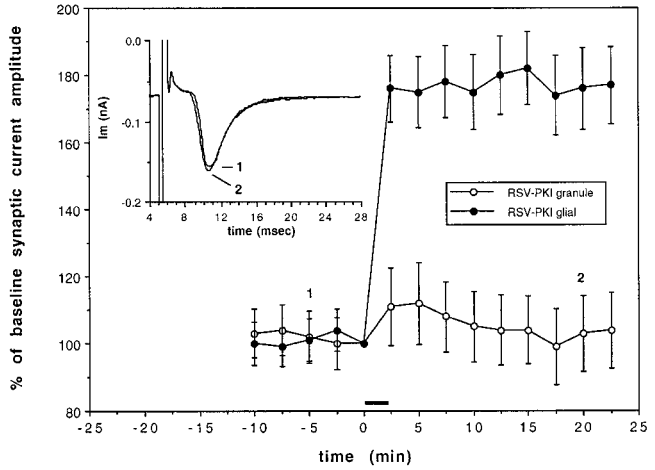

Figure 6. Transfection of a glial cell with a PKA inhibitor construct in a granule cell–glial cell pair has no effect on LTP induction. The PKA inhibitor peptide expression vector RSV-PKI was selectively transfected into either glial cells or granule cells before 4 Hz stimulation for 100 pulses. *Inset* illustrates current traces representing the average of 10 consecutive responses recorded from a granule cell–glial cell pair in which the granule cell was transfected with the RSV-PKI construct.

population, are consistent with a decrease in the probability of release. These include an increase in the failure rate, a decrease in the evoked EPSC amplitude, and an increase in paired-pulse facilitation. In addition, there was a small decrease in the mEPSC frequency with bath-applied, but not biolistic-transfected, PKA inhibitors. This last difference is not surprising given that a very small fraction of the synapses contributing to the net mEPSC frequency were likely to have been transfected.

If granule cell transfection with RSV-PKI is blocking cerebellar LTP induction through inhibition of PKA in the granule cell terminal, then it would be predicted that this treatment should also block induction of an LTP-like effect produced by bath application of an exogenous cAMP analog. Indeed, bath application of 100 μM Sp-8CPT-cAMP-S (at $t = 0$–5 min) produced a dramatic potentiation of granule cell-evoked EPSCs in cell pairs in which the granule cell was transfected with the control construct RSV-PKImut (217 ± 15.1% at $t = 22.5$ min, $n = 4$) (Fig. 5). This potentiation occluded the effect of subsequent stimulation at 4 Hz for 100 pulses (230 ± 17.0% at $t = 32.5$ min). However, when the granule cell was transfected with the PKA inhibitor construct RSV-PKI, neither Sp-8CPT-cAMP-S nor tetanic stimulation of the granule cell produced sustained potentiation (101 ± 7.6% at $t = 22.5$ min and 104 ± 8.8% at $t = 32.5$ min, $n = 6$).

Previous work from this lab has shown that action potentials evoked in granule cells in these cultures can give rise to synaptic currents recorded in nearby glial cells. When recordings were made with Cl-based internal salines, these currents were comprised of ~90% AMPA–kainate receptor-mediated current and ~10% current mediated by electrogenic glutamate transport. Both components of this glial synaptic current can be used as test pulses to detect cerebellar LTP in granule cell–glial cell pairs (Linden, 1997, 1998). To test a potential role of glial PKA in LTP induction, RSV-PKI was selectively transfected in glial cells and LTP was assessed in granule cell–glial cell pairs (Fig. 6), revealing that glial PKA inhibition was ineffective in blocking LTP (177 ± 11.5% at $t = 22.5$ min, $n = 5$). However, when granule cells were transfected in granule cell–glial cell pairs, LTP was blocked (104 ± 11.2% at $t = 22.5$ min, $n = 6$).

DISCUSSION

The main finding of this study is that activation of PKA in the presynaptic neuron is required for induction of cerebellar LTP in granule cell–Purkinje neuron pairs in culture. This is supported by the observation that transfection of granule cells with a PKA inhibitory peptide produces a nearly complete blockade of LTP induced by either granule cell stimulation or an exogenous cAMP analog, whereas transfection with a mutant peptide that does not inhibit PKA has no effect. Furthermore, PKA activation in other cellular compartments is unlikely to be involved because transfection of either postsynaptic Purkinje neurons or glial cells with the PKA inhibitory peptide had no effect. These results are consistent with the blockade of cerebellar LTP or LTP-like phenomena by bath-applied PKA inhibitors seen in this and other experiments (Salin et al., 1996; Chavis et al., 1998; Kimura et al., 1998; Storm et al., 1998). The blockade of LTP induction produced by PKA inhibitors herein is likely to be a relatively specific effect, because no major effects of these treatments on basal synaptic physiology was observed. The failure of PKA inhibitors acutely applied to the granule cell soma to block cerebellar LTP induction is most likely to result from inadequate perfusion of the granule cell presynaptic terminals within the time course of this experiment, consistent with the observation that QX-314 was unable to suppress back-propagating axonal spikes when applied in this manner.

In addition to revealing the locus of PKA activation in cerebellar LTP induction, this report reinforces a general caveat about presynaptic application of drugs. Although some laboratories have succeeded in rapidly modifying presynaptic processes with drugs applied in the somata of cultured neurons, one cannot assume that this will be the case in all model systems. Cerebellar granule cell axons may be unusually resistant to perfusion because they have small diameters (~0.1 μm). In addition, granule cell axons typically take a very circuitous path in culture, adding

Table 1. Effects of PKA manipulations on some basal properties of granule cell–Purkinje neuron synapses in culture

Measure	Control	KT5720 bath	Rp-8CPT-cAMP-S bath	RSV-PKI granule	RSV-PKImut granule
R_{input} (MΩ)	181 ± 28	174 ± 22	173 ± 30	190 ± 25	187 ± 21
mEPSC frequency (sec^{-1})	7.9 ± 2.6	6.2 ± 2.0	6.1 ± 2.3	7.8 ± 2.9	7.0 ± 3.0
mEPSC amplitude (pA)	27 ± 7	31 ± 8	30 ± 6	24 ± 7	22 ± 5
Failures (%)	35 ± 6	42 ± 5	41 ± 7	33 ± 7	34 ± 9
Evoked EPSC amplitude (pA)	102 ± 30	90 ± 26	92 ± 30	88 ± 33	106 ± 26
Paired-pulse facilitation (%)	164 ± 10	189 ± 15	183 ± 16	181 ± 13	161 ± 12

Values are mean ± SEM. $n = 10$ cell pairs per group. For each cell pair, measurements were made as follows. R_{input} was determined by measuring the sustained current deflection during a voltage step from −80 to −90 mV imposed on the Purkinje neuron. mEPSC frequency and amplitude measurements were made using the same 500-sec-long sample recorded in 0.5 μM tetrodotoxin. Failure rate was calculated from a sample of 100 pulses applied at 0.05 Hz. Mean evoked EPSC amplitudes were measured by averaging responses to 50 consecutive paired stimuli (50 msec interpulse interval) applied at 0.05 Hz and were then normalized to derive the percentage facilitation measure.

considerably to their length (data not shown). Interestingly, diffusion of marker dyes from a somatic patch pipette to axon terminals cannot be taken as a reliable indicator of effective perfusion of this compartment. In the present case, bis-fura-2 fluorescence could be clearly detected in granule cell presynaptic terminals within 10 min of initiating whole-cell recording (data not shown).

There are some caveats that should be sounded in relation to the present studies. First, although it is likely that the drugs and peptide we have applied are exerting their effects on LTP induction through PKA inhibition, it is worth noting that some of the drugs that have been used to inhibit PKA have side effects on cyclic nucleotide-gated ion channels, including the regulatory site binding Rp-cAMP-S derivatives (Kramer and Tibbs, 1996) used herein. To our knowledge, PKI peptide and KT5720 have yet to be screened for this side effect. Cyclic nucleotide-gated ion channels have been shown recently to be functionally expressed in a wide variety of brain regions, including cerebellum (El-Husseini et al., 1995; Bradley et al., 1997), and may have a role in induction of hippocampal LTP (Parent et al., 1998). Second, the organization of granule cell–Purkinje neuron synaptic connections in culture has some important differences with the intact cerebellum (or the slice preparation) that could potentially impact on mechanisms of cerebellar LTP induction. In the juvenile rat cerebellar slice, it has been reported that the average amplitude of current evoked in a granule cell–Purkinje neuron pair is 14.4 ± 16.1 pA (mean ± SD) (Barbour, 1993). In the present study, using similar conditions (Cs-based internal saline, similar V_{hold}), a mean evoked EPSC amplitude of 102 ± 30 pA (mean ± SEM) was observed. This value, although noticeably larger than that in the slice, is similar to that reported previously for granule cell–Purkinje neuron pairs in culture (Hirano and Hagiwara, 1988; Hirano, 1991). Two factors are likely to account for the slice versus culture difference. One is that the Purkinje neuron mEPSC amplitude reported in culture (in both this and previous studies) is approximately twofold larger than that seen in slices. Another is that, although a parallel fiber in the slice (or the intact cerebellum) typically makes only 1 or 2 synaptic contacts with a Purkinje neuron, no such anatomical constraint is present in culture. In the present cultures, we estimate that the mean number of synapses in a granule cell–Purkinje neuron pair is 4 ± 2.

A requirement for presynaptic PKA activation in cerebellar LTP induction integrates well with previous experimental observations. Cerebellar LTP induction appears to require presynaptic (but not postsynaptic) Ca influx and activation of Ca sensitive adenylyl cyclase, an enzyme that is concentrated in granule cell presynaptic terminals. This presynaptic cAMP elevation could then activate PKA is this same compartment, as indicated in the present study, leading to LTP induction. There are several lines of evidence suggesting that the expression of cerebellar LTP is presynaptic as well. First, induction of cerebellar LTP is associated with a decrease in the rate of synaptic failures (Hirano, 1991; Linden, 1997, 1998; Storm et al., 1998) and the extent of paired-pulse facilitation (Salin et al., 1996; Linden, 1998). Unfortunately, neither of these forms of evidence is definitive because postsynaptic scenarios have been proposed in which these parameters could be altered. Second, induction of an LTP-like effect by application of an exogenous cAMP analog was associated with an increase in presynaptic vesicular cycling as measured using an immunocytochemical technique (Chavis et al., 1998). This LTP-like effect is independent of alterations in axonal excitability or Ca influx into presynaptic terminals, suggesting a direct effect on the secretory apparatus (Chen and Regehr, 1997). Third, cerebellar LTP in culture can be detected using either AMPA–kainate receptor-mediated currents recorded in postsynaptic Purkinje neurons, AMPA–kainate receptor-mediated currents recorded in postsynaptic glial cells, or electrogenic glutamate transport currents recorded in postsynaptic glial cells, suggesting a common presynaptic locus of expression (Linden, 1997, 1998). Thus, the most parsimonious model for cerebellar LTP induction is that presynaptic Ca influx activates Ca-sensitive adenylyl cyclase, and the resultant cAMP transient activates presynaptic PKA, resulting in a phosphorylation event that potentiates glutamate release.

It is likely that cerebellar LTP is similar, if not identical, to LTP of the hippocampal mossy fiber–CA3 synapse. Both synapses have few, if any, NMDA receptors, and both presynaptic cells strongly express Ca-sensitive adenylyl cyclase type I. Two models have been proposed for LTP at this synapse. In one, the initial trigger for LTP is a postsynaptic Ca transient derived from two sources, influx through L-type voltage-sensitive Ca channels and mobilization via group I metabotropic glutamate receptors linked to phospholipase C activation and consequent production of inositol-1,4,5-trisphosphate. This Ca transient triggers activation of postsynaptic PKA (presumably via postsynaptic Ca-sensitive adenylyl cyclase), and this results in the production of a retrograde signal that ultimately acts on the presynaptic terminal to increase transmitter release (Johnston et al., 1992; Xiang et al., 1994; Kapur et al., 1998; Yeckel et al., 1999). Most recently, this model has been supported by experiments showing that mossy fiber LTP can be blocked by postsynaptic application of Ca chelator at unusually high concentration (>30 mM BAPTA), bath application of cocktail containing antagonists for both group I metabotropic glutamate receptors and ionotropic glutamate receptors, or postsynaptic application of PKA inhibitors (Yeckel et al., 1999).

A second model contends that mossy fiber–CA3 LTP does not require postsynaptic Ca influx or glutamate receptor activation but does require presynaptic Ca influx and subsequent activation of Ca-sensitive adenylyl cyclase and PKA (Zalutsky and Nicoll, 1990; Ito and Sugiyama, 1991; Katsuki et al., 1991; Huang et al., 1994; Weisskopf et al., 1994; Tong et al., 1996; Villacres et al., 1998). This model is further supported and extended by the finding that hippocampal mossy fiber LTP is strongly attenuated in slices taken from mutant mice in which the synaptic vesicle protein Rab3A, which is an effector for the PKA substrate rabphilin 3, has been rendered null (Castillo et al., 1997). In CA3 synaptosomes, stimuli that increase cAMP facilitate the action of Ca on the secretory apparatus (as assessed by measurement of glutamate release in response to ionomycin challenge), and this effect is blocked when Rab3A is deleted (Lonart et al., 1998). To our knowledge, all studies of cerebellar LTP published to date (including the present report), indicate that cerebellar LTP induction appears to use similar, if not identical, molecular mechanisms to those of the second hippocampal mossy fiber LTP model. Cerebellar LTP is not blocked by postsynaptic Ca chelator, even at very high concentrations (35 mM BAPTA) (Fig. 1). It can be induced when all glutamate receptors are blocked (both metabotropic and ionotropic) and the postsynaptic cell is voltage clamped at −80 mV (Linden, 1997). It may be induced when the postsynaptic cell is glial rather than neuronal (Linden, 1997, 1998) (Fig. 6). Finally, cerebellar LTP induction is blocked by presynaptic but not postsynaptic application of PKA inhibitors. These observations all argue strongly for a presynaptic induction mechanism. In the future, it will be useful to determine whether cerebellar LTP also requires rabphilin 3 and/or rab3A.

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