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Calcium-Dependent PKC Isoforms Have Specialized Roles in Short-Term Synaptic Plasticity

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SUMMARY

Posttetanic potentiation (PTP) is a widely observed form of short-term plasticity lasting for tens of seconds after high-frequency stimulation. Here we show that although protein kinase C (PKC) mediates PTP at the calyx of Held synapse in the auditory brainstem before and after hearing onset, PTP is produced primarily by an increased probability of release (*p*) before hearing onset, and by an increased readily releasable pool of vesicles (RRP) thereafter. We find that these mechanistic differences, which have distinct functional consequences, reflect unexpected differential actions of closely related calcium-dependent PKC isoforms. Prior to hearing onset, when PKCγ and PKCβ are both present, PKCγ mediates PTP by increasing *p* and partially suppressing PKCβ actions. After hearing onset, PKCγ is absent and PKCβ produces PTP by increasing RRP. In hearing animals, virally expressed PKCγ overrides PKCβ to produce PTP by increasing *p*. Thus, two similar PKC isoforms mediate PTP in distinctly different ways.

INTRODUCTION

Many forms of synaptic plasticity regulate neurotransmitter release by a combination of increasing the probability of release (*p*) and the size of the readily releasable pool of vesicles (RRP) (Pan and Zucker, 2009; Regehr et al., 2009; Zucker and Regehr, 2002). Whether increases in *p* or RRP underlie synaptic enhancement is important from a functional point of view, because these two mechanisms have very different effects on responses to stimulus trains (Pan and Zucker, 2009; Thanawala and Regehr, 2013). An increase in the RRP simply scales up the size of synaptic responses evoked by repetitive activation, and the effect is similar in many ways to increasing the number of postsynaptic receptors. In contrast, increasing *p* also increases use-dependent depression, and as a result for repetitive activation the initial synaptic response is more strongly enhanced than subsequent responses. Consequently, overall neurotransmitter release evoked by high-frequency stimulation is doubled if RRP doubles but is essentially unchanged if *p* doubles. It is particularly controversial whether changes in *p* or RRP underlie posttetanic potentiation (PTP), a form of short-term plasticity lasting tens of seconds to minutes after tetanic stimulation (Alle et al., 2001; Bao et al., 1997; Griffith, 1990; Magleby, 1979; Magleby and Zengel, 1975; Zucker and Regehr, 2002).

PTP is thought to be a neural mechanism that contributes to short-term memory, synaptic filtering, and information processing (Abbott and Regehr, 2004; Klug et al., 2012; Silva et al., 1996). High-frequency (tetanic) stimulation induces PTP by transiently increasing presynaptic calcium, which in turn activates downstream molecular effectors that elevate neurotransmitter release (Delaney and Tank, 1994; Delaney et al., 1989; Habets and Borst, 2006; Korogod et al., 2005; Regehr et al., 1994; Zucker and Regehr, 2002). A growing body of evidence supports a critical role for protein kinase C (PKC) in PTP (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Fioravante et al., 2011; Korogod et al., 2007; Lee et al., 2008; Wierda et al., 2007). The role of PKC in PTP has been most extensively studied at the calyx of Held synapse (Fioravante et al., 2011; Korogod et al., 2007; Lee et al., 2008), where it was established that in postnatal day 11–14 (P11–P14) animals, PTP is mediated primarily by PKCβ (Fioravante et al., 2011), one of the "classical" calcium-dependent isoforms (PKCα, PKCβ, and PKCγ), as opposed to the many calcium-insensitive "novel" and "atypical" isoforms (Newton, 1995, 2001; Steinberg, 2008). There is, however, considerable debate regarding whether PKC enhances release at the calyx of Held by increasing *p* or RRP. Most studies suggest that PKC mainly increases *p* (Habets and Borst, 2005, 2006; Korogod et al., 2007; Lou et al., 2005; Turecek and Trussell, 2001; Wu and Wu, 2001), but others suggest that PKC prominently increases RRP (Chu et al., 2012; Fioravante et al., 2011; Habets and Borst, 2007).

Because the calyx of Held synapse undergoes age-dependent anatomical and functional changes (Borst and Soria van Hoeve, 2012; Nakamura and Cramer, 2011; Rodríguez-Contreras et al., 2008; Taschenberger et al., 2002; von Gersdorff and Borst, 2002), we compared the properties of PTP before and after the onset of hearing, and we assessed the roles of the

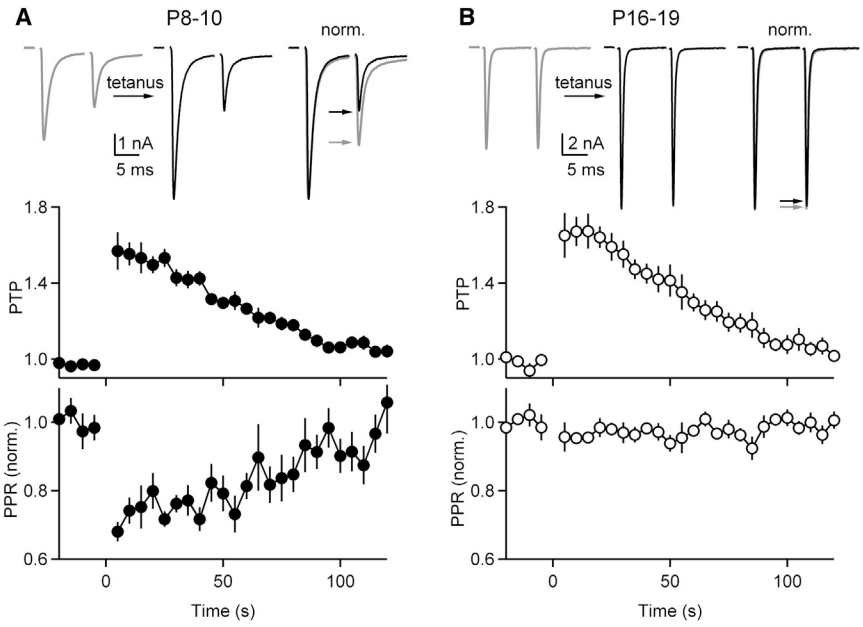


Figure 1. PTP Is Accompanied by a Large Decrease in Paired-Pulse Plasticity before the Onset of Hearing but Not after Hearing Onset

The calyx of Held was stimulated every 5 s with a pair of pulses separated by 10 ms, PTP was induced at t = 0 using a 4 s, 100 Hz train, and stimulation with pairs of pulses resumed. Results are shown for P8–P10 animals (A) and P16–P19 animals (B). Representative traces show the average baseline paired-pulse EPSC (A and B, top left, gray), the potentiated response during the peak of PTP (A and B, top middle, black), and traces normalized to the first EPSCs (A and B, top right). The EPSC amplitudes (A and B, middle) and the PPR are plotted as a function of time (A and B, bottom). Error bars represent SEM.

calcium-dependent PKC isoforms. We find that PKCγ produces PTP by increasing *p* before hearing onset and that PKCβ produces PTP by increasing RRP afterward. In prehearing PKCγ KO animals, PTP persists but is mainly due to PKCβ increasing RRP. This indicates that the key to whether PTP is due to an increase in *p* or RRP is whether PKCγ or PKCβ mediates synaptic enhancement. When both PKCγ and PKCβ are present, PTP is mediated by an increase in *p*, suggesting that PKCγ suppresses PKCβ-dependent regulation of the RRP. Thus, even though PKCγ and PKCβ are highly similar calcium-dependent isoforms that both mediate PTP, they do so by different synaptic mechanisms with different functional consequences.

RESULTS

Age-Dependent Properties of PTP

We initially determined whether the mechanisms of PTP are developmentally regulated by comparing the properties of synaptic plasticity before (P8–P10) and after (P16–P19) the onset of hearing (~P12) (Sonntag et al., 2009, 2011). A widely used approach to assess whether changes in *p* contribute to changes in neurotransmitter release is to determine whether synaptic changes are accompanied by alterations in the paired-pulse ratio (PPR) of two closely spaced stimuli. Typically, low *p* synapses facilitate, due to an accumulation of residual calcium, and high *p* synapses depress, probably as a result of significant depletion of the RRP during the first stimulus. If PTP reflects an increase in *p* it is expected to be accompanied by a decrease in PPR. We stimulated with pairs of pulses (Δt = 10 ms) every 5 s prior to and after tetanic stimulation (4 s, 100 Hz) (Figure 1A, top). On average, there was a significant reduction in PPR (−32% ± 3%; n = 6; $p < 0.01$) in P8–P10 animals (Figure 1A, bottom) during PTP (Figure 1A, middle). In P16–P19 animals, even though the basal properties of synaptic transmission were different from P8–P10 animals, as described previously (Iwasaki and Takahashi, 2001; Taschenberger et al., 2002), the magnitude and time course of PTP were comparable to that observed in P8–P10 animals (Table S1 available online). However, only a minimal reduction in PPR accompanied PTP (−3.1% ± 1.3%; p = 0.36) in P16–P19 animals (n = 8) (Figure 1B). These findings suggest that changes in *p* make a larger contribution to PTP in P8–P10 animals, but they cannot be used to precisely quantify the contributions of *p* and RRP to plasticity.

It is possible, however, to quantify the contributions of *p* and RRP to PTP from synaptic responses evoked by action potential trains in the presence of cyclothiazide and kynurenate to prevent postsynaptic receptor desensitization and saturation (Fioravante et al., 2011; Korogod et al., 2005; Lee et al., 2008; Schneggenburger et al., 1999). In the case of PTP, the responses to a stimulus train used to induce PTP and to a stimulus train 10 s later (at the peak of PTP), are compared. Such an approach is illustrated for prehearing and hearing animals (Figure 2). The amplitudes of the excitatory postsynaptic currents (EPSCs) can then be used to determine the size of the RRP in several different ways. A plot of the cumulative EPSC versus the stimulus number can be used to determine the size of the RRP (Moulder and Mennerick, 2005; Pan and Zucker, 2009; Stevens and Williams, 2007; Thanawala and Regehr, 2013). The key to this approach is that the EPSC amplitude eventually reaches a steady-state level, and under these conditions the RRP is depleted and the remaining release is due to replenishment from a reserve pool (Schneggenburger et al., 1999; Thanawala and Regehr, 2013). Extrapolation is then used to determine the size of the RRP (RRP_{train}). Although this approach is widely used, it is known to overestimate the amount of replenishment that occurs, which leads to an underestimate of the size of the RRP (Lee et al., 2008; Schneggenburger et al., 1999; Thanawala and Regehr, 2013). It is possible to refine this approach by correcting the estimate of the amount of replenishment that occurs early in the train to obtain a corrected estimate (RRP_{trainC}) (Thanawala and Regehr, 2013). Lastly, we estimated RRP using an approach that was first introduced by Elmqvist and Quastel (Elmqvist and Quastel, 1965; Grande and Wang, 2011; Taschenberger et al.,

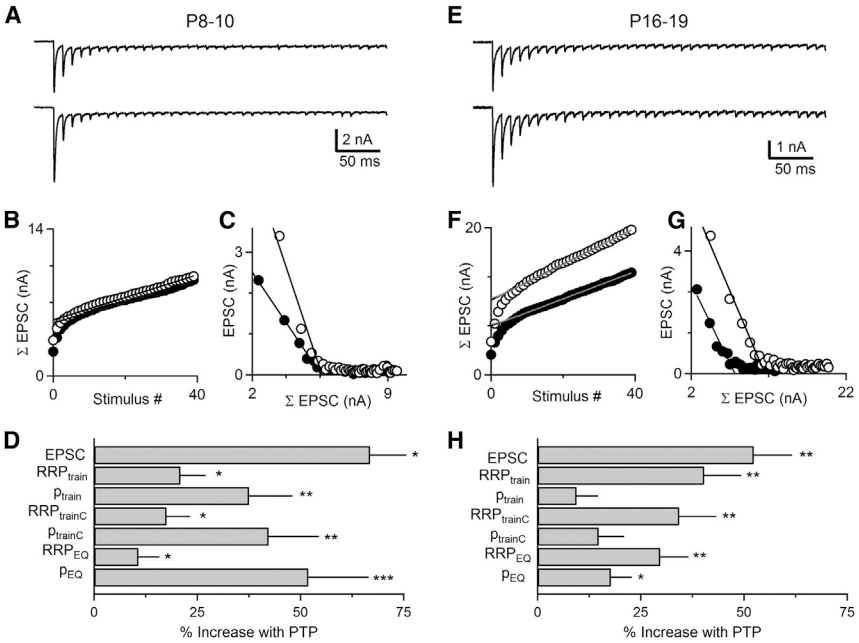


Figure 2. Assessing the Contributions of *p* and RRP to PTP before and after Hearing Onset

Mechanisms of PTP were examined using trains in the presence of kynurenate and CTZ to prevent receptor saturation and desensitization.

(A and E) Synaptic currents evoked by the first 40 stimuli of 4 s, 100 Hz train (top) and by a 40 pulse 100 Hz train (bottom) 10 s after tetanic stimulation (at the peak of PTP) are shown.

(B, C, F, and G) The change in pool size and *p* were determined using the cumulative EPSC method, the corrected EPSC method (B and F), and the EQ method (C and G).

(D and H) Summary of the changes in synaptic strength, RRP, and *p* determined using the three different quantification methods. Paired t tests were used to compare the changes in EPSC, RRP_{train}, p_{train}, RRP_{trainC}, p_{trainC}, RRP_{EQ}, and p_{EQ} during PTP to baseline, as indicated (*p < 0.05, **p < 0.01, ***p < 0.001). Error bars represent SEM.

2002, 2005; Thanawala and Regehr, 2013). This method is based on the assumption that during the train the synaptic currents get progressively smaller as the RRP depletes. A plot of the amplitude of the EPSCs in the train as a function of the cumulative EPSC is then used to estimate RRP (RRP_{EQ}). The amplitudes of the EPSCs were then used to determine the amount of synaptic enhancement, and *p* was computed from the equation EPSC = RRP × *p*. The use of these three approaches to quantify RRP and *p* at the calyx of Held has been addressed previously (Thanawala and Regehr, 2013). Our estimates of the RRP size were not confounded by large increases in the postsynaptic neurotransmitter sensitivity because there was only a very small increase in the size of quantal responses after PTP was induced (7.5% ± 6.5%, n = 12 in P8–P10 wild-type (WT) animals; 6.4% ± 6.6%, n = 10 in P16–P19 wild-type mice for the interval 6–16 s posttetanus).

We used all three of these approaches to determine the contributions of RRP and *p* to PTP in P8–P10 animals (Figures 2A–2D). The responses to 40 stimuli at 100 Hz during the initial conditioning train (a total of 400 stimuli at 100 Hz) that is used to induce PTP (Figure 2A, top) and during a second conditioning train 10 s after the end of initial train (Figure 2A, bottom) are shown for a representative experiment (Figures 2A–2C). As shown in this example, the responses to both trains showed prominent use-dependent depression, pool size increases were small (RRP_{trainC} 4%, RRP_{EQ} 2%), and the enhancement of the EPSC was primarily a consequence of an increase in *p* (p_{trainC} 41%, p_{EQ} 45%). On average, in P8–P10 wild-type animals, PTP was 67% ± 16%, RRP_{trainC} 18% ± 6%, RRP_{EQ} 11% ± 5%, p_{trainC} 42% ± 12%, and p_{EQ} 52% ± 15% (Figure 2D; Table S2).

We used the same approaches to determine the contributions of RRP and *p* to PTP in P16–P19 animals (Figures 2E–2H). In comparison to the responses observed in prehearing animals, the initial EPSCs were larger, the depression was less pronounced, and the steady-state EPSCs were larger (Figure 2E; Figure S1). The magnitude of PTP was comparable to that observed in prehearing animals, but the changes in RRP played a much larger role. In the illustrated example, the initial EPSC was enhanced by 64%, and there was a large increase in the size of the readily releasable pool (RRP_{trainC} 57%, RRP_{EQ} 52%; Figures 2F and 2G). A summary of the contributions of *p* and RRP, regardless of the method used to estimate them, revealed that the enhancement of the EPSC was primarily a consequence of an increase in RRP in P16–P19 animals (Figure 2H; Table S2). These findings support the hypothesis that prior to the onset of hearing PTP is mediated primarily by an increase in *p*, but after hearing onset it is mediated predominantly by an increase in RRP.

Age-dependent differences in the decrease of PPR that accompanied PTP were also observed when cyclothiazide and kynurenate were included in the bath (Fucile et al., 2006; Taschenberger et al., 2002). Under these conditions there was a significant reduction in PPR in P8–P10 mice (−42% ± 8%, n = 10, p < 0.05) and in P16–P19 mice (−14% ± 5%, n = 8, p < 0.05) but the reduction was larger in prehearing mice (p < 0.05).

Contributions of Different PKC Isoforms to PTP

To elucidate the mechanisms underlying PTP in prehearing and hearing animals, we performed a series of experiments using pharmacological and genetic manipulations. We characterized the basal properties of transmission and found that for all conditions tested, there were no differences in the initial amplitudes of the EPSC, initial PPRs, the initial sizes of RRP, and the initial probability of release (Figure S1). These data suggest that the basal properties of synaptic transmission among wild-type and knockout (KO) groups are not different within each age group of animals. For a subset of experimental conditions, we also

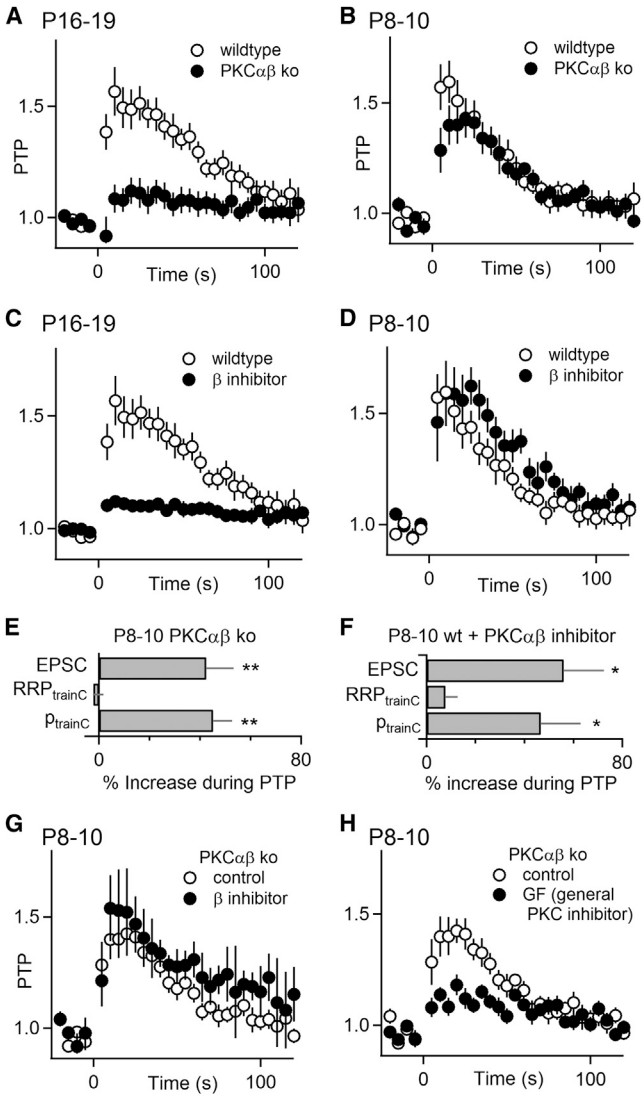


Figure 3. Age-Dependent Differences in the Roles of Calcium-Dependent PKC Isoforms in PTP

PTP as a function of time for P16–P19 animals (A and C) and P8–P10 animals (B, D, G, and H).

(A and B) PTP from wild-type and PKCαβ double KO cells are compared for P16–P19 (A) and P8–P10 (B) animals.

(C and D) The effects of the PKCβ inhibitor (Calbiochem 539654) on PTP in wild-type cells are shown for P16–P19 (C) and P8–P10 (D) animals.

(E and F) Summary plots of RRP and *p* contributions to PTP in prehearing PKCαβ double KO (E) and wild-type (F) animals. Experiments were performed in the presence of CTZ and kynurenate as shown in Figure S2.

(G and H) The effects of the PKCβ inhibitor (G) and a broad spectrum PKC inhibitor (GF109203X) (H) on PTP in P8–P10 PKCαβ double KO animals are shown. *p < 0.05, **p < 0.01. Error bars represent SEM.

performed experiments in the presence of cyclothiazide and kynurenate to quantify contributions of RRP and *p* to PTP, and to determine the changes in PPR that accompany PTP (Figure S1 and Table S2). We found that cyclothiazide and kynurenate did not significantly alter the amplitude of PTP (Tables S1 and S2). For the remainder of the paper, we also determined RRP and *p* by the three methods shown in Figure 2, but as there was good agreement between the corrected train method and the Elmqvist and Quastel method (Thanawala and Regehr, 2013), we present only RRP_{trainC} and p_{trainC} for simplicity.

We have previously shown that in P11–P14 mice, PKCα and PKCβ are both present in the calyx of Held, but PTP is mediated mainly by PKCβ-dependent increases in RRP (Fioravante et al., 2011). The age dependence of the properties of PTP could arise from differences in PKC signaling. There are two leading hypotheses by which PKC could account for the observed differences between prehearing and hearing animals: (1) PTP is mediated primarily by PKCβ at all ages but it enhances transmission through different mechanisms throughout development, or (2) in prehearing animals PTP is not mediated by PKCβ.

The role of PKC isoforms in prehearing and hearing animals was assessed with knockout animals and with an isoform-specific inhibitor. Whereas PTP was greatly reduced in P16–P19 PKCαβ knockout animals (8.3% ± 6.4%; p < 0.05; Figure 3A), it was largely intact in prehearing PKCαβ knockout animals (40% ± 9.0%; p = 0.15; Figure 3B). We also used a newly available PKCβ inhibitor to examine the role of PKCβ (Tanaka et al., 2004). Such a pharmacological approach compliments the use of knockout animals by allowing acute inhibition of PKCβ in a manner that is free from potential developmental complications that could occur in global knockout animals. This compound inhibits PKCα, PKCβI, PKCβII, and PKCγ, with K_ms of 330 nM, 21 nM, 5 nM, and >1 μM, respectively. These properties suggest that this inhibitor may be well-suited to our experiments in which we need to inhibit PKCβ without affecting PKCγ (although it is possible that this inhibitor could also partially inhibit PKCα, which plays a very minor role in PTP at the calyx of Held (Fioravante et al., 2011). We found that at a concentration of 250 nM, this drug reduced the magnitude of PTP from 57% ± 11% (n = 10) to 12% ± 1.5% (n = 7, p < 0.01) in P16–P19 animals (Figure 3C). Intriguingly, in P8–P10 wild-type animals, the PKCβ inhibitor did not attenuate PTP (59% ± 15%; n = 10; p = 0.97; Figure 3D). Moreover, the inhibitor did not disrupt PTP in P8–P10 PKCαβ KO mice (54% ± 15%; n = 3; p = 0.50; Figure 3G), suggesting that it does not have off-target effects. These findings indicate that PTP is dependent on PKCβ in hearing animals, but not in prehearing animals.

We went on to examine the contribution of *p* and RRP to synaptic enhancement in prehearing animals in which PKCβ was absent or inhibited. In PKCαβ KO animals, PTP is mediated exclusively by an increase in *p* and the small increase in pool size that is apparent in age-matched wild-type animals (Figure 2D) is absent (p_{trainC} 45% ± 8%, n = 9; Figure 3E: Figure S2A). For wild-type animals in the presence of a PKCβ inhibitor, there was a small but nonsignificant increase in RRP, and PTP was mediated by an increase in *p* (p_{trainC} 47% ± 16%, n = 14; Figure 3F; Figure S2B).

These results suggest that prior to the onset of hearing, PTP is mediated by a mechanism that is independent of PKCα and PKCβ. Alternatively, there could be a compensatory adaptation that only mediates PTP in the absence of PKCα and PKCβ. For example, at the granule cell to Purkinje cell synapse in the cerebellum, PTP is mediated by PKCα and PKCβ in wild-type animals, but it is mediated by a PKC-independent mechanism in

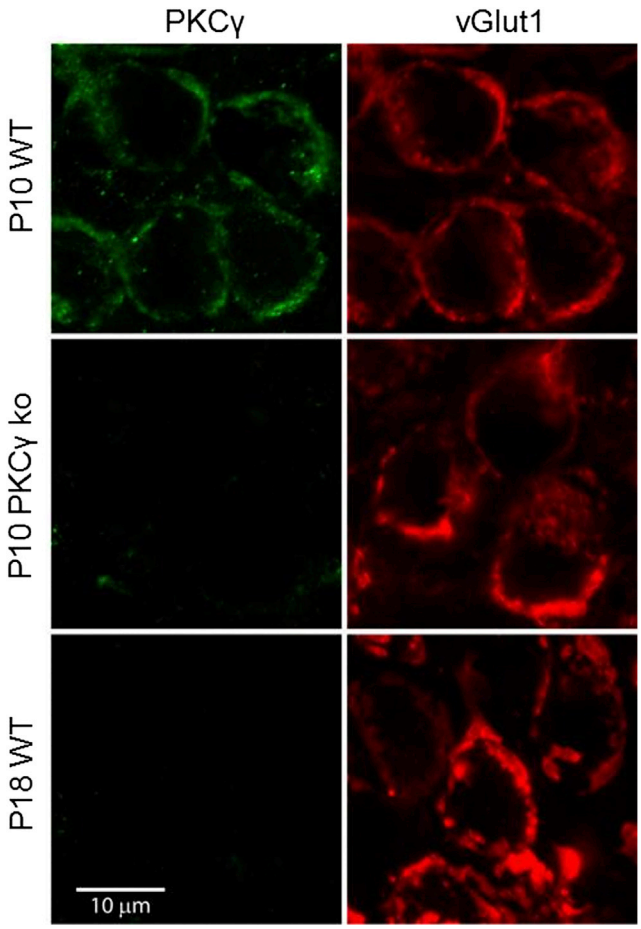


Figure 4. Immunohistochemical Localization of PKCγ at the Calyx of Held in Animals before and after Hearing Onset

Brain slices containing the MNTB region from wild-type (WT) and PKCγ KO animals prior to and after hearing onset were colabeled with antibodies to PKCγ (green) and an antibody to the presynaptic marker vGlut1 (red). Representative images are shown for P10 and P18 slices from WT and PKCγ KO animals.

PKCαβ KO mice (Fioravante et al., 2012). Although the observation that PTP is strongly attenuated in PKCαβ KO mice indicates that such a compensatory mechanism is not present at the calyx of Held in hearing animals, it is possible that a compensatory mechanism is present before the onset of hearing.

PTP in P8–P10 PKCαβ KO animals could be mediated either by a mechanism that involves other PKC isoforms, or one that is completely PKC independent. It is possible to distinguish between these possibilities by testing the effects of a broad spectrum PKC inhibitor on PTP in these animals. Previous studies have shown that in slices from wild-type mice a broad spectrum PKC inhibitor eliminates most of the PTP, and a small component remains that is mediated at least in part by myosin light chain kinase (Fioravante et al., 2011; Lee et al., 2008). We found that in P8–P10 PKCαβ KO mice, the pan-PKC inhibitor GF109203X (GF) greatly attenuates most PTP (14% ± 3%; n = 11; $p < 0.05$; Figure 3H). The small remaining enhancement is comparable to the enhancement mediated by myosin light chain kinase in P11–P14 wild-type mice (Fioravante et al., 2011). This observation suggests that the PKCαβ-independent PTP observed in prehearing mice could be mediated by other PKC isoform(s).

There are many PKC isoforms, but PKCα, PKCβ, and PKCγ are the only calcium-dependent ones (Newton, 1995, 2001; Steinberg, 2008). PKCγ therefore seemed like a reasonable candidate to mediate PTP in P8–P10 animals. Although previous studies suggested that PKCγ is not present at calyx of Held synapses in P13–P15 rats (Saitoh et al., 2001), the possibility that it could be present before hearing onset had not been assessed. We therefore used immunohistochemical techniques to determine whether PKCγ is present in P8–P10 animals. We colabeled glutamatergic calyces with an antibody against vesicular glutamate transporter 1 (vGlut1; red) and an anti-PKCγ antibody (green) (Figure 4). Confocal images through the center of MNTB neurons show a characteristic ring of vGlut1 labeling that demarcates the calyceal presynaptic terminals surrounding the cell bodies of MNTB neurons. In brainstem slices from a P10 representative animal, the PKCγ labeling showed a similar distribution, consistent with it being expressed presynaptically, and this labeling was absent in P8–P10 PKCγ KO animals (Figure 4, top and middle). These findings establish that PKCγ is indeed present at the calyx of Held in prehearing animals.

The lack of a contribution of PKCγ to PTP in hearing animals could arise either because PKCγ expression is downregulated during development, or because it is still present but no longer able to produce PTP. To distinguish between these possibilities, we tested for the presence of PKCγ in P16–P19 mice. We observed intense vGlut1 labeling with a pattern that was consistent with the morphology of adult calyces, but PKCγ was absent (Figure 4, bottom). This suggests that PKCγ cannot contribute to PTP in animals after hearing onset because its expression is significantly downregulated.

The finding that PKCγ is present at the calyx of Held in prehearing animals prompted us to test the role of this isoform in PTP. In P8–P10 PKCγ KO mice, PTP is present (54% ± 8%; n = 14) at a magnitude that is comparable to that observed in wild-type animals ($p = 0.65$; Figure 5A). This indicates that in prehearing animals PKCγ is not essential, and it suggests other PKC isoforms can mediate PTP in the absence of PKCγ. At first sight, the similarity in the amplitude and time course of PTP between prehearing wild-type and PKCγ KO animals does not seem to support an important role for PKCγ in PTP at this age. But an examination of the contribution of p and RRP to PTP indicates that PTP in PKCγ KO mice differs markedly from that observed in wild-type mice and indicates that PKCγ plays an important role in PTP prior to hearing onset. Remarkably, in P8–P10 PKCγ KO mice, the mechanism of PTP is very different from that seen in wild-type or in PKCαβ KO mice, and PTP is primarily a result of an increase in the size of the RRP (RRP_{trainC} 37% ± 13%; n = 8) rather than an increase in p (p_{trainC} 20% ± 11%; Figure 5B; Figure S2C). Indeed, the contributions of RRP and p are similar to those seen after the onset of hearing in wild-type animals (Figure 2H). The elimination of PKCγ from the synapses of prehearing

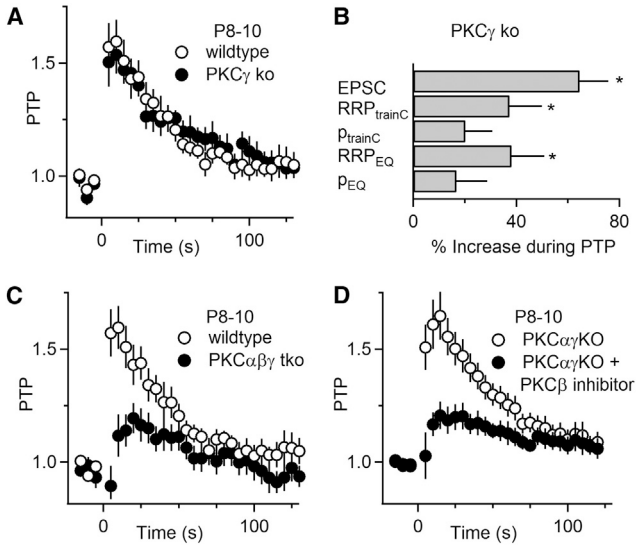


Figure 5. PKCγ Mediates PTP in Prehearing Animals at the Calyx of Held Synapse

(A) PTP in P8–P10 wild-type and PKCγ KO animals.

(B) Summary of the contributions of RRP and *p* to synaptic enhancement in P8–P10 PKCγ KO animals. Paired t tests were used to compare the changes in EPSC, RRP_{trainC}, p_{trainC} during PTP to baseline, as indicated (*p < 0.05).

(C) PTP in P8–P10 wild-type and PKCαβγ triple KO animals.

(D) PTP in PKCαγ KO animals in the absence and the presence of a PKCβ inhibitor (250 nM). Error bars represent SEM.

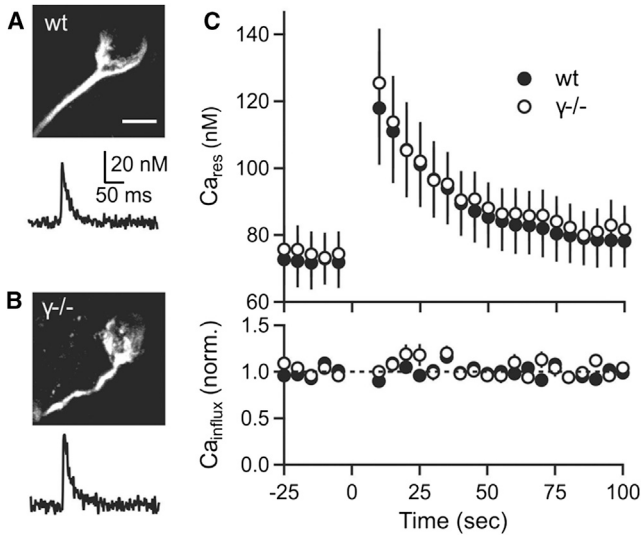


Figure 6. Tetanic Stimulation Produced Similar Presynaptic Residual Calcium Signals in Wild-Type and PKCγ KO Animals in Prehearing Animals

(A) Top: two-photon image of a calyx from a wild-type animal filled with Alexa 594 dextran and calcium-green dextran. Bottom: calcium transient evoked by a single stimulus for a wild-type calyx.

(B) Same as (A), but for a PKCγ KO animal.

(C) Plots of residual calcium (Ca_{res}, top) and calcium influx (bottom) in slices from wild-type (filled symbols) and PKCγ KO (open symbols) animals. Tetanic stimulation (4 s, 100 Hz) was at time t = 0. Scale bar for (A) and (B) indicates 10 μm. Error bars represent SEM.

animals has essentially transformed the properties of PTP to those typically observed only after the onset of hearing.

The important role of PKCγ in PTP prior to hearing onset is further supported by the strong attenuation of PTP when all three calcium-dependent isoforms are either genetically eliminated or inhibited. In contrast to PKCαβ KO animals in which PTP was present, PTP is strongly attenuated in P8–P10 PKCαβγ triple KO animals (12% ± 9%; n = 17; p < 0.01; Figure 5C). A PKCβ inhibitor attenuated the PTP in PKCαγ mice to a similar extent (17% ± 5%; n = 12; p < 0.01; Figure 5D). These observations indicate that before hearing onset a combination of PKCβ and PKCγ mediate PTP and that PKCγ plays a prominent role in wild-type animals.

Assessing the Contribution of Changes in Calcium Influx to PTP

Prior to the onset of hearing, PTP is primarily produced by PKCγ increasing *p*. One possible mechanism is that PKCγ could regulate calcium entry to increase *p*, because increases in calcium entry are known to contribute to some forms of short-term synaptic plasticity (Catterall and Few, 2008). Previously, we found that increases in calcium entry do not account for PKCβ-mediated PTP at the calyx of Held in P11–P14 animals (Fioravante et al., 2011), but at that age PKCβ-mediates PTP by increasing the RRP, whereas increasing action potential-evoked calcium influx is expected to act primarily by increasing *p* (although see Thanawala and Regehr, 2013). Thus, PTP mediated by PKCγ is a stronger candidate for the involvement of increases in calcium influx. Moreover, at the calyx of Held in prehearing animals, it is thought that increases in calcium influx contribute to PTP evoked by prolonged stimulation (Habets and Borst, 2006) and may also contribute following 4 s at 100 Hz (Korogod et al., 2007).

We therefore tested the hypothesis that PTP in prehearing animals is mediated by PKCγ-dependent increases in calcium influx in wild-type animals. We measured presynaptic calcium in the calyx of Held from P8–P10 animals as we had done previously for older animals (Fioravante et al., 2011; Figure S3). We loaded calyces with calcium green-1 dextran and Alexa 594 dextran, and labeled calyces were readily identified (Figure 6A, top). Single stimuli evoked rapid calcium transients of 31 ± 9 nM that decayed with a time constant of 66 ± 19 ms in wild-type calyces (representative trace shown in Figure 6A, bottom). After tetanic stimulation (4 s, 100 Hz), the residual calcium increased to over 100 nM and decayed to resting calcium levels with a time constant of 23 ± 7 s (Figure 6C, top). We used the calcium increase evoked by single stimuli as a means of detecting changes in calcium influx. Calcium increases evoked by single stimuli were unaltered by tetanic stimulation, indicating that tetanic stimulation does not result in an increase in calcium entry in prehearing animals (Figure 6C, bottom). We performed similar experiments in PKCγ KO animals in which PTP is mediated by an increase in RRP rather than *p*. Calcium signaling in PKCγ KO animals and wild-type animals was indistinguishable (Figures 6B and 6C). Thus, we find that PKCγ does not produce PTP by

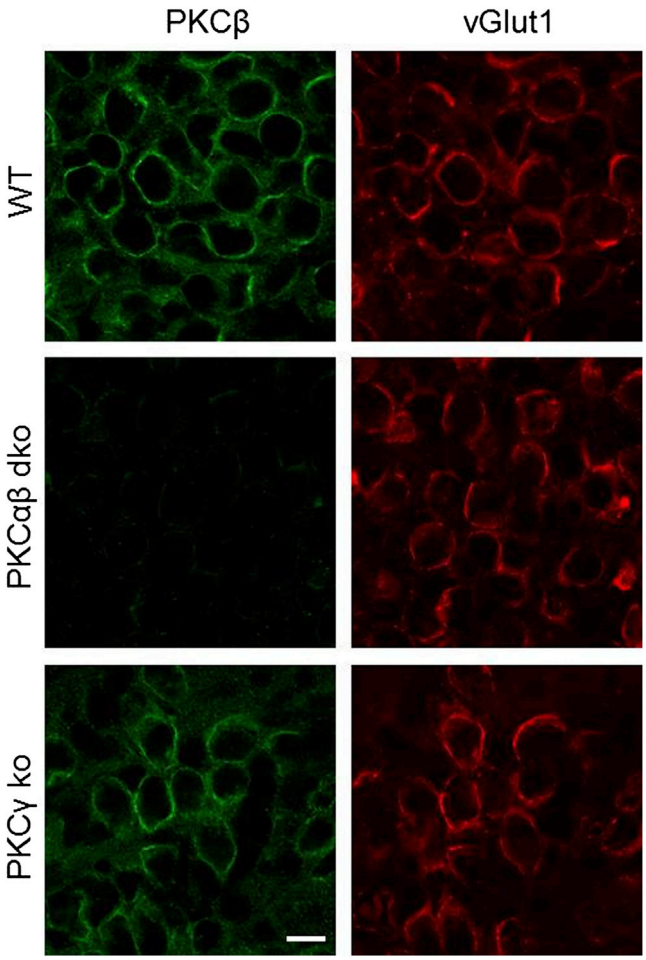


Figure 7. PKCβ Is Present at the Calyx of Held prior to Hearing Onset
Brain slices from P8–P10 animals containing the MNTB region were colabeled with antibodies to PKCβ (green) and an antibody to the presynaptic marker vGlut1 (red). Representative images are shown for P10 slices from wild-type (WT), PKCαβ KO, and PKCγ KO animals. Scale bar indicates 10 μm.

increasing action potential-evoked calcium entry, and PKCγ-dependent increases in calcium influx do not account for PTP in prehearing animals.

PKCγ Suppresses the Actions of PKCβ to Dictate the Mechanism of PTP

Based on the prominent contribution of *p* to PTP in wild-type animals prior to the onset of hearing, it seems that PTP is mediated primarily by PKCγ. However, in prehearing PKCγ KO animals, PTP is produced by an RRP increase mediated by PKCβ. Why does PKCβ mediate PTP in prehearing PKCγ KO animals but play such a minor role in P8–P10 wild-type animals?

To begin to address this question, we used immunohistochemistry to examine the expression of PKCβ at the calyx of Held prior to the onset of hearing. We found that PKCβ is present in wild-type animals (Figure 7, top). Immunofluorescence is eliminated in PKCαβ double KO animals (Figure 7, middle). In PKCγ knockout animals, PKCβ is still present and there is no obvious increase in expression levels (Figure 7, bottom). These findings indicate that in prehearing wild-type animals PKCβ is present, but it does not contribute significantly to PTP. This suggests that PKCγ somehow prevents PKCβ from increasing the RRP.

If PKCγ does indeed suppress the activity of PKCβ, then the expression of PKCγ in hearing animals should also suppress the increase in the RRP by PKCβ and lead to PTP mediated predominantly by an increase in *p*. We determined whether this is the case by using AAV to express PKCγ-YFP in globular bushy cells that give rise to calyx of Held synapses. Wild-type animals were used for these experiments. Calyces expressing PKCγ-YFP were readily identified by strong fluorescence (Figure 8A). Contributions of RRP and *p* to PTP were assessed (Figures 8B and 8C) and synapses expressing PKCγ-YFP showed robust PTP (75% ± 9%; n = 9) that was mediated primarily by an increase in *p* (p_{trainC} 58% ± 5%), with very little contribution from RRP (11% ± 5%) (Figure 8D). Synapses from the same animals in which PKCγ-YFP was not expressed exhibited PTP (60% ± 7%; n = 3) that was mediated mainly by an increase in RRP (RRP_{trainC} 45% ± 10%) (Figure 8E), which is typical of hearing wild-type animals. Although the magnitude of PTP was slightly larger for calyces infected with PKCγ-YFP, there was no significant difference in the amount of PTP between YFP-expressing and nonexpressing cells (p = 0.23). These experiments show that the presence of PKCγ prevents PKCβ from contributing to PTP by increasing the RRP.

The magnitude of PTP and the contributions of RRP and *p* to this enhancement in different knockout animals and in the presence of PKC inhibitors are summarized in Figure 9A and Table S2 for P8–P10 animals. The changes in PPR associated with PTP for all groups are summarized in Table S2. PTP in prehearing animals is accompanied by a large decrease in PPR only when PKCγ is present. This is qualitatively consistent with PKCγ mediating PTP by increasing the probability of release.

DISCUSSION

Our primary finding is that PKCβ and PKCγ both mediate PTP, but PKCγ predominantly enhances the probability of release and PKCβ mainly increases the size of the readily releasable pool. These findings indicate that the identity of the calcium-dependent PKC isoform that mediates PTP controls the mechanism and functional consequences of PTP.

After the onset of hearing, PTP is mediated largely by PKCβ, which governs plasticity primarily by increasing the size of the RRP. The situation is more complicated before the onset of hearing. Although all three calcium-dependent isoforms of PKC are present at the calyx of Held, PTP is due largely to an increase in *p* mediated by PKCγ. In PKCγ KO animals, PTP is still observed, but it is due largely to an increase in RRP mediated by PKCβ. In fact, the contributions of RRP and *p* in prehearing PKCγ KO animals are remarkably similar to those observed in wild-type animals after the onset of hearing. The elimination of PKCγ in P8–P10 animals essentially transforms the properties of PTP to those of wild-type animals after the onset of hearing, where PKCγ is absent from the calyx of Held. Moreover, the viral

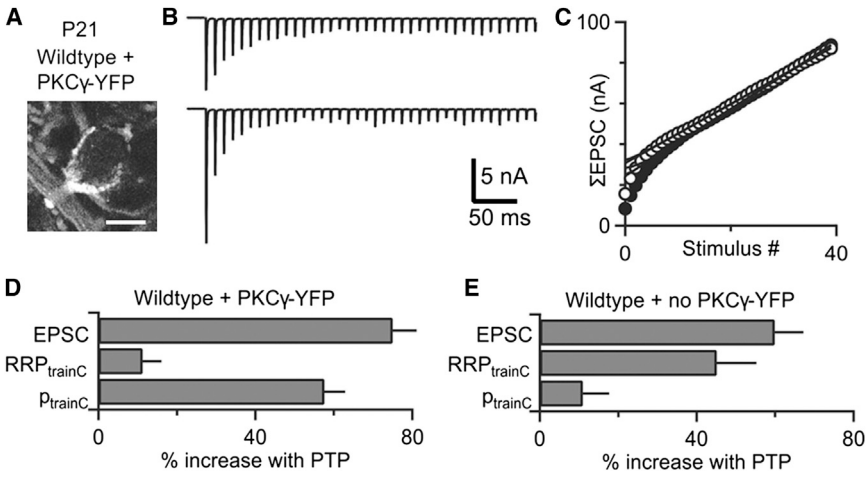


Figure 8. Viral Expression of PKCγ in the Calyx of Held of Hearing Animals Alters the Mechanism of PTP

An AAV expressing PKCγ-YFP was injected in the ventral cochlear nucleus at P4. Synaptic properties were examined at P19–P22 and calyces of Held expressing PKCγ-YFP were identified. Mechanisms of PTP were examined using trains in the presence of kynurenate and CTZ to prevent receptor saturation and desensitization.

(A) Representative fluorescence image from a P21 wild-type calyx showing PKCγ-YFP fluorescence.

(B) Synaptic currents evoked by the first 40 stimuli of 4 s, 100 Hz train (top) and by a 40 pulse, 100 Hz train (bottom) 10 s after tetanic stimulation (at the peak of PTP) are shown.

(C) Cumulative EPSCs for the initial train (closed circles) and the second train (open circles) are plotted against stimulus number.

(D) Summary of the contributions of RRP and *p* to PTP for synapses expressing PKCγ-YFP.

(E) Summary of the contributions of RRP and *p* to PTP for synapses not expressing PKCγ-YFP from the same animals as in (D). Scale bar for (A) indicates 10 μm.

expression of PKCγ after the onset of hearing leads to PTP with properties similar to prehearing wild-type animals. Thus, it is not simply the age of the animal that determines the properties of PTP; it is the complement of calcium-dependent PKC isoforms available to mediate PTP.

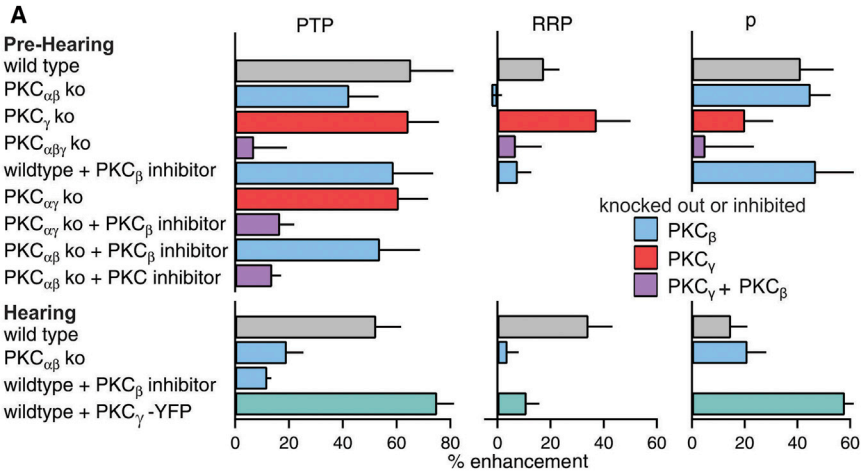
PKC Isoform-Specific Modulation

There is a growing appreciation that different PKC isoforms can perform specialized roles (Harper and Poole, 2007; Heemskerk et al., 2011; Sossin, 2007; Steinberg, 2008). An important factor in isoform-specific actions is that PKC isoforms are expressed differentially throughout the nervous system and often in a developmentally regulated manner (Huang et al., 1990; Kose et al., 1990; Roisin and Barbin, 1997). But even for cells that express multiple PKC isoforms, differential subcellular compartmentalization and differential activation of substrate targets can allow individual PKC isoforms to perform unique cellular functions (Dekker and Parker, 1994; Hofmann, 1997; Shirai and Saito, 2002; Steinberg, 2008). For example, the calcium-sensitive isoforms PKCα and PKCβ and the novel isoform PKCδ have opposing actions in platelet activation and aggregation (Gilio et al., 2010; Harper and Poole, 2007; Heemskerk et al., 2011; Strehl et al., 2007). Similarly, at *Aplysia* sensory-motor synapses, a calcium-independent PKC isoform mediates serotonin-induced recovery from depression (Manseau et al., 2001), whereas synaptic enhancement lasting for hours after stimulation is mediated by a calcium-dependent PKC isoform (Sossin, 2007; Zhao et al., 2006). As in these examples, isoform-specific actions within a cell often involve different classes of PKCs that are known to be activated by different types of signals. It is also known that different classes of PKCs are differentially effective at certain substrates. For example, calcium-dependent PKCs prefer basic residues N-terminal to the phosphorylation site, whereas novel PKCs prefer hydrophobic residues (Nishikawa et al., 1997; Sossin, 2007). The observation that PKC isoforms from different classes can act on different phosphorylation sites to differentially modulate L-type calcium channels (Yang et al., 2009) raises the possibility that PKC isoforms could act on the same protein but at different phosphorylation sites to produce PTP with different properties.

Although less is known about the ability of closely related calcium-dependent isoforms such as PKCβ and PKCγ to target different substrates, it is possible that anchoring proteins could allow such interactions to occur. PKCs have isoform-specific interactions with receptors for activated C kinase (RACKs), a family of membrane-associated anchoring proteins that function as molecular scaffolds to localize individual PKCs to distinct membrane microdomains so that PKC isoforms are in close proximity to their unique substrates. Cells may express unique RACKs for each PKC isoform and such PKC-RACK interactions could be essential for isoform-specific cellular responses (Csukai et al., 1997; Mackay and Mochly-Rosen, 2001; Schechtman et al., 2004). Different RACKS could localize specific PKC isoforms to different subdomains within the calyx of Held. Another possibility is that PKCβ and PKCγ could prefer different substrates (Nishikawa et al., 1997).

Possible Targets of PKCβ and PKCγ

Further studies are required to determine the molecular targets of PTP and the means by which PKCβ and PKCγ produce PTP with different functional properties. We tested and excluded the hypothesis that PKCγ modulates presynaptic calcium entry. This is consistent with the observation that even at synapses where changes in calcium influx contribute to short-lived forms of plasticity, they do not contribute to PTP (Korogod et al., 2007). A previous study (Habets and Borst, 2006) suggested a role for calcium influx modulation in short-term plasticity, but our findings differ from those results, probably because a much different induction protocol was used there to induce a longer-lasting form of short-term plasticity.



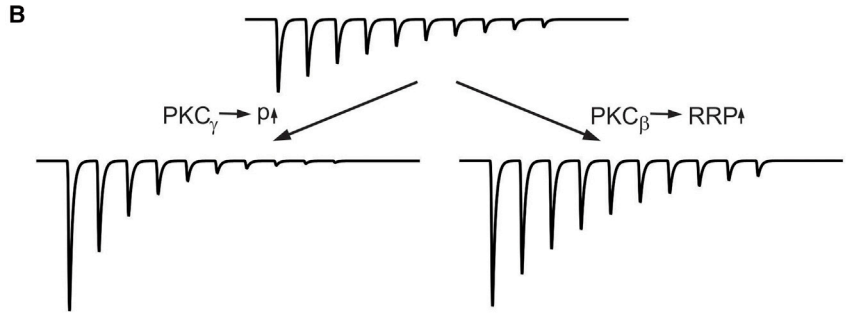


Figure 9. Summary and Schematic Showing the Effects of Different PKC Isoforms on PTP before and after Hearing Onset

(A) Summary bar graphs are shown for the magnitude of PTP, and the changes in RRP and *p* that occur after tetanic stimulation in wild-type and knockout mice and in the presence of pharmacological inhibitors. Bar graphs are color coded to indicate whether PKCβ, PKCγ, or both isoforms were knocked out or pharmacologically inhibited. Green bars indicate summaries of experiments in which PKCβ-YFP was expressed in wild-type animals.

(B) A schematic illustration of the differential ways PKCβ and PKCγ contribute to PTP and the functional consequences of their response to a stimulus train.

Munc18-1 remains a leading candidate effector molecule for PTP. For cultured hippocampal neurons, it was shown that synaptic enhancement produced by phorbol esters and a form of use-dependent plasticity following tetanic stimulation both rely on PKC phosphorylating Munc18-1 (Wierda et al., 2007). Munc18-1 has also been implicated in regulating pool size (Nili et al., 2006; Toonen et al., 2006) and the probability of release, and there are multiple PKC phosphorylation sites on Munc18-1 (Barclay et al., 2003; Fujita et al., 1996). Based on these observations, it is possible that PKCβ and PKCγ could both enhance transmission by phosphorylating Munc18-1, perhaps by phosphorylating different sites. A recent study of the calyx of Held synapse in P9–P12 mice found that replacing Munc18-1 with a mutant form of Munc18-1 lacking PKC phosphorylation sites reduced the magnitude and decreased the duration of PTP (Genc et al., 2014). This study establishes the importance of Munc 18-1 in PKC-dependent PTP at the calyx of Held, but the extent to which PKCγ and PKCβ enhance transmission by phosphorylating Munc18-1. One or both of these isoforms could also regulate transmission by phosphorylating other targets in the presynaptic bouton including SNAP-25 (Gonelle-Gispert et al., 2002; Houeland et al., 2007; Nagy et al., 2002; Zamponi et al., 1997).

PKCγ Suppresses the Actions of PKCβ to Dictate the Mechanism of PTP

We tested the hypothesis that PKCγ increases *p* and PKCβ increases RRP by independent mechanisms acting on different targets, with multiplicative effects. Our findings indicate that PKCβ-dependent increases in RRP and PKCγ-dependent increases in *p* are not independent mechanisms. If they were, then when both PKCγ and PKCβ are present, it would be expected that PKCγ would increase *p* by ~40%, PKCβ would increase RRP by ~40%, and the overall increase in EPSC amplitude would be ~96% [(1.4 × 1.4−1) × 100]. But when both PKCγ and PKCβ are present, either in prehearing wild-type animals or in calyces expressing PKCγ-YFP in hearing wild-type animals, increases in RRP are small, and the magnitude of PTP is smaller than expected if both *p* and RRP increased. These results indicate that PKCβ and PKCγ do not act through independent mechanisms. We conclude instead that PKCγ suppresses the PKCβ pathway. Further studies are required to determine how this occurs.

Functional Consequences of PTP Being Mediated by Increased *p* Prior to Hearing Onset and by Increased RRP Thereafter

Whether PTP is induced by an increase in *p* or RRP is important from a functional perspective, because these properties have very different effects on prolonged responses during firing of action potentials at the calyx of Held (Figure 9B). The properties of use-dependent plasticity at a synapse are probably tailored to the activity patterns of the presynaptic cell. In vivo, before the onset of hearing, neurons in the anteroventral cochlear nucleus (aVCN) fire spontaneous bursts of up to five action potentials often at more than 100 Hz (Sonntag et al., 2009, 2011). Such activity patterns could be crucial for setting up tonotopic maps prior to hearing onset (Kandler et al., 2009; Kandler and Friauf, 1993; Keuroghlian and Knudsen, 2007). After hearing onset, aVCN neurons fire more regularly and continuously, transmission reliability is high, and the calyx of Held synapse appears well-suited to convey auditory responses driven at high frequencies (Sonntag et al., 2011). It is perhaps not a surprise that the properties of short-term synaptic plasticity are regulated to respond

appropriately to such different patterns of presynaptic activity before and after the onset of hearing. Increasing *p* results in a more rapidly depressing synaptic response, and the overall release by a burst of activity is unaffected. It seems that such a mechanism of PTP would not be particularly effective after hearing onset when sustained activity predominates. Increasing RRP essentially scales up responses and enhances release even for sustained activity and may be a more suitable mechanism after the onset of hearing.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were completed in accordance with guidelines by the Harvard Medical Area Standing Committee on Animals. PKCαβ double KO mice were obtained through breeding of PKCα and PKCβ knockout animals generated by M. Leitges (Leitges et al., 1996, 2002). PKCγ KO mice (Abeliovich et al., 1993) were obtained from Jackson Laboratory. Because PKCβ and PKCγ are both located on chromosome 7, we first generated PKCβγ double KO mice through breeding of single PKCβ and PKCγ knockouts. PKCβγ double KO mice were then bred with PKCα KO mice to produce PKCαβγ triple KO animals. To prevent genetic drift in the inbred KO lines, we backcrossed them every second generation to C57BL/6J or 129S2. Because of the low probability of obtaining a double or triple knockout from heterologous crosses, we bred het-knockout animals together to increase the probability of getting desired animals.

Similarly, to increase the probability of obtaining wild-type mice, we crossed PKC het-het or het-wild-type mice to use as wild-type controls. Wild-type mice were derived from the same genetic line describe above. Animals of both sexes were used for experiments. Mice (C57BL/6J) from Charles River were used for all experiments in Figure 1 to test for changes in paired-pulse plasticity during PTP. For all other figures, mice of mixed background were used, and age-matched wild-type, PKCαβ, PKCγ, and PKCαβγ KO mice from our colony were interleaved for all experiments.

Brain Slices and Electrophysiology

Isoflurane was used to anesthetize animals, then their brains were dissected at 4°C using a solution containing 125 mM NaCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 25 mM glucose, 3 mM myo-inositol, 2 mM Na-pyruvate, and 0.4 mM ascorbic acid (pH 7.4) and continuously bubbled with 95% O_2/5% CO_2. Transverse slices (190–200 μm thick) containing the MNTB were cut from P8–P10 and P16–P19 mice using a Leica vibratome slicer (VT1000S). Slices were then incubated at 32°C for 30 min with a solution of the same composition as the cutting solution above but altered to have 2 $CaCl_2$ and 1 $MgCl_2$. Electrophysiological recordings were made as previously described (Fioravante et al., 2011) with the external solution containing 25 μM bicuculline and 1 μM strychnine to block inhibitory synaptic activity. Whole-cell patch-clamp recordings of ESPCs from MNTB neurons were made with an internal solution containing 140 mM Cs-gluconate, 20 mM CsCl, 20 mM TEA-Cl, 10 mM HEPES, 5 mM EGTA, 5 mM Na_2-phosphocreatine, 4 mM ATP-Mg, and 0.3 mM GTP-Na (pH 7.3), 315–320 mOsm.

A custom-made bipolar electrode was placed near the midline near the MNTB region to stimulate presynaptic calyceal fibers. PTP was induced using a tetanic train (4 s, 100 Hz), and the baseline and posttetanic EPSCs were measured at 0.2 Hz. To measure RRP and *p*, we induced a second high-frequency train (0.4 s, 100 Hz) 10 s after the first tetanus after washin of 0.1 mM cyclothiazide and 1 mM kynurenate to prevent AMPA receptor desensitization and saturation, respectively. In some experiments, slices were incubated for 30 min in the PKCβ inhibitor (*3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione*, Calbiochem) or for 60 min in the pan-PKC inhibitor (GF109203X HCl, Abcam).

Presynaptic Calcium Imaging

Calyces of Held were bulk loaded with calcium green-1 dextran (0.5%; 10 kDa, potassium salt, anionic, Invitrogen) and Alexa 594 dextran (0.025%) as previously described (Beierlein et al., 2004; Fioravante et al., 2011). Briefly, glass pipettes containing the loading dyes were placed next to calyceal fiber bundles arising from the midline. Loading times were 3–5 min at 32°C, and slices were incubated for 1 hr at 32°C after loading. Fluorescence calcium signals were obtained using a two-photon microscope and were converted to calcium by determining the R_{max}/R_{min} ratio (~5) in sealed pipettes. To determine R_{max}, we washed on 20 μM ionomycin at the end of the experimental session, and the R_{max} in ionomycin was used for all calcium calculations (see Figure S3A). The R_{train} determined using a high-frequency train did not reach the R_{max} value obtained with ionomycin. Calyces with bright green fluorescence at rest were rejected for further study, because they either had elevated resting calcium levels or were overloaded with calcium indicator.

Data Analysis

Custom-written programs in IgorPro (WaveMetrics) were used to analyze all data. PPR was calculated as $EPSC_2/EPSC_1$. The contributions of RRP and *p* to PTP were quantified using the cumulative EPSC method and the Elmqvist and Qaustel method (Elmqvist and Quastel, 1965; Fioravante et al., 2011; Thanawala and Regehr, 2013). Additionally, changes in RRP and *p* were also calculated using a corrected cumulative EPSC method (Thanawala and Regehr, 2013). Statistical analyses were completed using paired Student's t tests, or for multiple comparisons, one-way ANOVAs or Student's t tests with Bonferroni corrections. The level of significance was set at $p < 0.05$.

Immunohistochemistry

Transverse brainstem slices (190 μm thick) were obtained with a vibratome from P8–P10 and P16–P19 mice as described above. Slices were fixed in 2% paraformaldehyde for 1 hr at 4°C, washed three times in PBS (Sigma-Aldrich), and then incubated for 1 hr at room temperature in PBS with 0.25% Triton X-100 (PBST) and 10% normal goat serum (NGS). Slices were incubated afterward in primary antibodies (1:500 dilution) in PBST and 10% NGS overnight at 4°C. Slices were rinsed in PBS, incubated in secondary antibodies (1:500 dilution), then rinsed in PBS and mounted on Superfrost glass slides (VWR). The antibodies used were: anti-vGlut1 guinea pig polyclonal (Synaptic Systems), anti-PKCγ rabbit polyclonal (Santa Cruz Biotechnology), goat anti-guinea pig rhodamine-conjugated and goat anti-rabbit Alexa Fluor 488-conjugated secondaries (Santa Cruz Biotechnology). Images were obtained using an Olympus FluoViewTM FV1000 laser-scanning confocal microscope with a 63× oil objective. Excitation wavelengths were 543 nm for rhodamine (vGlut1) and 488 nm for Alexa Fluor 488 (PKCγ). Emission filters were LP560 for vGlut1 and BP505-530 for PKCγ. Stacked optical sections at 1,024 × 1,024 were obtained sequentially for each channel.

DNA Constructs and Viruses

Cloning was performed by Genscript. The adeno-associated virus for PKCγ-YFP was generated by the University of Pennsylvania Vector Core. All constructs were verified by sequencing. Mouse PKCγ was obtained through PCR from Addgene plasmid 21236, using the following primers: 5′-TAC AAGGCTGGTACCGAGCTCGGATCCGCGGGTCTGGGCCCTGGCGGAGGC GACT-3′; 5′-TGTTCGGGACACGGGCACGGACAGTACGAGCTCCGTGACCA -3′. To generate the AAV vector, we inserted PKCγ between the two YFP sequences of a custom pENN.AAV.CMV.YFP.YFP.RBG *cis*-plasmid (based on the University of Pennsylvania vector core plasmid pENN.AAV.CMV. TurboRFP.RBG) using SacII and SalI.

Surgery

P4 pups were stereotactically and unilaterally injected under isofluorane anesthesia into the VCN (from lambda: 1.3 mm lateral, 0.9 mm caudal, 3 mm ventral), where globular bushy cells that give rise to calyx of Held synapses in the contralateral MNTB reside. Injections (600 nl at a rate of 1 nl/s) were performed with an UltraMicroPump (UMP3, WPI) and Wiretrol II capillary micropipettes (Drummond Scientific) pulled to a fine tip (10–20 μm diameter). After the injection, pups were allowed to recover on a heating pad prior to returning to the home cage. We allowed 14–18 days for expression prior to slice preparation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.04.003.

AUTHOR CONTRIBUTIONS

Y.X.C. and D.F. contributed equally to this work. Specifically, Y.X.C. performed all electrophysiology experiments. D.F. made the viral constructs and completed animal surgery experiments. Both Y.X.C. and D.F. conducted the immunohistochemistry and imaging experiments.

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