The Role of Protein Kinase C in Short-Term Synaptic Plasticity

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YunXiang Chu

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Abstract

Short-term synaptic plasticity results from use-dependent activity, lasts on the timescale of milliseconds to minutes, and is thought to underlie working memory and neuronal information processing. Here, we focus on two forms of short-term plasticity: 1) post-tetanic potentiation (PTP), which is induced by high-frequency stimulation, and 2) presynaptic ionotropic receptor-activated synaptic enhancement, which can be produced by the activation of presynaptic glycine receptors. Potentiation of evoked and spontaneous responses is thought to arise from elevations in presynaptic residual Ca$^{2+}$, which activates one or more molecular targets to increase neurotransmitter release. However, the Ca$^{2+}$ sensor protein has not yet been identified. The overall goal of this work is to elucidate the Ca$^{2+}$-dependent mechanisms of short-term plasticity.

Pharmacological studies have implicated protein kinase C (PKC) in short-term plasticity. We overcame the limitations of problematic PKC pharmacology by using knockout (ko) animals to examine the roles of Ca$^{2+}$-dependent PKC (PKC$_{Ca}$) isoforms ($\alpha$, $\beta$, $\gamma$) in short-term plasticity at the calyx of Held synapse. We found that PKC$\alpha$$\beta$ isoforms predominantly mediate PTP and glycine-induced potentiation by increasing the size of the readily releasable pool (RRP) of vesicles in animals after hearing onset. Specifically, the PKC$\beta$ isoform appeared to mediate the bulk of PTP. However, PKC$\gamma$ mediates PTP by increasing the release probability ($p$) of vesicles in pre-hearing animals. Whether PTP is induced by an increase in $p$ or RRP is important from a functional perspective, because it has very different effects on prolonged responses during firing
of action potentials. Our results are the first to show that different PKC isoforms can perform specialized roles in mediating synaptic plasticity.

To definitively demonstrate that PKCβ functions as the Ca$^{2+}$-sensor for PTP, we performed rescue experiments by infecting PKCαβ double ko neurons with mutant PKCβ where the amino acids required for Ca$^{2+}$-coordination were mutated. We found that mutant PKCβ could not rescue PTP, but could rescue phorbol ester-induced synaptic potentiation, because the phorbol ester-activated domain remains fully functional. These results suggest that Ca$^{2+}$-dependent PKCβ isoforms act as a Ca$^{2+}$-sensor protein in PTP. Together our findings provide insight into the molecular mechanisms underlying short-term synaptic plasticity.
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Chapter 1

Introduction

Neuronal communication occurs through an electrochemical process at synapses and forms the cellular basis of memory, decision making, and motor control. A synapse is composed of a presynaptic terminal with docked vesicles containing neurotransmitter, a postsynaptic site with receptors for binding neurotransmitters, and a synaptic cleft separating the two apposing membranes (Sudhof, 2012). Neurotransmission occurs when an action potential (AP) arrives at the presynaptic terminal and triggers the opening of voltage-gated calcium (Ca$^{2+}$) channels (VGCCs). The rapid rise in intracellular Ca$^{2+}$ activates presynaptic proteins, such as synaptotagmin, to trigger the fusion of synaptic vesicles with the plasma membrane in less than a millisecond (Neher and Sakaba, 2008; Sudhof, 2013). As a result, neurotransmitters are released into the synaptic cleft and bind postsynaptic receptors. Synapses in the central nervous system (CNS) can be excitatory or inhibitory depending on the identity of the neurotransmitter and of the postsynaptic receptors (Sheng and Kim, 2011). Activation of AMPA and NMDA receptors by glutamate generates excitatory postsynaptic currents (EPSCs), while activation of GABA or glycine receptors induces inhibitory postsynaptic currents (IPSCs).

Synapses are not static entities; their performance adapts over time. The ability of synapses to strengthen or weaken in response to activity is called synaptic plasticity (Castillo, 2012; Regehr, 2012). This phenomenon is crucial because it helps to shape neural communication (Katz and Shatz, 1996), it is required during brain development to mediate the formation of immature synapses (Schinder and Poo, 2000), and it is thought to be the neurochemical basis of learning and memory (Mayford et al., 2012). Dysfunctional synaptic
plasticity has been implicated in numerous neurological diseases, including dementia, depression, ataxia, addiction, and anxiety disorders (Chen and Tonegawa, 1997; Duman, 2002; Sheng et al., 2012; Zoghbi and Bear, 2012). A better understanding of the basic mechanisms of plasticity will provide a foundation for understanding these brain disorders.

Synaptic plasticity can be classified based on its duration. Long-term plasticity reflects a change in synaptic efficacy lasting minutes to weeks, has been extensively studied, and is the best candidate mechanism for memory storage in the central nervous system (CNS) (Bliss and Collingridge, 1993; Kullmann and Lamsa, 2007; Lamsa et al., 2007; Malenka and Nicoll, 1993). Short-term plasticity lasts on the timescale of milliseconds to minutes, and is important because it underlies neuronal information processing (Abbott and Regehr, 2004; Zucker and Regehr, 2002). However, the molecular mechanisms mediating various forms of short-term plasticity still remain elusive despite decades of research.

**Short-term synaptic plasticity**

Short-term use-dependent plasticity is not a single process, but can be divided into four types: 1) depression, 2) facilitation, 3) augmentation, and 4) post-tetanic potentiation (PTP) (Regehr, 2012; Zucker and Regehr, 2002). Synaptic depression lasts hundreds of milliseconds to seconds and results in a decrease in synaptic strength. Facilitation lasts on a similar timescale, but induces an enhancement in the synaptic response evoked by closely-spaced stimuli. Augmentation (with a time decay on the order of seconds) and PTP (which lasts seconds to minutes) are both potentiated responses induced by sustained presynaptic activation. Most synapses exhibit multiple forms of pre-synaptic plasticity, and the resulting changes in the magnitude of evoked responses can represent a combination of several types of short-term plasticity (Dittman et al., 2000; Pan and Zucker, 2009; Varela et al., 1997).

Despite its detailed characterizations, the molecular mechanisms of short-term use-dependent plasticity are not well understood. What is known is that, at most synapses studied,
the mechanism of short-term plasticity involves presynaptic changes that are Ca^{2+}-dependent. The seminal experiments performed by Katz and Miledi (1968) (Katz and Miledi, 1968) showed that a lack of external Ca^{2+} during conditioning stimulation at the neuromuscular junction failed to facilitate release. Augmentation and PTP were subsequently shown to also depend on Ca^{2+} influx, as experiments with certain Ca^{2+} chelators eliminate synaptic potentiation (Regehr et al., 1994; Stevens and Wesseling, 1999; Delaney et al., 1989; Delaney and Tank, 1994). These observations led to the development of the “residual Ca^{2+} hypothesis,” which states that repetitive stimulation leads to an accumulation of Ca^{2+} in the presynaptic terminal, and an accompanying increase in the probability of release (Zucker and Regehr, 2002). Numerous studies have demonstrated a correlation between elevations in presynaptic Ca^{2+} and synaptic enhancement (Delaney and Tank, 1994; Delaney et al., 1989; Kretz et al., 1982; Regehr et al., 1994).

**Post-tetanic potentiation**

At many CNS synapses, a period of high-frequency (tetanic) stimulation can evoke a transient increase in synaptic strength known as post-tetanic potentiation (PTP) (Griffith, 1990; Magleby and Zengel, 1975; Zucker and Lara-Estrella, 1983; Zucker and Regehr, 2002). PTP is thought to provide an important means of synaptic regulation that can contribute to working memory and information processing (Abbott and Regehr, 2004; Silva et al., 1996). Tetanic stimulation also increases both the frequency and the magnitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Delaney and Tank, 1994; Habets and Borst, 2005; He et al., 2009; Korogod et al., 2005), but it is not known whether increases in the frequency and amplitude of spontaneous transmission and the increase in evoked release share a common presynaptic mechanism.

PTP has been investigated for over 60 years, but its mechanism has remained controversial. Numerous mechanisms could contribute to PTP. The residual Ca^{2+} hypothesis is
the leading explanation for the underlying mechanism of PTP. An elevation in residual Ca\textsuperscript{2+} (Ca\textsubscript{res}) could activate Ca\textsuperscript{2+} sensors other than synaptotagmin to induce an increase in the probability of release (Brager et al., 2003; Delaney and Tank, 1994; Delaney et al., 1989; Regehr et al., 1994; Zucker and Regehr, 2002). Other possibilities include an increase in the size of the vesicle pool containing release-ready vesicles (Habets and Borst, 2005; Habets and Borst, 2007; Lee et al., 2008), an enhancement in the size of mEPSCs as a result of vesicles fusing with each other before ultimately fusing with the plasma membrane (He et al., 2009), a change in action potential waveform (Eccles and Krnjevic, 1959; Habets and Borst, 2005), and an increase in Ca\textsuperscript{2+} entry (Habets and Borst, 2005; Habets and Borst, 2006; Korogod et al., 2005).

Several molecular candidates that could respond to Ca\textsubscript{res} have been proposed to mediate PTP. Pharmacological studies have implicated protein kinase C (PKC) in PTP at multiple synapses, but its role in PTP has been limited by the selectivity and effectiveness of pharmacological tools available to inhibit and activate PKC (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Brose and Rosenmund, 2002; Korogod et al., 2007; Lee et al., 2008). It has been hypothesized that the Ca\textsuperscript{2+}-dependent isoforms of PKC respond to Ca\textsubscript{res}, and produce PTP by phosphorylating Munc18-1 to increase the release probability and vesicle pool size (Wierda et al., 2007). Munc13, a Ca\textsuperscript{2+}-sensitive presynaptic protein involved in the priming step of vesicle exocytosis, has also been shown to be involved in tetanus-activated short-term plasticity (Junge et al., 2004; Shin et al., 2010). Other proteins implicated in PTP include calmodulin, Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) (Chapman et al., 1995; Junge et al., 2004; Wang and Maler, 1998), myosin light chain kinase (Lee et al., 2008), and synapsin (Fiumara et al., 2007). A long-standing question in the synaptic physiology field has been the identity of the Ca\textsuperscript{2+} sensor(s) mediating short-term plasticity.
Synaptic enhancement due to activation of presynaptic receptors

Another well-studied form of short-term synaptic plasticity is that mediated by the activation of presynaptic receptors, such as G-protein coupled receptors (GPCRs) (Blackmer et al., 2001; Schmitz et al., 2001; Wu and Saggau, 1997) or ionotropic receptors (Engelman and MacDermott, 2004; Kerchner et al., 2001; Turecek and Trussell, 2001). Synaptic modulation by GPCRs has been extensively characterized for GABA_B (Takahashi et al., 1998; Wu and Saggau, 1995), adenosine (Trussell and Jackson, 1985; Trussell and Jackson, 1987; Wu and Saggau, 1994), and cannabinoid receptors (Beierlein and Regehr, 2006; Carey et al., 2011; Safo and Regehr, 2005) at a variety of CNS synapses. Activation of these receptors inhibits transmitter release by inhibiting VGCCs near release site, by acting downstream of Ca^{2+}, or by enhancing K^+ channel activation (Hille, 1994). Presynaptic potentiation or inhibition of release by ionotropic receptors is a more recently discovered phenomenon, and its molecular mechanisms remain to be deciphered. Ionotropic receptors are typically thought of as postsynaptic components that open upon binding neurotransmitters. However, a wide variety of ionotropic receptors are found on presynaptic terminals (McGehee et al., 1995; McGehee and Role, 1996; McMahon et al., 1994; Turecek and Trussell, 2001), where they reside in proximity to vesicles and can influence release to modulate synaptic strength.

Presynaptic ionotropic receptors can enhance or inhibit transmission. One of the best studied examples of ionotropic receptor-mediated synaptic potentiation is at the calyx of Held synapse (Balakrishnan et al., 2009; Turecek and Trussell, 2001). Here, presynaptic glycine receptors can be activated by glycine spillover from nearby interneurons. Due to the relatively high chloride reversal potential at the calyx (~50 mV) (Huang and Trussell, 2008; Kim and Trussell, 2009; Price and Trussell, 2006), activation of this type of ionotropic receptor results in an efflux of Cl^- ions and a small presynaptic depolarization that is sufficient to open a small fraction of P/Q-type Ca^{2+} channels which elevates presynaptic Ca^{2+}. This process results in an
increase in the amplitude of evoked release and spontaneous transmission (Awatramani et al., 2005; Kim and Trussell, 2009; Trussell, 2002; Turecek and Trussell, 2001).

The Ca\textsuperscript{2+}-dependent mechanism of ionotropic receptor-mediated potentiation is similar to synaptic enhancement produced by sub-threshold somatic depolarization at cortical, hippocampal, and cerebellar synapses (Alle and Geiger, 2006; Christie et al., 2011; Glitsch and Marty, 1999; Shu et al., 2006; Yu et al., 2010). However, the mechanism through which presynaptic depolarizations enhance release remains unknown. The Ca\textsuperscript{2+}-dependent isoforms of PKC are excellent candidates for mediating glycine-induced synaptic enhancement, because these proteins respond to small increases in presynaptic Ca\textsuperscript{2+}, (Corbalan-Garcia et al., 1999; Corbin et al., 2007; Guerrero-Valero et al., 2007; Kohout et al., 2002) similar to the proposed mechanism underlying PTP. In addition, whether PTP and glycine-induced potentiation act through the same presynaptic Ca\textsuperscript{2+}-dependent mechanism remains to be explored.

**Protein kinase C in short-term synaptic plasticity**

Protein kinase C has been implicated in a wide variety of CNS functions, from the regulation of cell growth and differentiation (Clemens et al., 1992; Nishizuka, 1984; Nishizuka, 1992) to neuronal plasticity (Ben-Ari et al., 1992; Routtenberg, 1985; Sossin, 2007; Tanaka and Nishizuka, 1994). Isoforms of this kinase family are expressed ubiquitously throughout all cell types, but there is particularly high expression of PKCs in neuronal tissues. Activation of PKCs has been associated with modulation of ion channels (Kaczmarek, 1987; Shearman et al., 1989; Yang and Tsien, 1993; Yang et al., 2009), desensitization of receptors (Downing and Role, 1987; Huganir and Greengard, 1990), and enhancement of transmitter release (Malenka et al., 1987; Malenka et al., 1986). These factors make PKCs ideal candidates for mediating the mechanisms of short-term synaptic plasticity.

More than a dozen isoforms of PKC have been identified in mammalian tissues, but there are differences in their enzymatic properties, tissue expression, and subcellular
PKCs consist of a single polypeptide with an N-terminal regulatory region and a C-terminal catalytic region, and function by phosphorylating serine or threonine residues on its substrates (Newton, 1995; Newton, 2001b). Cloning of isoforms revealed four conserved domains, C1-C4, along with a shared pseudosubstrate sequence that autoinhibits the catalytic region and prevents PKC from being constitutively active (Newton, 1995). Activation requires the “unmasking” of the catalytic domain, which is achieved by binding of DAG/phorbols or Ca\(^{2+}\) (Takai et al., 1979). Binding of either co-factor induces a conformational change that causes the hinge to open and release the pseudosubstrate sequence from the catalytic region (Newton, 1995; Newton, 2010; Nishizuka, 1992). Upon activation, PKCs are translocated to the plasma membrane and are anchored by RACK (receptor for activated C kinase) proteins (Csukai et al., 1997; Mackay and Mochly-Rosen, 2001; Mochly-Rosen et al., 1991a; Mochly-Rosen et al., 1991b; Schechtman et al., 2004). In the CNS, the increase in intracellular Ca\(^{2+}\) is normally provided by the influx of Ca\(^{2+}\) through VGCCs or by release of Ca\(^{2+}\) stores from intracellular organelles like mitochondria. DAG is generated at the plasma membrane through the action of phospholipase C (PLC).

PKCs are divided into three subclasses based on their structure and co-factor regulation (Figure 1.1). Conventional or “classical” PKCs (α, βI, βII, γ) are activated by DAG/phorbols or Ca\(^{2+}\) at their C1 and C2 domains, respectively. Novel PKCs (δ, ε, η, θ, μ) are similar to classical PKCs, but their C2 domains do not bind Ca\(^{2+}\). Finally, atypical PKCs (ζ, λ) differ significantly in their C1 and C2 domains such that they are not activated by either DAG or Ca\(^{2+}\) (Newton, 1995; Tanaka and Nishizuka, 1994).

Immunohistochemical and in-situ studies show that neurons contain the highest levels of PKC expression, but that PKC isoforms have differential distributions throughout the CNS (Tanaka and Saito, 1992). For example, PKCγ is solely expressed in the brain and spinal cord, and predominantly in cerebellar Purkinje cells (Huang et al., 1990; Kikkawa et al., 1988a; Kikkawa et al., 1988b). PKCβI and βII isoforms are expressed in varying ratios in different
Protein kinase C subclasses and isoform structure. Depicted are simplified structural diagrams of the three PKC subclasses: classical PKCs (cPKC), novel PKCs (nPKC), and atypical PKCs (aPKC). The C1 domain contains a cysteine-rich that forms the diacylglycerol (DAG) binding site for cPKC and nPKC proteins. Phorbol esters are naturally occurring plant-derived DAG analogs that also bind the C1 domain, resulting in prolonged activation of PKCs. The C2 domain has aspartate residues that allow for Ca\(^{2+}\)-binding and subsequent PKC activation. The C3 and C4 domains comprise the ATP- and substrate-binding domains of the kinase. The regulatory and catalytic cores are separated by a hinge region. Note that nPKCs possess a “C2-like” domain and cannot be activated by Ca\(^{2+}\) binding. aPKCs have a shorter C1 domain (C1\(^*\)) that renders these isoforms unresponsive to DAG/phorbol ester activation.
neurons throughout development (Ase et al., 1988; Hirata et al., 1991), while the PKCα isoform is ubiquitously expressed in all tissues and cell types (Hashimoto et al., 1988; Ito et al., 1990; Shearman et al., 1988; Wood et al., 1986). Electron microscope studies have revealed that there are distinct intracellular localizations of different PKC isoforms in different brain regions (Kose et al., 1990; Kose et al., 1988). This differential pattern of distribution is of particular interest because it indicates that different isoforms may mediate distinct molecular processes upon activation depending on their proximity to substrates. PKCγ is distributed predominantly in the cytoplasm but is localized densely at presynaptic terminals of cerebellar Purkinje cells (Kose et al., 1990; Mori et al., 1990; Stichel et al., 1990; Tsujino et al., 1990). PKCα is located mostly in the somas of neurons (Ito et al., 1990; Kose et al., 1988). PKCβI and βII are located in perikarya, dendrites and axons of neurons, and cluster near the plasma membrane (Kose et al., 1990; Stichel et al., 1990; Tsujino et al., 1990).

Numerous pharmacological studies have implicated PKCs in mediating short-term synaptic plasticity. Application of phorbol esters, which activate PKC (Newton, 2001a), increase the amplitude of evoked synaptic responses and occlude PTP (Hori et al., 1999; Korogod et al., 2007; Lou et al., 2005; Malenka et al., 1986; Rhee et al., 2002; Wierda et al., 2007) and also enhance the frequency of spontaneous release (Hori et al., 1999; Lou et al., 2005; Oleskevich and Walmsley, 2000; Parfitt and Madison, 1993). At many synapses, PKC inhibitors decrease the magnitude of synaptic potentiation (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Korogod et al., 2007). However, the lack of selectivity and ineffectiveness of pharmacological tools have limited our ability to study the precise roles of PKCs in short-term plasticity. For instance, different PKC inhibitors have been shown to have variable effects on reducing the magnitude of PTP, where some inhibitors do not seem to affect PTP at all at certain synapses (Brose and Rosenmund, 2002; Lee et al., 2008). Phorbol esters are also known to activate other presynaptic proteins, such as Munc13 (Lou et al., 2008; Rhee et al.,
Identifying which PKC isoform(s) mediate short-term synaptic plasticity remains to be explored.

The calyx of Held synapse

The calyx of Held is a fast, sign-inverting relay synapse in the brainstem auditory pathway that is involved in sound localization (Figure 1.2) (Borst and Soria van Hoeve, 2012; von Gersdorff and Borst, 2002). The presynaptic terminals of globular bushy cells (GBCs) form the calyces of Held, which synapse onto principal neurons in the contralateral medial nucleus of the trapezoid body (MNTB) (Lenn and Reese, 1966; Smith et al., 1998). These contacts are monosynaptic with each calyx having ~700 active zones, or sites where tethered vesicles reside for transmitter release (Satzler et al., 2002). Due to the large size of the presynaptic terminal, the calyx of Held to MNTB principal cell synapse allows for paired electrophysiological recordings (Forsythe, 1994), and serves as a model synapse for the study of synaptic transmission and short-term plasticity.

Since the calyx plays a role in the auditory pathway, the onset of hearing in rodents (~P12) correlates closely with its development (Blatchley et al., 1987; Kandler and Friauf, 1993). The early MNTB principal cell at postnatal days 2-3 (P2-3) has several excitatory inputs that get quickly pruned by P4-5 to leave only one calyceal terminal in contact with each postsynaptic MNTB principal neuron (Rodriguez-Contreras et al., 2008). Over development, the calyx changes from a spoon-like structure to acquire a claw-like morphology that contain many release sites (Kandler and Friauf, 1993; Rowland et al., 2000).

Many forms of short-term plasticity leading to increases in synaptic strength have been identified at the calyx, including PTP which is induced by high-frequency (tetanic) stimulation (Habets and Borst, 2005; Korogod et al., 2005; Korogod et al., 2007). Brief trains of 100 Hz stimulation can produce PTP at the calyx, but the magnitude of PTP appears to be developmentally regulated (Korogod et al., 2005). The differences in inducing PTP at the calyx
The calyx of Held synapse in the auditory brainstem circuit. (A) Auditory signals activating cochlear hair cells are transmitted ipsilaterally to the anterior ventral cochlear nucleus (aVCN) by excitatory synapses onto globular bushy cells (GBCs) and spherical bushy cells (SBCs). The large diameter myelinated axons (4-12 µm) of the GBCs cross the brainstem midline to give rise to the calyx of Held, a glutamatergic synaptic terminal, which synapses onto the principal cells of the medial nucleus of the trapezoid body (MNTB). MNTB principal neurons are glycinergic and project to the lateral superior olive (LSO), which also receives excitatory input directly from SBCs of the ipsilateral aVCN. Therefore, action potential trains evoked by sound at the two cochleas converge onto LSO neurons as ipsilateral excitation, which is monosynaptic, and contralateral inhibition, which is disynaptic. The precise timing of these two inputs helps determine the response of the LSO to interaural intensity differences for sound localization in azimuth, while the calyx→MNTB synapse simply acts as a fast, sign-inverting relay station (reviewed in Borst and van Hoeve, 2012). (B) Calyces loaded with Alexa 594 from a P14 mouse show the distinct presynaptic morphology and large size of the presynaptic terminal. Scale bar is 10 µm.
could be the result of developmental changes in presynaptic Ca\textsuperscript{2+} buffering, Ca\textsuperscript{2+} channel kinetics, and/or differential expression of Ca\textsuperscript{2+}-sensor(s) that mediate plasticity. Such activity-dependent plasticity is functionally important because it could play a role in the development of the MNTB tonotopic map (Friauf, 1992; Grande et al., 2014; Grande and Wang, 2011).

A second form of short-term plasticity that has been characterized at the calyx of Held is small presynaptic depolarization-induced synaptic potentiation (Turecek and Trussell, 2001). Activation of presynaptic ionotropic receptors by glycine can cause a weakly depolarizing Cl\textsuperscript{-} current, because the reversal potential for Cl\textsuperscript{-} at the calyx is more positive (-50mV) than the resting membrane potential (-70 mV) (Kim and Trussell, 2009; Price and Trussell, 2006). This glycine-induced small depolarization enhances transmitter release by activating a small fraction of P/Q-type Ca\textsuperscript{2+} channels. Elevations in presynaptic residual Ca\textsuperscript{2+} concentrations appear to be necessary for increasing transmission, because intracellular buffering of Ca\textsuperscript{2+} using EGTA or blockade of VGCCs using Cd\textsuperscript{2+} blocks increases in evoked release (Turecek and Trussell, 2001). However, the Ca\textsuperscript{2+}-dependent molecular target involved in the mechanism for glycine-induced synaptic enhancement has not been identified. Moreover, because both PTP and glycine-induced potentiation at the calyx of Held depend on increases in presynaptic Ca\textsuperscript{2+}, it raises the possibility that the same Ca\textsuperscript{2+}-sensor protein could mediate both forms of short-term plasticity.

**Aims of this dissertation**

The main goal of this work is to study the roles of Ca\textsuperscript{2+}-dependent PKC isoforms in mediating short-term plasticity. I will investigate the mechanisms of PTP and glycine-induced enhancement at the calyx of Held synapse. Both forms of short-term plasticity have been well-characterized at the calyx, but the molecular mechanisms underlying these processes remain elusive. Whether or not Ca\textsuperscript{2+}-dependent PKC isoforms serve as the Ca\textsuperscript{2+} sensors for mediating synaptic plasticity also remains to be determined.
References


Chapter 2

Calcium-dependent isoforms of protein kinase C mediate post-tetanic potentiation at the calyx of Held synapse

Diasynou Fioravante*, YunXiang Chu*, Michael H. Myoga, Michael Leitges and Wade G. Regehr

*Y.X.C. and D.F. contributed equally to this work. Y.X.C. and D.F. conducted all electrophysiology and molecular experiments and analyzed the data. M.H.M. performed the two-photo microscopy experiments. M.L. generated the PKCa and PKCb mice. Y.X.C., D.F., and W.G.R. designed all experiments and wrote the paper.

Abstract

High frequency stimulation transiently increases spontaneous synaptic transmission and the amplitude of evoked synaptic transmission (known as post-tetanic potentiation, PTP). Here we examine the roles of the Ca^{2+}-dependent protein kinase C isoforms PKCα and PKCβ in PTP at the calyx of Held synapse. In PKCαβ double knockouts 80% of PTP is eliminated, whereas basal synaptic properties are unaffected. PKCα and PKCβ produce PTP by increasing the size of the readily-releasable pool of vesicles evoked by high-frequency stimulation, and by increasing the fraction of this pool released by the first stimulus. PKCα and PKCβ do not facilitate presynaptic Ca^{2+} currents. The small PTP remaining in double knockouts is mediated partly by an increase in mEPSC amplitude, and partly by a mechanism involving myosin light chain kinase. These experiments establish that PKCα and PKCβ are crucial for PTP, and suggest that long-lasting presynaptic Ca^{2+} increases produced by tetanic stimulation may activate these isoforms to produce PTP.
Introduction

At many synapses, a period of high-frequency (tetanic) stimulation can evoke a transient increase in synaptic strength known as post-tetanic potentiation (PTP) (Feng, 1941; Griffith, 1990; Magleby, 1987; Magleby and Zengel, 1975; Zucker and Lara-Estrella, 1983; Zucker and Regehr, 2002). PTP is thought to provide an important means of synaptic regulation that can contribute to working memory and information processing (Abbott and Regehr, 2004; Silva et al., 1996). Many high-frequency stimuli are needed to induce PTP, and the frequency and duration of tetanic stimulation regulate the magnitude and duration of the enhancement (which lasts tens of seconds to minutes) (Habets and Borst, 2005, 2007; Korogod et al., 2005; Lev-Tov and Rahamimoff, 1980; Magleby, 1979; Zucker, 1989). Tetanic stimulation also increases both the frequency and the magnitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) at many (Castillo and Katz, 1954; Delaney and Tank, 1994; Eliot et al., 1994; Habets and Borst, 2005; He et al., 2009; Korogod et al., 2005, 2007; Magleby, 1987), but not all synapses (Brager et al., 2003). It is not known whether increases in the frequency and amplitude of spontaneous transmission and the increase in evoked release share a common presynaptic mechanism.

Numerous mechanisms could contribute to PTP. According to the leading hypothesis, known as the residual Ca\textsuperscript{2+} hypothesis, tetanic stimulation leads to an accumulation of Ca\textsuperscript{2+} in the presynaptic terminal, and an accompanying increase in the probability of release that persists for tens of seconds (Brager et al., 2003; Delaney and Tank, 1994; Delaney et al., 1989; Regehr et al., 1994; Zucker and Regehr, 2002). Other possibilities include an increase in the size of the readily releasable pool of vesicles (Habets and Borst, 2005; Lee et al., 2008), an increase in the size of mEPSCs as a result of vesicles fusing with each other before ultimately fusing with the plasma membrane (He et al., 2009), a change in action potential waveform (Eccles and Krnjevic, 1959; Habets and Borst, 2005) and an increase in Ca\textsuperscript{2+} entry (Habets and Borst, 2005, 2006).
Pharmacological studies have implicated protein kinase C (PKC) in PTP. Phorbol esters, activators of PKC (Newton, 2001), increase the amplitude of evoked release and occlude PTP (Hori et al., 1999; Korogod et al., 2007; Lou et al., 2008; Lou et al., 2005; Malenka et al., 1986; Oleskevich and Walmsley, 2000; Rhee et al., 2002; Shapira et al., 1987; Virmani et al., 2005; Wierda et al., 2007). Phorbol esters also increase the frequency of mEPSCs (Hori et al., 1999; Lou et al., 2008; Lou et al., 2005; Oleskevich and Walmsley, 2000; Parfitt and Madison, 1993). In addition, PKC inhibitors reduce the magnitude of PTP at many synapses (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Korogod et al., 2007; Lee et al., 2007). Studies of the role of PKC in PTP have been limited by the selectivity and ineffectiveness of pharmacological tools available to inhibit and activate PKC (e.g., Lee et al., 2008) (for review see Brose and Rosenmund, 2002). Phorbol esters activate other synaptic proteins, including Munc13 (Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007). PKC inhibitors have highly variable effects on PTP: at the same synapse, different PKC inhibitors disrupt PTP to very different extents (Korogod, 2006; Lee et al., 2008); at some synapses, PKC inhibitors do not affect PTP (Eliot et al., 1994; Korogod, 2006; Lee et al., 2008; Reymann et al., 1988a; Reymann et al., 1988b); and in some cases PKC inhibitors and their inactive analogues have similar effects on PTP (Lee et al., 2008). In addition, other proteins have been implicated in PTP, including Munc13 (Junge et al., 2004; Shin et al., 2010), calmodulin and CamKII (Chapman et al., 1995; Fiumara et al., 2007; Junge et al., 2004; Khoutorsky and Spira, 2009; Reymann et al., 1988a; Wang and Maler, 1998) and myosin light chain kinase (Lee et al., 2008). These findings have cast doubt on the involvement of PKC in PTP.

If PKC is involved in PTP, identifying which PKC isoform mediates PTP is of fundamental importance. Is it a classical, Ca\(^{2+}\)-sensitive PKC isoform such as PKC\(\alpha\), PKC\(\beta\) or PKC\(\gamma\), or one of the eight Ca\(^{2+}\)-insensitive isoforms? The involvement of Ca\(^{2+}\)-sensitive PKCs would be compatible with PKC being a sensor of Ca\(^{2+}\) according to the residual Ca\(^{2+}\) hypothesis, whereas if Ca\(^{2+}\)-insensitive PKC isoforms regulate PTP, tetanic stimulation would have to
elevate presynaptic DAG or act through some unidentified pathway, and another presynaptic Ca\(^{2+}\) sensor would need to respond to residual Ca\(^{2+}\). At the calyx of Held, Ca\(^{2+}\)-insensitive PKCs have been implicated (Korogod, 2006; Saitoh et al., 2001), and inhibitors of Ca\(^{2+}\)-sensitive PKCs do not perturb PTP (Korogod, 2006). These results suggest that if PKC plays a role in PTP, it does not serve as a Ca\(^{2+}\) sensor.

Here we use knockout animals to examine the roles of PKC\(\alpha\) and PKC\(\beta\) in tetanus-induced enhancement of evoked and spontaneous transmission, and phorbol-ester-mediated enhancement at the calyx of Held synapse, where these forms of plasticity have been thoroughly characterized in wildtype animals (Habets and Borst, 2005, 2006, 2007; He et al., 2009; Hori et al., 1999; Korogod et al., 2005, 2007; Lee et al., 2008; Lou et al., 2008; Lou et al., 2005; Wu and Wu, 2001). We find that PKC\(\alpha\) and PKC\(\beta\) are both present at the calyx of Held. In PKC\(\alpha\)β double knockout animals, the calyx of Held is devoid of all Ca\(^{2+}\)-dependent PKCs, as PKC\(\gamma\) is not present at this synapse (Saitoh et al., 2001). In PKC\(\alpha\)β double knockouts, basal properties of synaptic transmission are normal but 80% of PTP is eliminated. PKC\(\alpha\) and PKC\(\beta\) produce PTP primarily by increasing the size of the readily releasable pool of vesicles that fuse in response to brief high-frequency stimulation; they do not facilitate presynaptic Ca\(^{2+}\) currents. The small PTP remaining in double knockout animals is mediated in part by an increase in mEPSC amplitude and in part by a mechanism involving myosin light chain kinase (MLCK). In contrast to PTP, the increase in the mEPSC frequency following tetanic stimulation does not depend on PKC\(\alpha\)β, suggesting that tetanic enhancement of evoked and spontaneous release are mediated by different mechanisms. Finally, phorbol ester-dependent enhancement is greatly reduced in slices from double knockout animals. These findings establish the crucial role of Ca\(^{2+}\)-sensitive PKCs in the enhancement of evoked synaptic responses induced by either tetanic stimulation or phorbol esters.
Results

Calcium-dependent PKC isoforms in the calyx of Held

We used immunohistochemistry to determine the localization of PKCα and PKCβ within the medial nucleus of the trapezoid body (MNTB) (Figure 2.1). Slices were co-labeled with antibodies to either PKCα or PKCβ (green) and to the vesicular glutamate transporter vGlut1 to label glutamate-containing synaptic vesicles within presynaptic terminals (red). vGlut1 labeling was used to identify the calyces of Held, the large presynaptic terminals that provide a synaptic contact between globular bushy cells in the anteroventral cochlear nucleus and the principle neurons in the MNTB. In slices from wildtype mice, antibody labeling for PKCα and PKCβ overlapped with vGlut1 labeling, consistent with presynaptic localization of these kinases at the calyx of Held synapse. No labeling was detected in MNTB primary neurons. Anti-PKCα antibody labeling appeared less restricted than anti-PKCβ antibody labeling, suggesting that PKCα might also be present in other structures in addition to the presynaptic terminals.

In PKCα-/- mice, labeling with antibodies to PKCβ and vGlut1 was similar to that observed in wildtype animals, but labeling with antibodies to PKCα was absent. Similarly, in PKCβ-/- mice labeling with antibodies to PKCα and vGlut1 was similar to that observed in wildtype animals, but labeling associated with antibodies to PKCβ was absent. In PKCα-/-β-/- double knockout mice no labeling was observed with antibodies to PKCα or PKCβ, but vGlut1 labeling was similar to that observed in wildtype littermate animals. These findings indicate that the targeted PKC isoforms are eliminated in knockout animals without noticeably affecting the synaptic distribution of vGlut1. They also suggest that the presence of one isoform is not required for the proper subcellular localization of the other.

Calcium-dependent PKC isoforms contribute to PTP

We investigated the role of Ca\(^{2+}\)-dependent PKCs in PTP by activating single inputs at 100 Hz for 4 s. Robust PTP was observed in wildtype mice. In a representative experiment,
Figure 2.1

Immunohistochemical localization of PKCα and PKCβ in the medial nucleus of the trapezoid body (MNTB). Brain slices containing the MNTB were labeled with antibodies to either PKCα or PKCβ (green), and co-labeled with an antibody to the presynaptic marker vGlut1 (red). Closely spaced pairs of images correspond to the same field of view imaged through the green and red channels. Representative images for PKCα/vGlut1 and PKCβ/vGlut1 are shown for slices from wildtype (wt), PKCα knockout (α-/-), PKCβ knockout (β-/-), and double knockout (α-/-β-/-) animals.
Figure 2.1 (continued)

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the amplitude of PTP was 1.9-fold and the time constant of decay was ~ 40 s (Figure 2.2A). Representative experiments show that somewhat smaller PTP was observed in PKCα-/- animals (Figure 2.2B), and PTP was greatly reduced in PKCβ-/- (Figure 2.2C) and PKCα/-β/- mice (Figure 2.2D). The varying extent of PTP within each group is shown in the cumulative histograms (Figure 2.2E). Significant differences between groups in the extent of PTP were apparent both in the cumulative histograms (Figure 2.2E) and in the summary plot of average potentiation (Figure 2.2F). In wildtype animals, the amplitude of enhancement ranged from 1.4-fold to 2.5-fold and averaged 1.81 ± 0.07-fold (n = 17), which is similar to what has been described previously (Korogod et al., 2005, 2007; Lee et al., 2008). The extent of PTP in slices from PKCα-/- animals (1.61 ± 0.06-fold, n = 13) was smaller than for wildtype animals (p < 0.01), and PTP was greatly reduced in PKCβ-/- (1.21 ± 0.02-fold, n = 15, p < 0.01) and PKCα/-β/- mice (1.16 ± 0.02-fold, n = 16, p < 0.01). These results suggest an important role for Ca^{2+}-dependent PKCs in PTP.

Properties of baseline synaptic transmission in slices from PKC knockout animals

To determine whether deletion of PKCαβ selectively impairs PTP or whether other aspects of transmission are also altered, we examined the properties of basal synaptic transmission in slices from wildtype and double knockout animals. The amplitude and frequency of mEPSCs was the same in wildtypes and PKC knockouts (Supplementary Figure S1A-B). We also measured the properties of use-dependent plasticity because changes in the initial probability of release alter the extent of use-dependent plasticity during high-frequency trains. These experiments were performed in the presence of kynurenate (1 mM) and cyclothiazide (0.1 mM) to reduce AMPA receptor saturation and desensitization, respectively, which can obscure changes in use-dependent plasticity. In Figure 2.3A, an example of EPSCs during 100 Hz train in a wildtype slice is shown. The average normalized EPSC amplitudes (Figure 2.3B) were similar in wildtype (black) (n=22) and PKCα/-β/- (purple) (n=18) animals. There was no
Calcium-dependent isoforms of PKC are necessary for post-tetanic potentiation (PTP) at the calyx of Held. (A through D), Plots of normalized EPSC amplitudes as a function of time from representative experiments. At t = 0 s, a train of 400 stimuli at 100 Hz (*inverted triangles*) was delivered to induce PTP. Insets show traces of the second potentiated response (*light traces*) superimposed to the average baseline response (*bold traces*). PTP elicited in PKCα knockouts (B), PKCβ knockouts (C), or PKCαβ double knockouts (D) was compared to wildtype littermate controls (A). These genetic manipulations significantly affected PTP amplitude (*p* < 0.01). (E), Cumulative histograms of normalized amplitudes of the 2nd potentiated response for wildtype (black), PKCα (*green*), PKCβ (*red*), and double knockout (*purple*) groups. (F), Average EPSC amplitudes (± SEM) for control and PKC knockout groups, plotted as a function of time. EPSCs were normalized to baseline before averaging.
Figure 2.2 (continued)

A. wt

B. α−/−

C. β−/−

D. α−/− β−/−

E. Cumulative fraction

F. EPSC (norm. vs. Time (s))
Figure 2.3

Deletion of PKCαβ impairs PTP-induced increases in the size of the readily releasable pool and release probability without affecting basal release properties. (A), Representative example of EPSCs elicited by 100 Hz train of stimuli. (B), Plot of normalized EPSC amplitude as a function of stimulus number during a 100 Hz, 0.4 s train delivered to slices from either wildtype (black) or double knockout (purple) animals. (C), Cumulative EPSC plotted as a function of stimulus number. The linear fit (gray line) to the last 15 points, back-extrapolated to the y-intercept, is used to obtain ΣEPSC₀, and provide a measure of the size of the readily releasable pool exocytosed by a train of stimuli (RRPₜrain). (D), Plot of normalized cumulative EPSCs for wildtype (black) and double knockout (purple) groups. (E through G), Basal ΣEPSC₀ (E), f₀ (the fraction of the RRPₜrain released by the first stimulus) (F), and slope (G) measurements for wildtype (black) and double knockouts (purple) groups. (H through I), Cumulative EPSCs in response to the first 40 stimuli of a PTP-inducing train (1ˢᵗ train; 100 Hz, 4 s; open symbols), and a 2ⁿᵈ train (100 Hz, 0.4 s) delivered 10 s later (closed symbols), for wildtype (black) (H) and double knockout (purple) (I) groups. (J), plots of ratios for ΣEPSC₀, f₀ and slope for wildtype (black) and double knockout (purple) groups. Deletion of PKCαβ significantly impaired the increase in RRPₜrain as well as the slope, and to a lesser extent the increase in f₀. * p < 0.01.
Figure 2.3 (continued)
significant difference in the use-dependent plasticity in wildtype and in PKCα-/β-/- mice (p = 0.24 for the second stimulus, p = 0.13 for the third stimulus, p = 0.08 for the average of the 31st to 40th stimuli) (Figure 2.3B).

Synaptic currents evoked by stimulus trains can also be used to quantify the size of the vesicle pool that is readily released by a train (RRP_{train}), as in Figure 2.3C. In this approach, the amplitudes of the EPSCs are measured and integrated. In the plot of the cumulative EPSC, after approximately the first 10 EPSCs, RRP_{train} is depleted, and the remaining steady-state EPSC is thought to reflect replenishment of RRP_{train}. The cumulative EPSC (∑EPSC₀) can then be determined by extrapolating back to the first EPSC in the train, as in Figure 2.3C. ∑EPSC₀ is proportional to RRP_{train} [RRP_{train} = ∑EPSC₀ / (average mEPSC size)]. The fraction of vesicles (f₀) within RRP_{train} that is liberated by the first action potential in a train can then be determined (f₀ = EPSC₀ / ∑EPSC₀). There was no significant difference in the size of RRP_{train} for wildtype and double knockout animals, because there was no difference in either ∑EPSC₀ (Figure 2.3D-E, p = 0.26) or in basal mEPSC size (Supplementary Figure S1). There was also no significant difference in basal f₀ (p = 0.66) (Figure 2.3F), or in the slope of the cumulative EPSC (p = 0.59) (Figure 2.3G). These findings indicate that the basal properties of synaptic transmission are similar in wildtype and double knockout animals.

**Tetanically-induced changes in RRP_{train} and f₀**

Our studies indicate that Ca^{2+}-dependent PKCs play a crucial role in PTP, but questions remain as to the mechanisms underlying this enhancement. Previous studies have shown that non-specific PKC activators cause little or no increase in the size of the readily releasable pool (RRP) as determined by a strong and prolonged depolarization (Lou et al., 2005; Wu and Wu, 2001). They do, however, produce large increases in RRP_{train} (Lou et al., 2008). One explanation for this apparent discrepancy is that the RRP consists of different pools of vesicles,
some that are located near Ca$^{2+}$ channels, and some that are located further from Ca$^{2+}$ channels (Neher and Sakaba, 2008). While prolonged depolarization or large presynaptic Ca$^{2+}$ signals can release the entire RRP, presynaptic action potentials produce brief and local Ca$^{2+}$ transients that trigger fusion of vesicles near Ca$^{2+}$ channels, but are not effective at triggering the fusion of more distant vesicles. Increasing the size of the Ca$^{2+}$ transient, as when external Ca$^{2+}$ levels are elevated, can increase RRP$_{\text{train}}$ by extending the spread of Ca$^{2+}$ entering through Ca$^{2+}$ channels to influence vesicle release. Alternatively, PKC could similarly extend the influence of Ca$^{2+}$ entering through Ca$^{2+}$ channels and increase RRP$_{\text{train}}$ by increasing the Ca$^{2+}$ sensitivity of release (lowering the Ca$^{2+}$ cooperativity) (Lou et al., 2008). Thus, if activation of Ca$^{2+}$-dependent PKCs produces PTP by increasing the Ca$^{2+}$ sensitivity of vesicles, it could lead to both an increase in RRP$_{\text{train}}$ and an increase in the fraction of those vesicles that are liberated by the first action potential in a train ($f_0$).

We tested this possibility by measuring the effect of tetanic stimulation on $\sum$EPSC$_0$ and $f_0$. Experiments were performed in the presence of CTZ and kynurenate to prevent receptor desensitization and saturation. Kynurenate and CTZ did not affect the magnitude of PTP in slices from either wildtype ($1.79 \pm 0.11$, $n = 9$, $p = 0.85$) (Supplementary Figure S2A) or double knockout mice ($1.14 \pm 0.08$, $n = 7$, $p = 0.78$) (Supplementary Figure S2B). To assess the effects of tetanization on $\sum$EPSC$_0$, synaptic currents were evoked by stimulating at 100 Hz for 4 seconds, followed by a brief train (100 Hz, 0.4 s) 10 s later. Plots of the cumulative EPSC were obtained for both trains, and used to calculate $\sum$EPSC$_0$ and $f_0$. As shown in representative experiments, tetanic stimulation increased $\sum$EPSC$_0$ in wildtype (Figure 2.3H), but not in double knockout mice (Figure 2.3I). Tetanic stimulation increased $\sum$EPSC$_0$ by 26 ± 0.7 % and 2 ± 3 % (Figure 2.3J, left; $p < 0.01$) and $f_0$ by 34 ± 5 % and 23 ± 6 % (Figure 2.3J, middle; $p = 0.14$), in wildtype and double knockout animals, respectively. Thus, the reduced PTP in double knockout mice arises primarily from decreases in the $\sum$EPSC$_0$ and perhaps $f_0$ (although the effect on $f_0$ is
not statistically significant). This finding is consistent with Ca\(^{2+}\)-dependent PKCs increasing the probability of release of vesicles located both near and far from Ca\(^{2+}\) channels (see Discussion). Moreover, in wildtype animals the slope of the cumulative EPSC vs. stimulus number was unaffected by tetanization (Figure 2.3H, 2.3J, right), but was reduced in double knockout animals (Figure 2.3I-J, right, p < 0.01). Impairment in the replenishment of the RRP\(_{\text{train}}\) or a decrease in steady-state release probability during the tetanus could contribute to decreased slope.

Previous studies suggest that myosin light chain kinase (MLCK) contributes to PTP through a mechanism that is distinct from Ca\(^{2+}\)-dependent PKCs, raising the possibility that the PTP remaining in double knockout animals could be mediated by MLCK. This kinase is thought to be responsible for an activity-dependent increase in the RRP\(_{\text{train}}\) that follows tetanic stimulation, but not the Ca\(^{2+}\)-dependent increase in the probability of release (Lee et al., 2010; Lee et al., 2008). The time course of the action of MLCK has not been thoroughly characterized, although it is thought to be independent of the slow mitochondrial-dependent decay of presynaptic Ca\(^{2+}\) following tetanic stimulation (Lee et al., 2008). According to a current model, Ca\(^{2+}\) increases during tetanic stimulation activate calmodulin and MLCK, which contribute to PTP by increasing RRP\(_{\text{train}}\) without affecting the overall RRP (Lee et al., 2010).

We tested this model by examining the contribution of MLCK to PTP in both wildtype and double knockout mice. In wildtype mice, the MLCK inhibitor ML9 reduced PTP from 87 ± 2 % (n = 17) to 26 ± 8 % (n = 10, p < 0.0001) 5 s after the train, and from 81 ± 2 % to 69 ± 2 % (p = 0.21) 10 s after the train (Figure 2.4A). These findings confirm that MLCK contributes to PTP. They also indicate that the contribution of MLCK is short-lived compared to overall PTP, and that by 10 s after stimulation the contribution is much smaller than that of Ca\(^{2+}\)-dependent PKCs. In double knockout animals, ML9 also influenced the magnitude of PTP, which at 10 s was reduced from 16 ± 2 % (n = 16) to 9 ± 2 % (n = 11, p < 0.05, Figure 2.4B). These findings
Figure 2.4

**Limited contribution of myosin light chain kinase to PTP.** (A through B), Plots of normalized EPSC amplitude as a function of time before and after PTP-inducing tetanization (*inverted triangles*) in slices from wildtype (black) and double knockout (*purple*) animals. Incubation with the MLCK inhibitor ML9 (*filled symbols*) partially affected PTP. (C through D), Plots of cumulative EPSCs as a function of stimulus number in slices from wildtype (C) and double knockout (D) animals, incubated with ML9. Gray lines are linear fits to the last 15 points. (E through F), Plots of ratios (2nd train / 1st train) for $\Sigma$EPSC$_0$, $f_0$, and the slope of the linear fits. Incubation with ML9 (*filled bars*) significantly decreased $f_0$ in both wildtypes (black) and double knockouts (*purple*). * $p < 0.05.$
Figure 2.4 (continued)
indicate that MLCK-dependent mechanisms account for some, but not all, of the PTP remaining in double knockout mice.

In order to compare the mechanisms of action of MLCK and Ca\(^{2+}\)-dependent PKCs in PTP, we examined the effect of MLCK inhibitors on \(f_0\) and \(\Sigma EPSC_0\) in both wildtype and double knockout mice. Inhibiting MLCK did not significantly change the basal properties of synaptic transmission in either wildtype or double knockout mice (Supplementary Figure S3). In wildtype mice, inhibiting MLCK did not alter the tetanus-induced increases in \(\Sigma EPSC_0\) (26 ± 7 %, \(n = 13\), compared to 21 ± 3 % in the presence of ML9, \(n = 10\), \(p = 0.51\), Figure 2.4C,E), it reduced the increase in \(f_0\) (35 ± 5 % compared to 16 ± 5 %, \(p < 0.05\)), and the slope was not statistically different (2 ± 4 % compared to -8 ± 4 %, \(p = 0.09\)). Similarly, in double knockout mice ML9 did not affect the tetanus-induced increase in \(\Sigma EPSC_0\) (2.2 ± 4.4 % \(n = 11\) compared to 4.3 ± 4.9 % \(n = 11\) in ML9, \(p = 0.72\), Figure 2.4D,F), but it reduced the increase in \(f_0\) (23 ± 6 % compared to 5 ± 4 %, \(p < 0.05\)), and the slope was not statistically different (-13 ± 3 % compared to -15 ± 7 %, \(p = 0.82\)). Therefore, under our experimental conditions, MLCK did not contribute to PTP-induced increases in RRP\(_{\text{train}}\) but did contribute to increases in \(f_0\). This finding indicates that Ca\(^{2+}\)-dependent PKCs contribute to PTP through mechanisms that are distinct from MLCK.

**Tetanus-induced presynaptic calcium signals**

To further elucidate the manner in which Ca\(^{2+}\)-dependent PKCs contribute to PTP, we examined the role of presynaptic Ca\(^{2+}\) signaling in PTP. Previous studies suggest that presynaptic Ca\(^{2+}\) could be involved in PTP in different ways. First, PTP could involve the small but long-lasting presynaptic residual Ca\(^{2+}\) (Ca\(_{\text{res}}\)) signals that follow tetanic stimulation (Zucker and Regehr, 2002). At the calyx of Held, PTP and Ca\(_{\text{res}}\) decay with similar time courses, and roughly linear relationship between Ca\(_{\text{res}}\) and PTP has been suggested (Habets and Borst,
This is consistent with the hypothesis that \( \text{Ca}_{\text{res}} \) activates proteins that respond to modest \( \text{Ca}_{\text{res}} \) levels to increase synaptic efficacy. \( \text{Ca}^{2+} \)-dependent PKCs could be the \( \text{Ca}_{\text{res}} \) sensor that produces PTP. Another possibility is that tetanic stimulation increases presynaptic \( \text{Ca}^{2+} \) entry by modulating \( \text{Ca}^{2+} \) channels (Catterall and Few, 2008). At the calyx of Held, prolonged tetanic activation can increase presynaptic \( \text{Ca}^{2+} \) entry under some circumstances (Habets and Borst, 2006). Indeed, tetanic stimulation for 4 s at 100 Hz can result in a 15% increase in presynaptic \( \text{Ca}^{2+} \) entry (Korogod et al., 2007). \( \text{Ca}^{2+} \)-dependent PKCs could mediate this \( \text{Ca}^{2+} \) channel facilitation. There is controversy over the relative importance of \( \text{Ca}_{\text{res}} \) and \( \text{Ca}^{2+} \) channel facilitation, but there is agreement that presynaptic \( \text{Ca}^{2+} \) signaling plays an important role in PTP.

We therefore examined \( \text{Ca}^{2+} \) signaling in calyces of Held from wildtype and double knockout animals. We introduced a low concentration of a \( \text{Ca}^{2+} \) indicator (Calcium Green-1 dextran, \( K_D = 326 \text{ nM} \)) into the calyx of Held, as described previously for other synapses (Beierlein et al., 2004), and quantified \( \text{Ca}^{2+} \) signals using established methodology (Brenowitz et al., 2006; Maravall et al., 2000). Brief loading times were used so that a small amount of indicator was introduced in order to minimize perturbations of presynaptic \( \text{Ca}^{2+} \) signaling. A red dye (Alexa 594-dextran) was also used to allow visualization of calyces (Figure 2.5A), because basal Calcium Green-1 fluorescence is faint. As shown for an example experiment, single stimuli evoked fluorescence transients that decayed rapidly (Figure 2.5B). Single stimuli produced \( \text{Ca}^{2+} \) increases of \( 18 \pm 4 \text{ nM} \) and \( 17 \pm 3 \text{ nM} \) in wildtype and double knockout animals, respectively. Following tetanic stimulation of 100 Hz for 4 seconds, \( \text{Ca}_{\text{res}} \) was \( 146 \pm 25 \text{ nM} \) 5 s after the end of stimulation, and \( \text{Ca}_{\text{res}} \) decayed with a time constant of 24.6 s. In double knockout animals the \( \text{Ca}_{\text{res}} \) was \( 142 \pm 31 \text{ nM} \) and decayed with a time constant of 24.7 s. (Figure 2.5C top). This indicates that diminished PTP in double knockout animals is not a result of perturbed \( \text{Ca}_{\text{res}} \) signals following tetanic stimulation.
Figure 2.5

Presynaptic residual calcium signals and calcium entry in wildtype and PKCα-/-β-/- animals. (A), 2-photon image of a calyx filled with Alexa 594-dextran and Calcium Green-1 dextran. Scale bar: 20 μm. (B), Fluorescence Ca$^{2+}$ transient evoked by a single stimulus. (C), Plots of residual Ca$^{2+}$ ($Ca_{res}$, top) and Ca$^{2+}$ influx (bottom) in slices from wildtype (black) and double knockout (purple) animals. Inverted triangle indicates 100 Hz, 4 s tetanus delivered to induce PTP.
Figure 2.5 (continued)
We tested whether Ca\textsuperscript{2+} channel facilitation contributes to PTP by measuring the effect of tetanic stimulation on increases in Ca\textsuperscript{2+} transients evoked by single stimuli (Figure 2.5C bottom). In wildtype animals tetanic stimulation elevated the Ca\textsuperscript{2+} increases evoked by single stimuli, but the enhancement was short-lived. This suggests that under our experimental conditions, tetanus-induced increases in Ca\textsuperscript{2+} influx make only a short-lived contribution to PTP. In double knockout animals the Ca\textsuperscript{2+} increases evoked by single stimuli show a similar short-lived increase following tetanic stimulation. This suggests that the impairment of PTP in double knockout animals is not due to impaired facilitation of Ca\textsuperscript{2+} currents in response to tetanic stimulation.

**Activity-dependent increases in mEPSC frequency**

In addition to enhancing the amplitude of evoked synaptic transmission, tetanic stimulation also enhances the frequency of spontaneous release (Castillo and Katz, 1954; Eliot et al., 1994; Habets and Borst, 2005; Korogod et al., 2005, 2007; Magleby, 1987). We tested whether PKC\textalpha{} and PKC\textbeta{} mediate this activity-dependent increase in mEPSC frequency. In these experiments the same tetanic stimulation was used as in our PTP experiments (4 s, 100 Hz), but without test stimuli. This allowed us to monitor mEPSCs before and after tetanic stimulation. Our tetanic stimulation protocol resulted in a large increase in mEPSC frequency, which is illustrated in a representative experiment in which the mEPSC frequency before and 2-6 s after stimulation increased from 1.0 to 3.5 Hz (Figure 2.6A, black). The extent of enhancement ranged from 1.7-fold to 6.7-fold (Figure 2.6B, black, the ratio of mEPSC frequency 2 - 6 s following tetanic stimulation to basal frequency). In wildtype animals the time course of mEPSC frequency enhancement decayed with a time constant of ~12 s (Figure 2.6C). Tetanic stimulation also increased the frequency of spontaneous events in PKC\textalpha{}/-, PKC\textbeta{}/-, and PKC\textalpha{}/-\textbeta{}/- mice, as shown in representative experiments in which enhancement was 4.2-fold (increased from 0.67 to 2.8 Hz), 3.7-fold (0.74 to 2.77 Hz), and 3.9-fold (0.80 to 3.1
The increase in spontaneous transmission after tetanization is independent of PKCα and PKCβ. (A), Representative recordings of spontaneous transmission before (basal; left) and after (right) tetanization from slices from wildtype (black), PKCα (green), PKCβ (red), and double knockout (purple) animals. (B), Cumulative histograms of average mEPSC frequency after tetanization, normalized to that before the tetanus. (C), Plot of normalized average mEPSC frequency as a function of time for wildtype (○), PKCα (○), PKCβ (○) and double knockout (○) groups. At t = 0 s, a 4 s, 100 Hz tetanus was delivered (inverted triangle). No statistical difference was observed among the 4 groups.
Figure 2.6 (continued)
Hz), respectively (Figure 2.6A). There was no significant difference in the enhancement of mEPSC frequency among wildtype (3.5 ± 0.3, n = 15), PKCα−/− (3.5 ± 0.5, n = 10), PKCβ−/− (4.4 ± 0.5, n = 16) and PKCα−/β−/− groups (4.3 ± 0.6, n = 13) (p = 0.43) (Figure 2.6B-C). These results suggest that at the calyx of Held synapse, PTP and the enhancement of spontaneous release arise from different mechanisms. Ca²⁺-dependent PKCs are crucial to PTP, but they do not mediate tetanus-evoked increases in mEPSC frequency.

Activity-dependent increases in mEPSC amplitude

We tested whether PKCα and PKCβ mediate the increase in mEPSC amplitude that follows tetanic stimulation (He et al., 2009). In wildtype mice, tetanic stimulation altered the distribution of mEPSC sizes, and after tetanic stimulation the fraction of small mEPSCs was reduced and the fraction of large mEPSCs increased, as shown in a representative experiment (Figure 2.7A). In slices from PKC knockout animals, tetanic stimulation also increased mEPSC amplitude and produced similar effects on the mEPSC distributions, as illustrated in representative experiments from slices from PKCα−/− (Figure 2.7B), PKCβ−/− (Figure 2.7C), and PKCα−/β−/− (Figure 2.7D) mice. As shown in the cumulative histograms (Figure 2.7E), tetanic stimulation significantly increased the mEPSC amplitude in slices from wildtype, PKCα−/−, PKCβ−/−, and PKCα−/β−/− mice compared to their respective baseline (p < 0.05 for all paired comparisons). On average, enhancement was somewhat smaller in PKCβ−/− (10.1 ± 2.8 %), and PKCα−/β−/− (10.9 ± 4.7 %) compared to wildtype (13.1 ± 3.5 %) and PKCα−/− (18.7 ± 2.5 %), but these differences were not statistically significant (p = 0.34). The time courses of the enhancement of mEPSC amplitude in the different genotypes (Figure 2.7F) can be approximated by single exponential decays with time constants of 47 ± 9 s, 39 ± 4 s, 67 ± 17 s and 35 ± 8 s for wildtype, PKCα−/−, PKCβ−/− and PKCα−/β−/− groups, respectively.
Figure 2.7

The increase in mEPSC size following tetanic stimulation is independent of PKCα and PKCβ. (A through D), Representative distributions of mEPSC amplitudes recorded before (light traces, 25 s interval prior to stimulation) and after a 4 s, 100 Hz tetanus (bold traces, 2-12 s after tetanus) are shown for wildtype (A, black), PKCα knockout (B, green), PKCβ knockout (C, red) and double knockout (D, purple) groups. To facilitate comparison of the distributions, the mEPSC distributions are expressed as fractional contribution of each 2-pA bin to the total number of events detected. (E), Cumulative histograms of average mEPSC amplitude after tetanization, normalized to that before the tetanus. (F), Plot of normalized average mEPSC frequency as a function of time for wildtype (○), PKCα (○), PKCβ (○) and double knockout (○) groups.
Figure 2.7 (continued)
Synaptic enhancement mediated by phorbol esters

Phorbol esters activate PKC by binding to the diacylglycerol (DAG) binding site (Newton, 2001), leading to large synaptic enhancement that mimics and occludes PTP (Korogod et al., 2007; Malenka et al., 1986). Synaptic enhancement by phorbol esters has been studied extensively to provide insight into the mechanism mediating PTP. We examined the role of PKCα and PKCβ in phorbol ester-induced enhancement. As shown in representative experiments, the phorbol ester PDBu (1 μM) enhanced EPSC amplitude in slices from wildtype (Figure 2.8A, 2.5-fold), PKCα-/- (Figure 2.8B, 1.7-fold), PKCβ-/- (Figure 2.8C, 1.4-fold), and PKCα-/-β-/- (Figure 2.8D, 1.4-fold) mice, but the degree of enhancement was smaller in the knockout groups. Although there was variability in the extent of enhancement in the different genotypes (Figure 2.8E), the average extent of enhancement was clearly reduced in the PKC knockout groups (Figure 2.8F), and there was a significant difference in the PDBu-dependent enhancement between wildtype (2.22 ± 0.14, n = 17) and PKCα-/- (1.80 ± 0.12, n = 13, p < 0.05), PKCβ-/- (1.46 ± 0.05, n = 13, p < 0.01), and PKCα-/-β-/- (1.44 ± 0.09, n = 9, p < 0.01) groups. These experiments establish that Ca²⁺-dependent PKCs play an important role in phorbol ester-dependent enhancement at the calyx of Held. Compared to baseline, there is still significant enhancement remaining in slices from PKCα-/-β-/- mice (p < 0.01), which indicates that other target(s) of phorbol esters (Brose and Rosenmund, 2002; Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007) are engaged at this synapse.

In addition to enhancing the amplitude of evoked EPSCs, phorbol esters increase mEPSC frequency. This is illustrated in a representative experiment by comparing spontaneous mEPSCs recorded in control conditions and in the presence of PDBu (Figure 2.8G, black). We tested whether PKCα and PKCβ also contribute to this enhancement of mEPSC frequency. As shown in the representative experiments, PDBu increased the mEPSC frequency in slices from PKCα-/- (Figure 2.8G, green), PKCβ-/- (Figure 2.8G, red), and PKCα-/-β-/- (Figure 2.8G, purple) mice. The range of mEPSC frequency enhancement was quite broad in all genotypes.
Figure 2.8

The contribution of calcium-dependent isoforms of PKC to synaptic plasticity mediated by phorbol esters. The phorbol ester PDBu (1 μM) was bath-applied at t = 0 s and the effect on the amplitudes of evoked EPSCs (A through F) and mEPSC frequencies (G through I) were monitored. Experiments corresponding to different genotypes are displayed in different colors: wildtype (black), PKCα (green), PKCβ (red), and double knockout (purple) groups. Representative experiments show the effects on the evoked EPSCs for wildtype (A), PKCα knockouts (B), PKCβ knockouts (C), and PKCαβ double knockouts (D). (E), Cumulative histograms of EPSC enhancement in PDBu. (F), Average EPSC amplitudes (± SEM) plotted as a function of time. (G), Representative experiments show mEPSCs before (left) and during (right) the application of PDBu. (H), Cumulative histograms of mEPSC enhancement in PDBu. (I), Average mEPSC frequency (± SEM) plotted as a function of time.
Figure 2.8 (continued)

A  wt  

B  α -/-  

C  β -/-  

D  α -/- β -/-  

E  

F  

G  

H  

I  

Figure 2.8 (continued)
(Figure 2.8H). The average enhancement was 5.6 ± 0.7 in wildtype (Figure 2.8I, black, n = 13), 4.9 ± 0.4 in PKCα/-/ (Figure 2.8I, green, n = 14), 4.4 ± 0.5 in PKCβ/-/ (Figure 2.8I, red, n = 14), and 3.1 ± 0.6 in PKCα/-/β/-/ (Figure 2.8I, purple, n = 7) groups. Although there was a trend suggesting that PKCα and PKCβ contributed to the phorbol ester-dependent enhancement in mEPSC frequency, the differences did not reach statistical significance (p = 0.054), despite the relatively large sample sizes. However, a pair-wise comparison using a Kolmogorov-Smirnov 2-sample test indicated that the mEPSC frequency distributions for wildtype and double knockout groups were significantly different (p < 0.05).

**Summary of the properties of synaptic transmission in wildtype and knockout animals**

Our findings indicate that PKCα and PKCβ play important roles in synaptic transmission at the calyx of Held synapse. Although there are no discernable effects on basal properties of synaptic transmission, there are profound differences in synaptic plasticity, with various synaptic properties affected differentially. This is illustrated in Figure 2.9, where the plasticity in different genotypes is expressed relative to the plasticity in the wildtype group. The largest effect was on PTP. In PKCα/-/-, PKCβ/-/- and PKCα/-/β/-/- groups PTP was 75 ± 10 %, 26 ± 4 % and 20 ± 3 %, respectively of that observed in the wildtype group (Figure 2.9A, top). The primary effect of PKCαβ was on ΣEPSC0, which in double knockouts was reduced to 8 ± 13 % of wildtype (Figure 2.9B, top). Deletion of PKCαβ also modulated f0, which was reduced to 66 ± 19 % of wildtype. The increases in mEPSC frequencies following tetanic stimulation in PKCα/-/-, PKCβ/-/-, and PKCα/-/β/-/- were 87 ± 30 %, 140 ± 41 % and 137 ± 43 %, respectively of that observed in the wildtype group (Figure 2.9C, top). Thus, the same stimulus train profoundly reduced PTP without reducing the frequency of spontaneous mEPSCs. Furthermore, the amplitude of mEPSC was also not significantly affected by the absence of Ca2+-sensitive PKCs (Figure 2.9D, top). The mEPSC amplitude changes in PKCα/-/-, PKCβ/-/-, and PKCα/-/β/-/- after the tetanus were 143 ± 42 %, 77 ± 29 % and 83 ± 42 % respectively, of the wildtype group.
Summary of the properties of synaptic transmission in slices from wildtype and PKC knockout animals. (A through E, top), The tetanus-induced (A through D, top) and PDBu-induced (E, top) changes in the properties of synaptic transmission are compared for wildtype (black), PKCα knockouts (green), PKCβ knockouts (red), and double knockouts (purple). The synaptic enhancement in the different genotypes was normalized to that in wildtype animals. PTP and the enhancement of mEPSC frequency and amplitude were evoked by 4 s, 100 Hz stimulation. (A through E, bottom), Examples of the different types of synaptic modulation are shown, and their dependence on PKCα and PKCβ is indicated.
Figure 2.9 (continued)

A  
PTP

B

\[ \text{EPSC}_0 \int_{f_0} \]

\[
\begin{array}{c}
\text{wt} \\
\alpha{-/-} \\
\beta{-/-} \\
\alpha{-/-}\beta{-/-} \\
\end{array}
\]

C  
mEPSC frequency

D  
mEPSC amplitude

E  
PDBu potentiation

PKC\(\alpha/\beta\) dependent

PKC\(\alpha/\beta\) independent

PKC\(\alpha/\beta\) dependent
Following application of PDBu, the increase in the amplitude of evoked synaptic responses in PKCα-/-, PKCβ-/-, and PKCα/-β/- was 66 ± 12 %, 38 ± 6 % and 36 ± 9 %, respectively, of that observed in the wildtype group (Figure 2.9E, top). In the PKCα-/-β/- group a higher percentage of enhancement remains for PDBu-dependent enhancement (36%) than for PTP (20%).

Discussion

Here we report that in the absence of both PKCα and PKCβ, PTP is 20% of that observed in wildtype animals, indicating that Ca^{2+}-dependent PKCs mediate most of PTP at the calyx of Held synapse. The remaining PTP appears to be mediated in part by an MLCK-dependent mechanism and in part by an increase in mEPSC size. Ca^{2+}-dependent PKCs enhance transmission primarily by increasing RRP_{train}, and to a lesser extent by increasing the fraction of vesicles released in response to a stimulus; they also influence replenishment of RRP_{train} following tetanic stimulation. Similar to PTP, phorbol ester-dependent enhancement was greatly reduced in slices from double knockout animals. The differential effects of PKCα and PKCβ on evoked and spontaneous synaptic transmission are summarized in Figure 2.9 (top: group averages, bottom: individual examples).

PTP is mediated primarily by PKCα and PKCβ

Our finding that PTP is greatly reduced in the absence of PKCα and PKCβ establishes an important role for these kinase isoforms in PTP at the calyx of Held. Our results resolve a long-standing controversy over whether PKC plays a role in PTP. Previous observations that phorbol esters occlude PTP (Korogod et al., 2007; Malenka et al., 1986) were thought to support a role for PKC in PTP until it was realized that in addition to activating PKC, phorbol esters activate other proteins such as Munc13 (Brose and Rosenmund, 2002; Lou et al., 2008; Rhee et al., 2002; Wierda et al., 2007). Similarly, the finding that PKC inhibitors reduce the
magnitude of PTP (Alle et al., 2001; Beierlein et al., 2007; Brager et al., 2003; Korogod et al., 2007; Lee et al., 2007) supported a role for PKC in PTP, but the observation that inactive analogues have similar effects on PTP (Lee et al., 2008) and that some PKC inhibitors do not affect PTP at all (Eliot et al., 1994; Korogod, 2006; Lee et al., 2008; Reymann et al., 1988a; Reymann et al., 1988b) have blurred the role of PKC in PTP. Using a molecular genetic approach allowed us to overcome the limitations associated with the lack of selectivity of PKC inhibitors and activators and establish that PKC plays a crucial role in PTP.

Our results establish that Ca\(^{2+}\)-dependent PKC isoforms mediate most of the PTP at the calyx of Held, with PKC\(\beta\) playing a more prominent role than PKC\(\alpha\) (Figure 2.9A, top). This challenges the previously-held view that a Ca\(^{2+}\)-independent PKC isoform mediates PTP (Korogod, 2006; Saitoh et al., 2001). Previous studies at the calyx of Held found that phorbol esters induce translocation of PKC\(\varepsilon\) and suggested that this Ca\(^{2+}\)-independent isoform mediates PKC-dependent plasticity at this synapse (Saitoh et al., 2001). Moreover, different PKC inhibitors were found to have very different effects on PTP. A broad-spectrum inhibitor (bisindolylmaleimide, BIS) and one that preferentially targets Ca\(^{2+}\)-independent isoforms (Ro31-8220) reduced PTP (expressed as fraction of PTP in control conditions) to approximately 40% and 20%, respectively, whereas an inhibitor that targets Ca\(^{2+}\)-dependent isoforms (Gö-6976) did not affect PTP (Korogod, 2006; Korogod et al., 2007). One interpretation of these results is that PTP involves Ca\(^{2+}\)-independent PKCs, which might be activated by a tetanus-dependent elevation of DAG rather than by Ca\(^{2+}\). This interpretation is, however, complicated, because PKC inhibitors do not readily penetrate brain slices, and slices must be soaked in high concentrations of the inhibitors for long periods of time prior to the experiment. In some cases, broad-spectrum inhibitors (chelerythrine) do not reduce the magnitude of PTP (Lee et al., 2008). Limitations associated with the use of PKC inhibitors in slice preparation raise the possibility that the differential efficacy of PKC inhibitors may reflect their ability to penetrate the slice, rather than their isoform selectivity (Brose and Rosenmund, 2002). This seems to be a plausible
explanation for the differential effects of PKC inhibitors, in light of our observation that the Ca$^{2+}$-dependent isoforms PKCα and PKCβ account for most of the PTP.

Mechanisms of PTP mediated by PKCα and PKCβ

Our experiments provide new insight into the mechanisms underlying PTP. Calcium measurements suggest that although Ca$^{2+}$ channels are briefly facilitated, this facilitation makes a short-lived contribution to PTP (Figure 2.5C). Facilitated Ca$^{2+}$ entry is still present in double knockout animals, indicating that it is not mediated by Ca$^{2+}$-dependent PKCs. PTP at cultured superior cervical ganglion neurons is also mediated primarily by mechanisms that are independent of Ca$^{2+}$ channel facilitation (Mochida et al., 2008).

The effect of tetanic stimulation on responses evoked by brief 100 Hz stimulus trains was revealing with respect to how Ca$^{2+}$-dependent PKCs contribute to PTP. The use-dependent increase in the size of RRP$\text{train}$ is absent in double knockout animals (Figure 2.3I), suggesting that PKCα and PKCβ mediate the increase in the size of the pool of vesicles following tetanic activation. This appears to be the primary mechanism by which Ca$^{2+}$-dependent PKCs produce PTP, although they also appear to be partially responsible for the increase in the fraction of vesicles exocytosed by an action potential. The substantial increase in RRP$\text{train}$ is compatible with the observation that phorbol esters have only minor effects on the overall RRP size, provided the properties of different vesicle pools at the calyx of Held are considered (Lou et al., 2008). When an action potential invades a presynaptic bouton, vesicles that are located near Ca$^{2+}$ channels are exposed to a larger Ca$^{2+}$ signal than more distant vesicles (Neher and Sakaba, 2008). Any increase in the sensitivity of a vesicle to Ca$^{2+}$ could increase both the size of the vesicle pool that can be exocytosed by a train of action potentials, and the fraction of the vesicles that are released by the first action potential (Lou et al., 2008). The relative contributions of these two mechanisms depend upon the detailed ultrastructure of the synapse, the spatiotemporal Ca$^{2+}$ signal, and the Ca$^{2+}$ sensitivity of the vesicles (Branco and
Staras, 2009; Neher and Sakaba, 2008). In the case of PTP, our findings suggest that PKCαβ act primarily to increase the size of the readily releasable pool.

The involvement of Ca$^{2+}$-dependent PKC isoforms in PTP raises the question: are PKCα and PKCβ the Ca$^{2+}$ sensors that, according to the residual Ca$^{2+}$ hypothesis of PTP, detect presynaptic Ca$^{2+}$ signals evoked by tetanic stimulation to phosphorylate downstream targets thus increasing the probability of release? We find that Ca$_{\text{res}}$ decays ($\tau \sim 25$ s) more quickly than PTP ($\tau \sim 45$ s), suggesting that for our experimental conditions PTP is longer-lived than Ca$_{\text{res}}$ at the calyx of Held, as is the case at hippocampal and cerebellar synapses (Beierlein et al., 2007; Brager et al., 2003). Furthermore, we find that PTP is produced by tetanic stimulation that increases Ca$_{\text{res}}$ by several hundred nanomolar. Can Ca$^{2+}$-dependent PKCs respond to such small Ca$^{2+}$ increases? In the absence of lipid membranes, the Ca$^{2+}$-binding affinities for PKCα and PKCβ are $\sim 40$ μM (Kohout et al., 2002), which is much higher than the observed residual Ca$^{2+}$ signals. However, in the presence of phosphatidylserine and/or PIP2-containing membranes or in model systems, cooperative Ca$^{2+}$ binding is observed for both isoforms, and Ca$^{2+}$ affinities range from 0.1 to 5 μM (Corbalan-Garcia et al., 1999; Corbin et al., 2007; Guerrero-Valero et al., 2007; Kohout et al., 2002). It is also possible that factors in the intracellular milieu raise the binding affinity of PKCs for Ca$^{2+}$, as is the case for calmodulin (Xia and Storm, 2005). Thus, it is plausible that PKCα and PKCβ could be sufficiently sensitive to detect residual Ca$^{2+}$. Alternatively, PKCs could be initially activated by the Ca$^{2+}$ signals during the train and then, because of positive cooperative binding, become sensitive to residual Ca$^{2+}$. Once activated, PKC could phosphorylate proteins such as Munc18 to increase the probability of release (Wierda et al., 2007). Further studies are needed to determine if PKCα and PKCβ are indeed the Ca$^{2+}$ sensors in PTP, and if they increase the probability of release by phosphorylating Munc18.
Increases in mEPSC frequency and amplitude evoked by tetanic stimulation

Tetanic stimulation increases the frequency of mEPSCs several-fold at the calyx of Held synapse and at other synapses (Figure 2.6) (Castillo and Katz, 1954; Eliot et al., 1994; Groffen et al., 2010; Habets and Borst, 2005; Korogod et al., 2005, 2007; Magleby, 1987). The increase in the frequency of spontaneous release and PTP are both dependent on presynaptic Ca\textsuperscript{2+} increases (Bao et al., 1997; Korogod et al., 2005; Nussinovitch and Rahamimoff, 1988; Zucker and Lara-Estrella, 1983), suggesting that they share a common mechanism. However, the elevation in mEPSC frequency does not last as long as the enhancement of evoked EPSCs (τ ~ 12 s and 45 s respectively) (see also Korogod et al., 2007). In addition, pharmacological inhibitors of PKC that reduce the increase in evoked EPSC amplitude do not prevent the increase in mEPSC frequency at calyx of Held synapses (Korogod et al., 2007). Here, using a genetic approach, we also find that the frequency of mEPSC and the amplitude of evoked EPSCs are regulated independently. Indeed, potentiation of evoked EPSCs is reduced by 80% in slices from PKCα-/-β-/- double knockout animals compared to controls (Figure 2.9A) whereas the increase in mEPSC frequency is largely unaffected (Figure 2.9C). Therefore, the activity-dependent regulation of mEPSC frequency is not mediated by PKCs, and is likely regulated by other Ca\textsuperscript{2+}-sensitive proteins in the presynaptic terminal, such as Doc2a and Doc2b (Groffen et al., 2010).

Tetanic stimulation also results in increased mEPSC amplitude in slices from wildtype animals (Figure 2.7). Although modest, this increase has a time course (τ ~ 47 s) that is similar to that of PTP (τ ~ 45 s, compare Figures 2.2F and 2.7F), and it is thought to contribute to PTP (He et al., 2009). The increase in mEPSC amplitude appears to reflect the fusion of vesicles with each other prior to ultimate fusion with the plasma membrane (He et al., 2009). We find that the increase in mEPSC amplitude persists in the absence of PKCα, PKCβ, or both isoforms (Figure 2.7). This suggests that Ca\textsuperscript{2+}-dependent isoforms of PKC do not regulate vesicle-to-vesicle fusion within the calyx of Held, in contrast to their prominent role in regulating vesicle

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fusion with the plasma membrane that underlies evoked EPSCs (*Figure 2.2*). The 10% increase in mEPSC amplitude that remains in PKCαβ double knockout animals could account for some of the remaining PTP observed in this group (*Figure 2.9A*).

**Synaptic enhancement by phorbol esters**

Previous studies have supported a role for PKCs in the phorbol ester-induced enhancement of evoked EPSCs at the calyx of Held (Hori et al., 1999; Korogod, 2006; Lou et al., 2008), but the isoform responsible for this enhancement was not known. A broad-spectrum PKC inhibitor, though with questionable selectivity (Lee et al., 2008), reduced enhancement by PDBu to approximately 40% of control (Hori et al., 1999; Korogod, 2006), and inclusion of a more selective PKC blocking peptide in the presynaptic terminal reduced the enhancement to less than 20% of that observed in control conditions (Hori et al., 1999). Previous studies suggested that the Ca²⁺-insensitive isoform PKCε mediates this enhancement because it is present at the calyx of Held and is activated by phorbol esters (Saitoh et al., 2001). However, our observation that phorbol ester-induced potentiation of evoked EPSCs is reduced by ~ 70% in PKCαβ double knockouts compared to controls indicate that these two isoforms account for the bulk of the contribution of PKCs to EPSC enhancement by phorbol esters. Moreover, our results are consistent with the observation that ~ 50% of phorbol ester-induced potentiation in the hippocampus is impaired in PKCβ knockout mice (Weeber et al., 2000). The component of phorbol ester-induced enhancement that is not mediated by PKCs is likely mediated by the synaptic protein Munc13, either as a result of phorbol esters directly activating Munc13, or as a result of phorbol ester binding to the N-terminal domain of Doc2α, thereby allowing it to interact with Munc13 (Hori et al., 1999; Lou et al., 2008).

Phorbol esters enhance mEPSC frequency ~ 6-fold in wildtype animals (*Figure 2.8*). In the absence of PKCα and PKCβ, this enhancement is reduced by ~ 50% (compare black and purple traces in *Figure 2.8I*). This result agrees with previous observations using pharmacology
(Lou et al., 2008; Oleskevich and Walmsley, 2000) and suggests that PKC plays a less
important role in potentiating spontaneous release compared to evoked release. In double
knockout animals, the impairment of the phorbol ester-induced increase in mEPSC frequency
(Figure 2.8I), although moderate, contrasts the lack of effect on tetanus-induced increase in
mEPSC frequency (Figure 2.9C). Further studies are needed to understand this potential
difference in the regulation of spontaneous activity.

Materials and Methods

Animals

PKCα (in 129S2 genetic background) and PKCβ (in C57BL/6J genetic background)
single knockout animals, generated by M. Leitges (Leitges et al., 2002; Leitges et al., 1996),
were bred together to obtain offspring heterozygous for both genes (het-het animals). Crosses
of het-het animals generated α+/+β+/+ (wt), α−/−β+/+ (αKO), α+/+β−/− (βKO), and α−/−β−/− (double
knockout) animals with a frequency of 1:16 each. All animal handling and procedures abided by
the guidelines of the Harvard Medical Area Standing Committee on Animals.

Preparation of brain slices

Transverse 190- to 200-μm-thick brainstem slices containing the region of the medial
nucleus of the trapezoid body (MNTB) were made with a vibratome slicer (VT1000S, Leica)
from postnatal day P11-14 mice deeply anesthetized with isoflurane. Brains were dissected and
sliced at 4 °C in cutting solution consisting of the following (in mM): 125 NaCl, 25 NaHCO₃, 1.25
NaH₂PO₄, 2.5 KCl, 0.1 CaCl₂, 3 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic
acid, continuously bubbled with 95% O₂/5% CO₂ (pH 7.4). Slices were incubated at 32 °C for at
least 30 min in a bicarbonate-buffered solution composed of the following (in mM): 125 NaCl, 25
NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate,
0.4 ascorbic acid, continuously bubbled with 95 % O₂/5 % CO₂ (pH 7.4).
**Electrophysiology**

Slices were transferred to a recording chamber at room temperature (21-24 °C) in an upright microscope (Olympus, Center Valley, PA) equipped with a 60X, 0.9 N.A. objective. During recordings, the standard perfusion solution consisted of the bicarbonate-buffered solution (see above) with 1 μM strychnine and 25 μM bicuculline to block inhibitory synaptic transmission. Slices were superfused at 1-3 ml/min with this external solution. Whole-cell postsynaptic patch-clamp recordings were made from visually identified cells in the MNTB region using glass pipettes of 2-3 MΩ resistance, filled with an internal recording solution of the following (in mM): 20 CsCl, 140 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 EGTA, 5 Na$_2$-phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, pH: 7.3, 315-320 mOsm. Series resistance ($R_s$) was compensated by up to 70 % and the membrane potential was held at -70 mV.

Excitatory postsynaptic potentials (EPSCs) were evoked by stimulating presynaptic axons with a bipolar stimulating electrode (custom-made or from FHC, Bowdoin ME) placed midway between the medial border of the MNTB and the midline of the brainstem. Multiclamp 700A and 700B (Axon Instruments/Molecular Devices, Union City, CA) amplifiers were used. Recordings were digitized at 20 KHz with an ITC-18 A/D converter (Instrutech Corp., Port Washington, NY) using custom macros (written by M.A. Xu-Friedman) in Igor Pro (Wavemetrics, Lake Oswego, OR) and filtered at 8 kHz.

The protocol for inducing PTP was as follows: an estimate of baseline synaptic strength was obtained through low-frequency stimulation at 0.2 Hz for 25 s. PTP was induced with a 4-s stimulus train at 100 Hz, followed by low-frequency stimulation to test for PTP. Changes in miniature EPSCs (mEPSCs) were measured by delivering the same PTP-inducing train, but without the low-frequency stimulation. For phorbol ester experiments, basal synaptic strength was evaluated by paired (50 ms interval) stimuli, repeated every 20 s. During the intertrial intervals, 10 s stretches of postsynaptic current were recorded to assess the frequency and
amplitude of mEPSCs. For all recordings, the access resistance and leak current were monitored, and experiments were rejected if either of these parameters changed significantly.

**Presynaptic calcium measurements**

Alexa 594 dextran and Calcium Green-1 dextran (10 kDa, Invitrogen) were loaded into presynaptic boutons as described previously (Beierlein et al., 2004). Loading times were 3-5 min and the loading solution contained 0.025-0.1 % Alexa 594 dextran and 0.5 % Calcium Green-1 dextran. Fluorescence transients from calyces were monitored with a 2-photon microscope as described previously (Brenowitz et al., 2006). Fluorescence signals were converted to Ca\(^{2+}\) by determining the \(\frac{F_{\text{max}}}{F_{\text{min}}}\) ratio (\(\frac{F_{\text{max}}}{F_{\text{min}}} = 5.5\)) in a cuvette, determining \(F_{\text{max}}\) using high frequency stimulation according to the approach presented previously (Maravall et al., 2000). In general, calyces that had bright green fluorescence at rest were found to be unsuitable for further study, either because they had elevated resting Ca\(^{2+}\) levels, or they were overloaded with Ca\(^{2+}\) indicator and the Ca\(^{2+}\) transients were slowed.

**Data analysis**

Data analysis was performed using routines written in IgorPro (WaveMetrics, Lake Oswego, OR). PTP magnitude was calculated as the ratio of EPSC amplitude 10 s after the 100 Hz train over the average baseline. mEPSCs were detected using a threshold (average peak-to-peak noise in the baseline) of the first derivative of the raw current trace, and confirmed visually. mEPSC frequency measurements were made during the baseline (25 s before PTP induction) and starting 6 s after PTP induction. The observed increases in mEPSC size cannot be attributed to the near synchronous fusion of 2 vesicles because, assuming a Poisson distribution and a peak mEPSC frequency (\(\nu\)) of 12 events/s (as observed following tetanic stimulation), we estimate that only \((1-\exp(-\Delta t^*\nu))= 2.4 \%\) of mEPSCs occur within 2 ms of each
other following tetanic stimulation (a conservative upper bound for the timing of 2 closely spaced mEPSCs that can be both detected). Statistical analyses were done using one-way ANOVA tests for multiple group comparisons followed by Tukey post-hoc analysis. Pair-wise comparisons were performed with Student’s paired t-tests or Wilcoxon signed rank tests. Level of significance was set at p < 0.05.

**Immunohistochemistry**

150 μm-thick transverse brainstem slices were prepared from P12 animals as described above and fixed with 4 % paraformaldehyde for 2 h at 4 °C. At the end of fixation, slices were transferred to phosphate buffer (Sigma-Aldrich, St Louis, MO) and stored at 4 °C until further processing. Slices were then incubated in blocking solution [phosphate buffered solution + 0.25 % Triton X-100 (PBST) + 10 % normal goat serum] for 1 h at room temperature. Slices were incubated with primary antibodies in PBST overnight at 4 °C, followed by incubation with secondary antibodies in PBST for 2 h at room temperature. Slices were mounted to Superfrost glass slides (VWR, West Chester PA) and air-dried for 30 min. Following application of DAPI-containing Prolong anti-fade medium (Invitrogen, Carlsbad CA), slices were covered with a top glass coverslip (VWR) and allowed to dry for 24 h prior to imaging. The following antibodies were used: anti-vGlut1 guinea pig polyclonal (Synaptic Systems, Göettingen Germany), anti-PKCα rabbit monoclonal (Abcam, Cambridge, MA), anti-PKCβI rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz CA), goat anti-guinea pig rhodamine-conjugated and goat anti-rabbit FITC-conjugated secondaries (Santa Cruz Biotechnology, Santa Cruz CA). All antibodies were used at 1:500 dilution.

Images were acquired with a Zeiss 510 Meta confocal microscope using a Plan-apochromat 63X 1.4 N.A. oil lens. Excitation was set at 543 nm for rhodamine (vGlut1) and 488 nm for FITC (PKCs). Emission filters were LP560 for vGlut1 and BP505-530 for PKCs. An optical zoom of 2 was used. Single optical sections at 1024x1024 (Kalman average of 4 scans)
were obtained sequentially for the different channels. Experiments with slices from different animals of all genotypes were repeated 3 times.

References


Chapter 3

Calcium-dependent isoforms of protein kinase C mediate glycine-induced synaptic enhancement at the calyx of Held

YunXiang Chu, Diasynou Fioravante, Monica Thanawala, Michael Leitges and Wade G. Regehr


Author contributions:
Y.X.C. and W.G.R. designed all experiments and interpreted the findings. D.F. generated and maintained the transgenic mice, and provided technical and conceptual advice. M.T. conducted and analyzed data for experiments in 3 mM external Ca\(^{2+}\), and wrote codes for data analysis. Y.X.C. performed all other experiments and analyzed the results. M.L. generated the original PKC\(\alpha\) and PKC\(\beta\) mice. Y.X.C. and W.G.R. wrote the paper.
Abstract

Depolarization of presynaptic terminals that arises from activation of presynaptic ionotropic receptors, or somatic depolarization, can enhance neurotransmitter release; however, the molecular mechanisms mediating this plasticity are not known. Here we investigate the mechanism of this enhancement at the calyx of Held synapse, where presynaptic glycine receptors depolarize presynaptic terminals, elevate resting Ca$^{2+}$ levels and potentiate release. Using knockout mice of the Ca$^{2+}$-sensitive PKC isoforms (PKC$_{Ca}$), we find that enhancement of evoked but not spontaneous synaptic transmission by glycine is mediated primarily by PKC$_{Ca}$. Measurements of Ca$^{2+}$ at the calyx of Held indicate that deficits in synaptic modulation in PKC$_{Ca}$ knockout mice occur downstream of presynaptic Ca$^{2+}$ increases. Glycine enhances synaptic transmission primarily by increasing the effective size of the pool of readily-releasable vesicles. Our results reveal that PKC$_{Ca}$ can enhance evoked neurotransmitter release in response to Ca$^{2+}$ increases caused by small presynaptic depolarizations.
Introduction

Like G protein-coupled receptors (Wu and Saggau, 1997; Blackmer et al., 2001; Schmitz et al., 2001), presynaptic ionotropic receptors modulate synaptic transmission (Kerchner et al., 2001; Turecek and Trussell, 2001; Engelman and MacDermott, 2004). The latter form of synaptic regulation can involve small depolarizations of presynaptic boutons that enhance neurotransmitter release by increasing presynaptic Ca$^{2+}$ (Turecek and Trussell, 2001; Awatramani et al., 2005; Christie et al., 2011). In this way, synaptic enhancement by ionotropic receptors is similar to synaptic enhancement produced by subthreshold somatic depolarizations at cortical, hippocampal and cerebellar synapses (Glitsch and Marty, 1999; Alle and Geiger, 2006; Shu et al., 2006; Christie et al., 2011; Yu et al., 2011). In general, it is not understood how presynaptic depolarizations enhance neurotransmitter release.

Glycinergic enhancement of evoked and spontaneous release at the calyx of Held synapse is a well-studied form of plasticity that involves a small presynaptic depolarization arising from activation of presynaptic ionotropic receptors (Turecek and Trussell, 2001; Balakrishnan et al., 2009). Glycine, which is released by interneurons in the medial nucleus of the trapezoid body (Turecek and Trussell, 2001), activates presynaptic ionotropic receptors that depolarize presynaptic terminals due to the relatively high chloride reversal potential at this synapse (-50 mV) (Price and Trussell, 2006; Huang and Trussell, 2008; Kim and Trussell, 2009). This depolarization is sufficient to open a small fraction of P-type voltage-gated Ca$^{2+}$ channels, elevating presynaptic Ca$^{2+}$ and, ultimately, increasing the amplitude of evoked excitatory postsynaptic currents (EPSCs) and miniature EPSC (mEPSC) frequency (Turecek and Trussell, 2001; Trussell, 2002; Awatramani et al., 2005; Kim and Trussell, 2009). This form of enhancement can be mimicked by direct depolarization of the presynaptic bouton (Awatramani et al., 2005), suggesting that it can provide general insight into enhancement arising from both presynaptic ionotropic receptor activation and depolarization of presynaptic boutons conveyed from the soma. The molecular mechanisms that respond to elevated Ca$^{2+}$ to
produce such synaptic enhancement are not known. The increases in presynaptic Ca$^{2+}$ associated with glycine-induced enhancement are too small (tens to hundreds of nanomolar (Turecek and Trussell, 2001; Awatramani et al., 2005)) to effectively activate synaptotagmin, the low affinity Ca$^{2+}$ sensor that mediates vesicle fusion (Schneeggenburger and Neher, 2005; Sudhof and Rothman, 2009). This suggests that an unidentified Ca$^{2+}$ sensor provides the link between presynaptic Ca$^{2+}$ elevations induced by glycine and enhanced vesicle fusion.

The Ca$^{2+}$-sensitive PKC isoforms, PKCα and PKCβ, are good candidates to mediate glycine-induced enhancement. They are present at the calyx of Held and they respond to modest Ca$^{2+}$ increases following tetanic stimulation (post-tetanic potentiation, PTP) to enhance evoked synaptic responses (Fioravante et al., 2011). Because PTP and glycine-induced synaptic enhancement both depend on small increases in presynaptic Ca$^{2+}$, we hypothesize that PKCα and PKCβ also mediate glycine-induced enhancement.

Here we test the involvement of PKCα and PKCβ in synaptic enhancement mediated by glycine at the calyx of Held synapse. By using PKCα and PKCβ knockout mice, and PKCαβ double knockout (dko) mice, we find that most of the glycine-induced enhancement of evoked synaptic responses is mediated by PKCα and PKCβ, but that these isoforms do not mediate the increase in mEPSC frequency following glycine application. Presynaptic Ca$^{2+}$ signaling is unaltered in dko mice as compared to wildtype animals, indicating that the effect of PKC$\text{ca}$ is downstream of Ca$^{2+}$ influx. Blocking glycine receptors with strychnine, however, prevents normal Ca$^{2+}$ increases in wildtype mice in response to glycine application. We find that glycine enhances synaptic transmission primarily by increasing the effective size of the pool of readily-releasable vesicles. Thus, by depolarizing the calyx of Held, glycine opens presynaptic Ca$^{2+}$ channels to activate PKCα and PKCβ and thus increases neurotransmitter release. Moreover, these findings establish that PKCα and PKCβ can play central roles in increasing synaptic strength following small presynaptic depolarizations.
Results

Our ultimate goal was to use knockout mice to study the mechanism of glycine-induced enhancement at the calyx of Held, but previously this form of synaptic enhancement had only been described in rats (Turecek and Trussell, 2001). We therefore examined the effect of glycine on transmission in the mouse, and found that it enhanced transmission at calyx of Held synapses, as shown in a representative experiment (Figure 3.1A). In this experiment, glycine increased the EPSC by about 1.5-fold, and this enhancement reversed when glycine was washed out of the slice. We found that glycine enhances synaptic transmission by 40 ± 4 % (n=23) in wildtype mice. Thus, in both rats and mice glycine enhances synaptic transmission at calyx of Held synapses.

Previous studies have shown that Ca$^{2+}$ acts presynaptically to enhance release in both glycine-induced enhancement and in PTP (Turecek and Trussell, 2001; Fioravante et al., 2011), raising the possibility that they could be mediated by the same Ca$^{2+}$-dependent target. If these forms of enhancement share a mechanism they should partially occlude each other. We determined whether this is the case by measuring the amount of PTP induced by tetanic stimulation (4 s, 100 Hz) under control conditions and in the presence of glycine. In control conditions there was a 92 ± 11 % (n=8) increase in the EPSC amplitude after tetanic stimulation. Glycine not only potentiated the synaptic response, but also reduced PTP to 54 ± 13 % (n=8) (Figure 3.1B). The reduction of the magnitude of PTP by glycine is consistent with the two forms of enhancement sharing a mechanism leading to occlusion, but saturation of release could also contribute to a reduction in PTP in the presence of glycine. We therefore repeated these experiments in 1.5 mM external Ca$^{2+}$ (Figure 3.1C), which decreases the initial EPSC by ~50%, and thereby greatly reduces the possibility that release is saturated. We found that under these conditions glycine significantly reduced the magnitude of PTP from 121 ± 19 % to 53 ± 11 % (n=6, Figure 3.1C, middle). The finding that glycine-induced potentiation
**Figure 3.1**

**Glycine-induced presynaptic enhancement partially occludes PTP.** (A) A representative experiment showing that the bath application of glycine reversibly enhances synaptic strength at the calyx of Held synapse in mice. Top traces depict the average baseline EPSC response (*control*), potentiated responses in glycine, and responses after washout of glycine. (B-C) Experiments were performed to assess whether the enhancement produced by glycine interacted with the enhancement of synaptic strength produced by tetanic stimulation (post-tetanic potentiation, PTP). The presynaptic calyceal terminal was stimulated at low frequency (0.2 Hz) to monitor synaptic strength, and the effect of tetanic stimulation (4 s, 100 Hz, inverted triangles) was assessed in control conditions and in the presence of glycine. (B, left) The time course of synaptic amplitudes is the average of 8 experiments performed in 2 mM Ca$_{\text{e}}$. EPSCs were normalized to the average EPSC recorded in 2 mM Ca$_{\text{e}}$ prior to tetanic stimulation. (B, middle) The synaptic enhancement produced by tetanic stimulation in (B, left) is shown for control conditions and in the presence of glycine. EPSCs are normalized to the average EPSC amplitude recorded prior to tetanic stimulation. (B, right) The effect of glycine on PTP is summarized for 8 individual experiments (open circles) and the average for each group (filled circles). (C) Similar experiments to (B) were performed but in 1.5 mM external Ca$^{2+}$ to decrease the initial release probability by approximately 50%, thereby allowing us to assess whether glycine reduced the amplitude of PTP by saturating the probability of release. In (C, left) EPSCs are normalized to the EPSC amplitude in 2 mM Ca$_{\text{e}}$. Results shown are the average of 6 cells. (*: p<0.05, paired t-test). Errors bars are SEM.
Figure 3.1 (continued)
partially occludes PTP, even for conditions under which release is not saturated, supports the hypothesis that glycine-induced plasticity and PTP act through similar presynaptic mechanisms.

**The involvement of PKCαβ in glycine-induced enhancement**

The observation that glycine partially occludes PTP, combined with our previous finding that Ca\(^{2+}\)-dependent PKC isoforms PKCα and PKCβ play a crucial role in PTP, suggested the hypothesis that PKCα and PKCβ could also mediate glycine-induced enhancement. We tested this hypothesis by studying wildtype and PKCαβ double knockout (dko) mice. Application of glycine enhanced evoked synaptic transmission in wildtype mice, and this enhancement was blocked by the ionotropic glycine receptor antagonist strychnine (Figure 3.2A). We found that this form of synaptic enhancement was significantly reduced in PKCαβ dko mice (p<0.002) (Figure 3.2B). The extent of glycine-induced enhancement was variable for all groups (Figure 3.2C). On average, glycine enhanced synaptic strength by 40 ± 4 % in wildtype mice (n = 23), by 25 ± 7 % in PKCα ko mice (n = 8), by 25 ± 6 % in PKCβ ko mice (n = 11), and by 10 ± 7 % (n = 13) in PKCαβ dko mice (Figure 3.2D). These results suggest that PKCα and PKCβ together mediate the bulk of glycine-dependent enhancement of evoked transmission at the calyx of Held, and that PKCα and PKCβ make comparable contributions.

Previous studies found that the glycine-induced increase in mEPSC frequency is prevented by blocking voltage-gated Ca\(^{2+}\) channels, suggesting that it is a consequence of elevated presynaptic Ca\(^{2+}\) entry leading to small increases in basal Ca\(^{2+}\) levels (Turecek and Trussell, 2001). Although increases in evoked transmission and mEPSC frequency are both produced by elevated presynaptic Ca\(^{2+}\) levels, it was not known if these two forms of synaptic enhancement were produced by the same molecular mechanism. We therefore tested whether, in addition to enhancing evoked release, Ca\(^{2+}\)-dependent PKCs mediate increases in mEPSC frequency. We found that glycine produced a significant increase in mEPSC frequency in wildtype mice (Figure 3.3A) and had a similar effect in PKCαβ dko mice (Figure 3.3B). Glycine
Figure 3.2

Calcium-dependent isoforms of PKC are necessary for glycine-induced presynaptic potentiation. (A and B), From representative experiments, top traces show the average baseline EPSC response (left), potentiated response in glycine (middle) and response with strychnine application (right). Bottom, Plots of normalized EPSC amplitudes as a function of time. At t = 0 s, glycine wash-in was initiated, eliciting potentiated EPSC amplitudes in wildtype controls (A) but not littermate PKCαβ double knockouts (B). (C), Cumulative histograms of normalized amplitudes of the potentiated response in glycine for wildtype (black), PKCα (green), PKCβ (red) and double knockout (purple) calyces. (D), Percent EPSC enhancement in glycine (± SEM) for wildtype and PKC knockout groups. EPSCs were normalized to baseline before determining the amount of synaptic enhancement (*, p<0.05).
Figure 3.2 (continued)
Figure 3.3

PKCα and PKCβ are not required for glycine-induced increases in spontaneous transmission. (A and B), Representative traces of spontaneous transmission before (control; top) and after (bottom) glycine application from wildtype (black) and PKCαβ double knockout (purple) slices. (C), Average mEPSC frequencies and mEPSC amplitudes recorded in the presence of glycine, normalized to the values measured prior to the application of glycine.
Figure 3.3 (continued)

A  wt
control

25 pA
2 s
glycine

B  α−/−β−/−
control

glycine

C  mEPSC Frequency (norm.)

0  1
wt α−/β+/− α−/+β−/− α−/−β−/−

mEPSC Amplitude (norm.)

0  1
wt α−/β+/− α−/+β−/− α−/−β−/−
increased mEPSC frequencies to similar extents in wildtype (2.9 ± 0.4 fold increase, n = 21), PKCα (2.8 ± 0.4 fold increase, n = 8), PKCβ (2.7 ± 0.6 fold increase, n = 10), and PKCαβ dko mice (2.8 ± 0.5 fold increase, n = 13; Figure 3.3C, left). The increases in mEPSC frequency were not significantly different among groups (p = 0.99) and glycine did not significantly alter the amplitudes of mEPSCs for all groups (Figure 3.3C, right, p = 0.85).

The lack of an effect of PKC Ca deletion on mEPSC enhancement by glycine is important in two ways. First, it suggests that different molecular mechanisms mediate glycine-induced enhancement of evoked and spontaneous release. Second, it shows that in PKCαβ dko mice, glycine still activates presynaptic glycine receptors and depolarizes the terminals sufficiently to open voltage-gated Ca²⁺ channels and enhance spontaneous synaptic transmission, suggesting that presynaptic glycine signaling is intact in dko animals.

Presynaptic Calcium Signaling

We explicitly tested whether glycine signaling is intact in PKCαβ dko mice by measuring the effects of glycine on basal Ca²⁺ levels and stimulus-evoked Ca²⁺ signals. We used dextran-conjugated Ca²⁺ indicators and 2-photon microscopy to measure presynaptic Ca²⁺ influx and basal Ca²⁺ concentrations (Fioravante et al., 2011). In wildtype mice, glycine elevated basal presynaptic Ca²⁺ at the calyx of Held by 56 ± 13 nM (Figure 3.4A, left; B, C), and these Ca²⁺ increases were prevented by strychnine (4.5 ± 3.8 nM) (Figure 3.4A, middle; B, C). This was shown previously for rats (Turecek and Trussell, 2001). We also found that Ca²⁺ increases evoked by single stimuli ([Ca]ᵢ) were not significantly altered by glycine in wildtype mice ([Ca]ᵢ increased by 4.4 ± 2.5 nM, n = 7, p = 0.22; Figure 3.4A, left, C, right) and in wildtype mice in the presence of strychnine [Ca]ᵢ increased by 3.1 ± 3.7 nM (n = 4, p = 0.21; Figure 3.4A, middle; C, right). If Ca²⁺ signals were the same in wildtype and dko mice, it would indicate that glycine receptors are functional in the presynaptic terminals of dko mice, and when activated they continue to depolarize the calyx and elevate Ca²⁺; if not, it would suggest that PKCαβ act
Figure 3.4

Glycine elevates presynaptic calcium levels to a similar extent in wildtype and PKCαβ double knockout animals. The effects of glycine on the resting Ca\(^{2+}\) concentration and on Ca\(^{2+}\) transients following single stimuli at the calyx of Held were measured for wildtype mice (black), wildtype mice in the presence of the glycine receptor antagonist strychnine (blue), and for PKCαβ double knockout (purple) mice. Representative Ca\(^{2+}\) signals from individual experiments (A), the average time courses of glycine on the resting presynaptic Ca\(^{2+}\) concentration (B), and the average effect of glycine on the resting Ca\(^{2+}\) concentration and the amplitude of presynaptic Ca\(^{2+}\) signals evoked by single stimuli (C) are shown.
Figure 3.4 (continued)
upstream of presynaptic Ca\textsuperscript{2+} signaling. We found that in PKCαβ dko mice, glycine elevated basal Ca\textsuperscript{2+} levels to the same degree as in wildtype mice (53 ± 7 nM; n= 8; p = 0.51; Figure 3.4A, right; B, C) and that [Ca]\textsubscript{i} was not significantly altered by glycine (an increase of 4.2 ± 4.1 nM, n = 8; p = 0.27; Figure 3.4A, right; C, right). The consistent lack of an effect of glycine on [Ca]\textsubscript{i} indicates that glycine does not significantly alter stimulus-evoked Ca\textsuperscript{2+} entry. These findings indicate that glycine elevates basal Ca\textsuperscript{2+} in dko mice and suggest that the reduced ability of glycine to enhance evoked transmission must occur downstream of the elevation in presynaptic Ca\textsuperscript{2+} concentration.

**Mechanisms of Glycine-Induced Enhancement**

To determine whether changes in \( p \) contribute to the glycine-induced enhancement observed under our experimental conditions, we examined the effects of glycine on paired-pulse ratio (PPR=EPSC\textsubscript{2}/EPSC\textsubscript{1}) (Figure 3.5). These experiments were conducted in the presence of cyclothiazide (CTZ) and kynurenate (Kyn) to minimize AMPA receptor desensitization and saturation, respectively. Glycine did not significantly alter PPR in wildtype mice (PPR\textsubscript{glycine}/PPR\textsubscript{control} = 0.96 ± 0.03, n = 13, p = 0.44; Figure 3.5, black) suggesting that glycine did not change \( p \). We tested the sensitivity of PPR to changes in \( p \) by assessing the effect of elevating external Ca\textsuperscript{2+} from 2 mM to 3 mM. This manipulation produced a synaptic enhancement similar in amplitude to glycine and significantly decreased PPR (PPR\textsubscript{3Ca}/PPR\textsubscript{control} = 0.85 ± 0.01, n = 6, p <0.05; Figure 3.5, red). Glycine did not alter PPR in dko mice (PPR\textsubscript{glycine}/PPR\textsubscript{control} = 0.97 ± 0.02, n = 15, p = 0.87; Figure 3.5, purple), and in wildtype mice in the presence of strychnine (PPR\textsubscript{glycine}/PPR\textsubscript{control} = 0.99 ± 0.03, n = 4, p = 0.98; Figure 3.5, blue). These findings suggest that increases in \( p \) do not make large contributions to glycine-induced enhancement in mice.

If synaptic enhancement is not mediated by increases in \( p \), then the effective size of the readily releasable pool must be increased. We therefore measured the effective pool size by
**Figure 3.5**

Glycine-induced synaptic potentiation is not accompanied by an increase in paired-pulse ratio (PPR).  **A.** Representative traces from the four groups show superimposed EPSCs measured in two different conditions, with traces normalized to the first EPSCs.  **B.** The effects of the indicated experimental manipulations on PPR are summarized by plotting the ratio of the PPR measured in the indicated condition to the PPR measured under control conditions.
Figure 3.5 (continued)
using high-frequency trains (100 Hz, 0.4 s) to evoke a series of EPSCs in the presence of CTZ and Kyn. We calculated the cumulative EPSC and determined the effective pool size (RRP$_{\text{train}}$) by fitting the last 15 points and back-extrapolating to the y-axis to correct for replenishment of the readily-releasable pool (Figure 3.6A). In this method, the probability of release in response to the first stimulus was determined by $p_{\text{train}} = \text{EPSC}/\text{RRP}_{\text{train}}$ (Schneggenburger et al., 1999). In wildtype mice, glycine increased the EPSC size (57 ± 10 %, n = 13) primarily by increasing RRP$_{\text{train}}$ (increased by 44 ± 8%), and only slightly increasing $p_{\text{train}}$ (increased by 5.8 ± 3.5%) (Figure 3.6B, black). We compared the effects of glycine on synaptic transmission to the effects of raising the external Ca$^{2+}$ concentration from 2 mM to 3 mM and found that increasing the external Ca$^{2+}$ concentration elevated synaptic strength by 48 ± 7 % (n = 6), primarily by increasing $p_{\text{train}}$ (39 ± 6 %), with only a small contribution from the effective pool size (13 ± 8 %) (Figure 3.6B, red). The increases in synaptic strength (10 ± 3 %, n = 17) and RRP$_{\text{train}}$ (8 ± 3 %) by glycine were largely eliminated in PKCαβ dko mice (Figure 3.6B, purple) and in the presence of strychnine (6 ± 5 % increase in EPSC; 3.8 ± 0.8% increase in RRP$_{\text{train}}$) (Figure 3.6B, blue).

We further tested the contributions of different mechanisms to glycine-mediated enhancement by using an alternate method to estimate effective pool size and $p$ that was introduced by Elmqvist and Quastel (Elmqvist and Quastel, 1965) (Figure 3.6C). In this approach, the reduction in the amplitudes of EPSCs during a stimulus train is attributed to vesicle depletion, and the decrease in amplitude of the first few EPSCs in the train is used to estimate the effective pool size (RRP$_{\text{EQ}}$). Although this approach and that described above both rely on the use of stimulus trains, the Elmqvist and Quastel method uses the initial EPSCs in the train and provides an upper-bound estimate of the effective pool size because it ignores the contribution of replenishment of the readily-releasable pool from reserve pools during the first few EPSCs to the measured RRP size. In contrast, the cumulative method used previously relies on the synaptic responses measured late in the train and provides a lower-bound estimate.
Figure 3.6

Glycine-induced synaptic potentiation occurs primarily by increases in the effective pool size. Calyces were stimulated with a 100 Hz, 40-pulse train in the presence of CTZ and kynurenic, and two methods were used to determine the mechanism by which glycine enhances synaptic transmission. (A) In the first approach the cumulative EPSC was plotted against stimulus number, where back-extrapolation using a linear fit of the last 15 points to the y-intercept yielded the $RRP_{\text{train}}$ as an estimate of the effect pool size. (B) This method was used to determine the effect of the indicated manipulations on EPSC amplitude, $RRP_{\text{train}}$ and $p_{\text{train}}$. Representative experiments are shown above and summary data are shown below for the indicated conditions. (C) The same experiments were reanalyzed using an approach introduced by Elmqvist and Quastel. In this approach the reduction in the amplitude of the EPSC is attributed to depletion and is used to estimate $RRP_{\text{EQ}}$. (D) Representative experiments are shown above and summary data are shown below for the indicated conditions. Paired t-tests were used to compare the EPSC, $RRP_{\text{train}}$, $p_{\text{train}}$, $RRP_{\text{EQ}}$ and $p_{\text{EQ}}$ amplitudes in control and in the presence of either glycine or elevated external Ca$^{2+}$, as indicated (*: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$). All other values were not statistically significant.
Figure 3.6 (continued)

A. Cumulative EPSC (nA) vs Stimulus #

B. Cumulative EPSC (nA) for different treatments:
- **wt**
- **wt + strychnine**
- **α+/β−−**

C. EPSC (nA) vs Cumulative EPSC (nA)

D. Cumulative EPSC (nA) for different treatments:
- **wt**
- **α+/β−−**
- **α−/β−−**

% increase in EPSC for different conditions:
- **wt**
- **wt + strychnine**
- **α+/β−−**
- **α−/β−−**
of the effective pool size because it assumes a maximal steady-state replenishment rate throughout the train and probably over estimates recovery at the beginning of the train (see Discussion). As expected, the estimates of pool size with the Elmqvist and Quastel method are consistently higher than those obtained with the cumulative method. According to the Elmqvist and Quastel method, glycine enhances synaptic transmission in wildtype mice largely by increasing $\text{RRP}_{\text{EQ}} (35 \pm 8 \%)$, although small increases in $p_{\text{EQ}} (8.2 \pm 2.1\%)$ also contribute (Figure 3.6D, black). Increases in external $\text{Ca}^{2+}$ enhance synaptic strength exclusively by increasing $p_{\text{EQ}} (47 \pm 9 \%)$ rather than increasing $\text{RRP}_{\text{EQ}} (1.3 \pm 2.2\%;$ Figure 3.6D, red). Finally, only very small increases in EPSC size (10 ± 3 %), $\text{RRP}_{\text{EQ}} (8 \pm 3\%)$ and $p_{\text{EQ}} (3.3 \pm 2.5\%)$ were observed in PKCαβ dko mice (Figure 3.6D, purple) and in wildtype mice treated with strychnine (EPSC: 6 ± 5 %; $\text{RRP}_{\text{EQ}}: 4 \pm 1 \%, p_{\text{EQ}}: 6 \pm 7\%;$ Figure 3.6D, blue). Thus we find that glycine-induced increases in EPSC size are produced primarily by an increase in the effective pool size.

The use of a one-way ANOVA for wildtype, PKCαβ dko, and strychnine- treated wildtype mice revealed that the glycine-induced increase in EPSC amplitude ($p<0.01$), $\text{RRP}_{\text{train}} (p<0.01)$ and $\text{RRP}_{\text{EQ}} (p<0.01)$ were all reduced in dko mice compared to control. Similarly the enhancement of EPSC amplitude ($p<0.05$), $\text{RRP}_{\text{train}} (p<0.01)$ and $\text{RRP}_{\text{EQ}} (p<0.01)$ were all reduced in strychnine-treated wildtype mice compared to control mice. There was no significant difference between $p_{\text{train}}$ and $p_{\text{EQ}}$ among groups, and no significant difference in any synaptic properties among PKCαβ dko and strychnine- treated wildtype mice.

Previous studies of glycine-induced enhancement performed in rats reported that glycine decreases the magnitude of paired-pulse plasticity (Turecek and Trussell, 2001), suggesting that glycine increases $p$. Moreover, it was found that glycine does not alter the overall size of the pool of vesicles released by a large presynaptic voltage step (Turecek and Trussell, 2001). These findings suggested that in rats, increases in $p$ make a prominent contribution to glycine-induced enhancement. We therefore re-examined the mechanism of glycine-induced
Figure 3.7

Glycine-induced synaptic potentiation in rats primarily reflects an increase in the effective pool size, but increases in the probability of release also contribute. Experiments were conducted in the presence of CTZ and kynurenic acid to assess the mechanism of glycine-induced enhancement in rats (P11-P14), where previous studies have suggested that increases in $p$ contribute to glycine-induced enhancement. Glycine led to an average synaptic enhancement of $45 \pm 4\%$ ($n = 6$). (A) Comparison of pairs of EPSCs recorded in control conditions and in the presence of glycine (the EPSCs in glycine were normalized to the amplitude of the first EPSC in control) showed that glycine decreased the paired-pulse ratio. Experiments with stimulus trains (40 pulses, 100 Hz) were used to determine the contributions of $p$ and the effective RRP to glycine-induced enhancement using the cumulative EPSC method (B) and the Elmqvist and Quastel method (C). (D) The effects of glycine on synaptic properties are summarized ($n = 6$, ± SEM). In these experiments glycine decreased PPR in rats by $12 \pm 3\%$. The use of trains to determine the contribution of effective RRP and $p$ revealed that glycine produced small increases in $p$ ($p_{\text{train}}: 15 \pm 4\%; p_{\text{EQ}}: 17 \pm 4\%, n=6$), but produced larger increases in effective RRP ($\text{RRP}_{\text{train}}: 26 \pm 2\%; \text{RRP}_{\text{EQ}}: 24 \pm 3\%, n=6$). Thus, in rats, glycine increases $p$ in addition to increasing the effective pool size, but as in mice, increased neurotransmitter release is mainly due to an increase in the effective pool size.
Figure 3.7 (continued)
enhancement in rats and found that glycine decreased PPR (Figure 3.7A), which is consistent with glycine increasing \( p \) in rats. The use of trains to determine the contribution of effective RRP and \( p \) revealed that glycine increases \( p \) but produces larger increases in effective RRP (Figure 3.7B-D). This was found to be the case for both the cumulative EPSC method and the Elmqvist and Quastel method. Thus, for both rats and mice, two distinct methods of measuring pool size and \( p \) support the conclusion that glycine enhances transmission primarily by increasing the effective pool size rather than by increasing \( p \), although in rat there is also an appreciable contribution from increases in \( p \).

**Discussion**

Our primary finding is that glycine-induced enhancement of evoked synaptic currents is significantly reduced in mice lacking PK\( \text{C}\alpha \) and PK\( \text{C}\beta \), indicating that these Ca\(^{2+}\)-dependent PKC isoforms mediate the bulk of this form of enhancement at the calyx of Held synapse. In contrast, Ca\(^{2+}\)-dependent PKCs are not required for glycine-induced increases in mEPSC frequency, indicating that spontaneous and evoked release are differentially regulated. Activated Ca\(^{2+}\)-dependent PKCs enhance transmitter release primarily by increasing the effective pool size, with only a modest increase in the probability of vesicle release.

**Glycine-induced enhancement is mediated primarily by PK\( \text{C}\alpha \) and PK\( \text{C}\beta \)**

Previous studies of glycine-induced enhancement have shown that the opening of chloride channels, and activation of persistent sodium channels, depolarizes the calyx of Held sufficiently to activate a small fraction of P-type Ca\(^{2+}\) channels (Turecek and Trussell, 2001; Price and Trussell, 2006; Huang and Trussell, 2008). Although glycine receptor activation drives chloride efflux, the observation that glycine-induced synaptic enhancement can be mimicked by small sustained presynaptic depolarizations suggests that changes in presynaptic chloride levels are not important in this form of plasticity (Turecek and Trussell, 2001). Previous studies
have shown, however, that introducing the Ca\(^{2+}\) chelator EGTA into the calyx of Held abolished synaptic enhancement caused by glycine, indicating that Ca\(^{2+}\) increases are crucial to this form of plasticity (Turecek and Trussell, 2001).

Our findings indicate that PKC\(\alpha\) and PKC\(\beta\) are required to allow glycine-induced presynaptic Ca\(^{2+}\) increases to enhance evoked neurotransmitter release. Based on our observation that glycine continues to elevate presynaptic Ca\(^{2+}\) levels and increase mEPSC frequency at the calyx of Held synapse in PKC\(\alpha\)\(\beta\) dko mice, we conclude that PKC\(\alpha\) and PKC\(\beta\) act downstream from increases in Ca\(^{2+}\) entry.

The involvement of Ca\(^{2+}\)-dependent PKC isoforms in glycine-induced synaptic potentiation raises the possibility that these isoforms are directly activated by elevated presynaptic Ca\(^{2+}\) levels to produce synaptic enhancement. Although the Ca\(^{2+}\)-binding affinities for PKC\(\alpha\) and PKC\(\beta\) (~40 \(\mu\)M (Kohout et al., 2002)) are much higher than the average increase in presynaptic Ca\(^{2+}\) levels evoked by glycine (50-100 nM), other intracellular factors can raise the binding affinity of PKCs for Ca\(^{2+}\) to 0.1-5 \(\mu\)M or less (Corbalan-Garcia et al., 1999; Kohout et al., 2002; Corbin et al., 2007; Guerrero-Valero et al., 2007), a range that would allow PKC\(\alpha\) and PKC\(\beta\) to respond to the Ca\(^{2+}\) increases produced by glycine.

**The Mechanism of Synaptic Enhancement**

Several lines of evidence suggest that glycine increases synaptic transmission primarily by increasing the effective size of the readily-releasable pool of vesicles. First, despite the fact that the application of glycine and increasing Ca\(_e\) to 3 mM produced the same synaptic enhancement, glycine did not affect paired-pulse plasticity whereas alterations in external Ca\(^{2+}\) decreased PPR in a manner that is consistent with an increase in \(\rho\). The lack of an effect of glycine on paired-pulse plasticity suggests that glycine does not alter the initial \(\rho\).

Moreover, plots of the cumulative EPSC as a function of stimulus number revealed that glycine increased the effective size of the readily-releasable pool. Both the synaptic
enhancement and the increase in the effective pool size were eliminated when ionotropic glycine receptors were blocked pharmacologically and in PKCαβ dko mice. There are challenges associated with measuring effective pool size and $p$. For example, determining the effective pool size using the cumulative EPSC is complicated by replenishment of the pool during stimulation (Schneggenburger et al., 1999) (Figure 3.6). Extrapolation corrects for replenishment during the train, but assumes that the rate of replenishment is constant throughout the train. This cumulative EPSC method provides a lower-bound estimate of the effective pool size because the assumed rate of replenishment is calculated from the steady-state rate of replenishment at the end of the train, which is likely higher than the initial rate of replenishment.

Finally, we also estimated the effective pool size and $p$ with a method introduced by Elmqvist and Quastel (Elmqvist and Quastel, 1965). With this approach the progressive decrease in the amplitude of EPSCs during a stimulus train is attributed to vesicle depletion, and the decreases in amplitude of the initial EPSCs in the train can be used to estimate the effective pool size. Because this approach assumes there is no replenishment, it serves as an upper-bound estimate of the effective pool size. As expected, the estimates of pool size with this approach were consistently higher than those obtained with the cumulative method. Nonetheless, this approach also indicated that glycine increased synaptic strength primarily by increasing the effective pool size, and that synaptic enhancement and the increase in effective pool size were eliminated in dko mice, whereas increasing extracellular $\text{Ca}^{2+}$ increased synaptic strength primarily by increasing $p$. These results suggest that in mice, glycine increases EPSC size largely by increasing the effective size of the RRP.

A comparison of PTP and glycine-induced enhancement

Glycine-induced enhancement and PTP share many features. Both forms of plasticity are mediated by an increase in presynaptic $\text{Ca}^{2+}$ levels (Turecek and Trussell, 2001; Kim et al.,
2005; Korogod et al., 2007) and by two Ca$^{2+}$-sensitive isoforms of PKC, PKC$\alpha$ and PKC$\beta$ (Fioravante et al., 2011) (Figure 3.1). In addition, increases in mEPSC frequency following either tetanic stimulation (Habets and Borst, 2005; Kim et al., 2005; Korogod et al., 2007) or the application of glycine were mediated by Ca$^{2+}$ increases (Zucker and Lara-Estrella, 1983; Bao et al., 1997; Kim et al., 2005), but did not involve PKC$\alpha$ and PKC$\beta$ (Fioravante et al., 2011). Thus, in both cases spontaneous vesicle fusion and evoked release are differentially regulated, and other Ca$^{2+}$-sensitive proteins must mediate enhancement of mEPSC frequency (Groffen et al., 2010; Pang et al., 2011; Yao et al., 2011). In addition, PKC$\alpha$ and PKC$\beta$ enhance neurotransmitter release primarily by increasing the effective size of the readily-releasable pool for both forms of synaptic enhancement (Fioravante et al., 2011) (Figure 3.6). It is likely that once activated, PKC$\alpha$ and PKC$\beta$ phosphorylate the same target, possibly Munc 18-1 (Wierda et al., 2007), to produce this increase in effective RRP size.

There are also several interesting differences between PTP and glycine-induced enhancement. Notably, PTP tetanic stimulation increases mEPSC size by 10 – 20 %, and this contributes to synaptic enhancement (He et al., 2009; Fioravante et al., 2011), but in glycine-induced enhancement no such increase in mEPSC size is observed. This suggests that the high Ca$^{2+}$ levels evoked by tetanic stimulation are needed to increase mEPSC size. In addition, PKC$\alpha$ and PKC$\beta$ are differentially effective in these two phenomena. PKC$\beta$ makes a much larger contribution to PTP than PKC$\alpha$ (Fioravante et al., 2011), whereas PKC$\alpha$ and PKC$\beta$ make comparable contributions to glycine-induced enhancement. The different Ca$^{2+}$ signals that induce these forms of plasticity may underlie the differential contributions of these isoforms.

PTP is induced by a presynaptic Ca$^{2+}$ signal that decays more rapidly than PTP (Regehr et al., 1994; Brager et al., 2003; Beierlein et al., 2007; Fioravante et al., 2011). At the calyx of Held, tetanic stimulation increases presynaptic Ca$^{2+}$ by several hundred nanomolar and it decays to resting levels with a time constant of ~22 s, compared to synaptic enhancement that decays with a time constant of ~45 s (Fioravante et al., 2011). For glycine-induced enhancement the
opening of voltage gated Ca\textsuperscript{2+} channels leads to a sustained elevation of residual Ca\textsuperscript{2+} signal by ~50 nM, and larger local Ca\textsuperscript{2+} increases near open Ca\textsuperscript{2+} channels. Together these findings indicate that despite being similar in many ways, PKC\textalpha and PKC\textbeta can make differential contributions to synaptic plasticity.

**Implications for Related Forms of Synaptic Plasticity**

Related forms of synaptic plasticity have been described at a number of synapses. For example, somatic depolarization can lead to depolarization of presynaptic boutons that can increase neurotransmitter release. This has been described at cortical (Sjostrom et al., 2003; Corlew et al., 2007; Christie and Jahr, 2009), hippocampal (Vogt and Regehr, 2001), and cerebellar (Glitsch and Marty, 1999) synapses. These forms of plasticity are similar to glycine-mediated modulation, which can be effectively mimicked with small steady presynaptic depolarizations (Awatramani et al., 2005). The similarity of these forms of plasticity to glycine-induced modulation raises the possibility that Ca\textsuperscript{2+}-dependent PKCs could also contribute to synaptic plasticity arising from somatic depolarization.

**Materials and Methods**

**Animals**

PKC\textalpha and PKC\textbeta single knockout animals, and PKC\textalpha\textbeta double knockout mice were generated as described previously (Leitges et al., 1996; Leitges et al., 2002). All animal handling and procedures were completed in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.

**Brain Slices and Electrophysiology**

Postnatal day P11-14 mice were deeply anesthetized with isoflurane and transverse 190 \mu m-thick brainstem slices containing the medial nucleus of the trapezoid body (MNTB) region...
were cut using a vibratome slicer (VT1000S, Leica). Brains were dissected and cut at 4 °C in slicing solution containing (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 0.1 CaCl₂, 3 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O₂/5% CO₂ (pH 7.4). Slices were incubated at 32 °C for 30 min in a solution consisting of (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O₂/5% CO₂ (pH 7.4). After incubation, slices were transferred to a recording chamber at room temperature. All recordings were made using an upright microscope (Olympus, Center Valley, PA) with a 60x, 0.9 N.A. objective.

Slices were perfused at 2-3 ml/min during the recording with a standard perfusion solution that contained 25 μM bicuculline and 2 μm CGP 55845 [(2S)-3-[(15)-1-(2,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)-phosphinic acid] to block transmission mediated by GABA_A and GABA_B receptors, respectively. Whole-cell postsynaptic patch-clamp recordings were made from visually identified MNTB principle cells using glass pipettes with 2-3 MΩ resistance, filled with internal solution containing (in mM): 135 CsF, 5 CsCl, 5 EGTA, 10 HEPES, 2 QX314 (N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride), 1 DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate) (300 mOsm) at pH 7.4 with CsOH. Whole-cell recordings were compensated electronically by 70% and voltage clamped to -60 mV.

Excitatory postsynaptic currents (EPSCs) were evoked by stimulating presynaptic axons with a custom-made bipolar stimulating electrode placed midway between the midline of the brainstem and the medial border of the MNTB. Multiclamp 700A and 700B (Axon Instruments/Molecular Devices, Union City, CA) amplifiers were used. All recordings were digitized at 20 kHz with an ITC-18 A/D converter (Instrutech, Port Washington, NY) using custom macros (written by M.A. Xu-Friedman) in Igor Pro (Wavemetrics, Lake Oswego, OR)
and filtered at 8 kHz. The access resistance and leak current were monitored, and experiments were rejected if either of these parameters changed significantly.

Glycine-induced changes in EPSC and the probability of release \((p)\) were measured by first recording the baseline synaptic strength with paired stimuli (20 ms interval) repeated every 20 s for 3-5 min followed by wash-in of 1 mM glycine with or without 1 μM strychnine. Changes in miniature EPSCs (mEPSCs) were measured by recording spontaneous activity for 10 s before each paired-pulse stimuli. The size of the readily releasable pool (RRP) and \(p\) were estimated via two methods. First, RRP due to a train of stimuli (RRP\(_{\text{train}}\)) elicited at 100 Hz, 0.4 s was obtained by plotting the cumulative EPSC as a function of the stimulus number (Schneggenburger et al., 1999); back-extrapolation to the y-axis using a linear fit of the last 15 points yielded the \(\Sigma\)EPSC\(_0\). The fraction of the RRP\(_{\text{train}}\) released by the first stimulus (\(p\)) was calculated by dividing the first EPSC by the \(\Sigma\)EPSC\(_0\). The second method, referred to as the RRP\(_{\text{EQ}}\) method (Elmqvist and Quastel, 1965), involved plotting the EPSC amplitude as a function of the cumulative EPSC for the synaptic response obtained with each 100 Hz, 0.4 s train. Extrapolation to the x-axis using a linear fit of the first 7 points in the plot yielded the \(\Sigma\)EPSC\(_0\) as an estimate of the RRP, as previously described by Elmqvist and Quastel (Elmqvist and Quastel, 1965).

**Presynaptic Calcium Measurements**

Presynaptic calyceal terminals were loaded for 5 min with 0.025%-0.1% Alexa 594 dextran and 0.5% Calcium Green-1 dextran (10 kDa, Invitrogen, Carlsbad, CA) and incubated at 32 °C for 30 min before imaging with a 2-photon microscope as previously described (Beierlein et al., 2004; Fioravante et al., 2011). Fluorescence signals from calyces were converted to calcium by determining the \(F_{\text{max}}/F_{\text{min}}\) ratio (4.8) in a blank pipette and by determining \(F_{\text{max}}\) using high frequency stimulation as previously described (Maravall et al., 2000). Calyces that
exhibited bright green fluorescence at rest were found to be unhealthy for further study because they either had elevated resting Ca\(^{2+}\) levels or were overloaded with Ca\(^{2+}\) indicator.

**Data Analysis**

All data analyses were performed using macros written in Igor Pro (Wavemetrics). Glycine-induced synaptic changes were calculated as the ratio of EPSC amplitude 3-5 min after wash-in of 1 mM glycine over the average baseline. mEPSCs were detected as described previously (Fioravante et al., 2011). Statistical analyses were performed using one-way ANOVA tests for multiple group comparisons followed by Tukey post-hoc analysis. Student’s paired t-tests were used for pairwise comparisons. Level of significance was set at p < 0.05.

**References**


Chapter 4

Calcium-dependent PKC isoforms have specialized roles in short-term synaptic plasticity

YunXiang Chu*, Diasynou Fioravante*, Michael Leitges and Wade G. Regehr

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Author contributions:

Y.X.C. and D.F. contributed equally to this work by conducting all experiments and analysis.

M.L. provided reagents and technical assistance. Y.X.C., D.F., and W.G.R. wrote the paper.
Abstract

Post-tetanic 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 Ca$^{2+}$-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 post-tetanic potentiation (PTP), a form of short-term plasticity lasting tens of seconds to minutes following 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 $Ca^{2+}$, 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 (P)11-14 animals PTP is mediated primarily by PKCβ (Fioravante et al., 2011), one of the “classical” Ca\textsuperscript{2+}-dependent isoforms (PKCα, PKCβ and PKCγ, as opposed to the many Ca\textsuperscript{2+}-insensitive “novel” and “atypical” isoforms (Newton, 2001; Newton, 1995; 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; Habets and Borst, 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 Ca\textsuperscript{2+}-dependent PKC isoforms. We find that PKCγ produces PTP by increasing p before hearing onset, and that PKCβ produces PTP by increasing RRP afterwards. In pre-hearing 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 Ca\textsuperscript{2+}-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 (P8-10) and after (P16-19) the onset of hearing (~P12) (Sonntag et al., 2009; Sonntag et al., 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 \( \text{Ca}^{2+} \), and high \( p \) synapses depress, likely 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 (\( \Delta t = 10 \text{ ms} \)) every 5 s prior to and following tetanic stimulation (4s, 100 Hz) (Figure 4.1A, top). On average there was a significant reduction in PPR (-32 ± 3%; n=6; \( p<0.01 \)) in P8-10 animals (Figure 4.1A, bottom) during PTP (Figure 4.1A, middle). In P16-19 animals, even though the basal properties of synaptic transmission were different from P8-10 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 P8-10 animals (Supplemental Table S1). However, only a minimal reduction in PPR accompanied PTP (-3.1±1.3%; \( p=0.36 \)) in P16-19 animals (n=8) (Figure 4.1B). These findings suggest that changes in \( p \) make a larger contribution to PTP in P8-10 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 seconds later (at the peak of PTP), are compared. Such an approach is illustrated for pre-hearing and hearing animals.
Figure 4.1

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 seconds 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 P8-10 animals (A) and P16-19 animals (B). Representative traces show the average baseline paired-pulse EPSC (A, B, top left, gray), the potentiated response during the peak of PTP (A, B, top middle, black), and traces normalized to the first EPSCs (A, B, top right). The EPSC amplitudes (A, B, middle) and the PPR are plotted as a function of time (A, B, bottom). Plots are mean ± SEM.
Figure 4.1 (continued)
(Figure 4.2). The amplitudes of the 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 ($\text{RRP}_{\text{train}}$). 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 ($\text{RRP}_{\text{trainC}}$) (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., 2002; Taschenberger et al., 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 ($\text{RRP}_{\text{EQ}}$). The amplitudes of the EPSCs were then used to determine the amount of synaptic enhancement, and $p$ was computed from the equation $\text{EPSC} = \text{RRP} \ast p$. 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 ($7.5 \pm 6.5\%$, $n=12$ in P8-10 wildtype animals; $6.4 \pm 6.6\%$, $n=10$ in P16-19 wildtype mice for the interval 6-16 s post-tetanus).
We used all three of these approaches to determine the contributions of RRP and p to PTP in P8-10 animals (Figure 4.2A-D). 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 4.2A, top) and during a second conditioning train 10 seconds after the end of initial train (Figure 4.2A, bottom) are shown for a representative experiment (Figure 4.2A-C). As shown in this example, the responses to both trains showed prominent use-dependent depression, pool size increases were small (RRP$_{\text{train C}}$ 4%, RRP$_{\text{EQ}}$ 2%) and the enhancement of the EPSC was primarily a consequence of an increase in p ($p_{\text{train C}}$ 41%, $p_{\text{EQ}}$ 45%). On average, in P8-10 wildtype animals PTP was 67 ± 16 %, RRP$_{\text{train C}}$ 18 ± 6%, RRP$_{\text{EQ}}$ 11 ± 5%, $p_{\text{train C}}$ 42 ± 12%, and $p_{\text{EQ}}$ 52 ± 15% (Figure 4.2D, Supplemental Table S2).

We used the same approaches to determine the contributions of RRP and p to PTP in P16-19 animals (Figure 4.2E-H). In comparison to the responses observed in pre-hearing animals the initial EPSCs were larger, the depression was less pronounced, and the steady-state EPSCs were larger (Figure 4.2E, Supplemental Figure S4). The magnitude of PTP was comparable to that observed in pre-hearing 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$_{\text{train C}}$ 57%, RRP$_{\text{EQ}}$ 52%; Figure 4.2F,G). 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 P16-19 animals (Figure 4.2H, Supplemental 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 kynurenicate were included in the bath (Fucile et al., 2006; Taschenberger et al., 2002). Under these conditions there was a significant reduction in PPR in
Figure 4.2
Assessing the contributions of $p$ and RRP to PTP before (A-D) and after (E-H) hearing onset. Mechanisms of PTP were examined using trains in the presence of kynurenate and CTZ to prevent receptor saturation and desensitization. (A, E) Synaptic currents evoked by the first 40 stimuli of 4s, 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. The change in pool size and $p$ were determined using the cumulative EPSC method, the corrected EPSC method (B, F) and the EQ method (C, G). (D, 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, $\text{RRP}_{\text{train}}, \text{RRP}_{\text{trainC}}, \text{RRP}_{\text{EQ}}$, and $p_{\text{EQ}}$ during PTP to baseline, as indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Plots are mean ± SEM.
Figure 4.2 (continued)
P8-10 mice (−42 ± 8 %, n=10, p<0.05) and in P16-19 mice (−14 ± 5%, n=8, p<0.05) but the reduction was larger in pre-hearing mice (p<0.05).

**Contributions of different PKC isoforms to PTP**

To elucidate the mechanisms underlying PTP in pre-hearing 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 (**Supplemental Figure S4**). These data suggest that the basal properties of synaptic transmission among wildtype and knockout groups are not different within each age group of animals. For a subset of experimental conditions we also 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 (**Supplemental Figure S4 and Table S2**). We found that cyclothiazide and kynurenate did not significantly alter the amplitude of PTP (**Supplemental Tables S1 and S2**). For the remainder of the paper we also determined RRP and p by the three methods shown in **Figure 4.2**, 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 P11-14 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 pre-hearing 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 pre-hearing animals PTP is not mediated by PKCβ.
The role of PKC isoforms in pre-hearing and hearing animals was assessed with knockout animals and with an isoform-specific inhibitor. Whereas PTP was greatly reduced in P16-19 PKCαβ knockout animals (8.3 ± 6.4%; p<0.05; Figure 4.3A), it was largely intact in pre-hearing PKCαβ knockout animals (40 ± 9.0%; p=0.15; Figure 4.3B). 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_m values 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 P16-19 animals (Figure 4.3C). Intriguingly, in P8-10 wildtype animals the PKCβ inhibitor did not attenuate PTP (59 ± 15%; n=10; p=0.97; Figure 4.3D). Moreover, the inhibitor did not disrupt PTP in P8-10 PKCαβ ko mice (54 ± 15%; n=3; p=0.50; Figure 4.3G) suggesting that it does not have off-target effects. These findings indicate that PTP is dependent on PKCβ in hearing animals, but not in pre-hearing animals.

We went on to examine the contribution of p and RRP to synaptic enhancement in pre-hearing 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 wildtype animals (Figure 4.2D) is absent (p_trainC 45 ± 8%, n=9; Figure 4.3E, Supplemental Figure S5A). For wildtype animals in the presence of a PKCβ inhibitor there was a small but non-significant increase in RRP, and PTP was mediated by an increase in p (p_trainC 47 ± 16%, n=14; Figure 4.3F, Supplemental Figure S5B).
Figure 4.3

Age-dependent differences in the roles of calcium-dependent PKC isoforms in PTP. PTP as a function of time for P16-19 animals (A, C) and P8-10 animals (B, D, G, H). (A, B) PTP from wildtype and PKCαβ dko animals are compared. (C, D) The effects of the PKCβ inhibitor (Calbiochem 539654) on PTP in wildtype animals are shown for the corresponding age groups. (E, F) Summary plots of RRP and p contributions to PTP in pre-hearing animals. Experiments were performed in the presence of CTZ and kynurenate as shown in Supplementary Figure S2. (G, H) The effects of the PKCβ inhibitor (G) and a broad spectrum PKC inhibitor (GF109203X) (H) on PTP in P8-10 PKCβ dko animals are shown. *p<0.05, **p<0.01. Plots are mean ± SEM.
Figure 4.3 (continued)

A P16-19

B P8-10

C P16-19

D P8-10

E P8-10 PKCαβ ko

F P8-10 wt + PKCβ inhibitor

G P8-10

H P8-10
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 wildtype animals, but it is mediated by a PKC-independent mechanism in 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 P8-10 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 wildtype 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 P8-10 PKCαβ ko mice the pan-PKC inhibitor GF109203X (GF) greatly attenuates most PTP (14 ± 3%; n=11; p<0.05; Figure 4.3H). The small remaining enhancement is comparable to the enhancement mediated by myosin light chain kinase in P11-14 wild type mice (Fioravante et al., 2011). This observation suggests that the PKCαβ-independent PTP observed in pre-hearing mice could be mediated by other PKC isoform(s).

There are many PKC isoforms, but PKCα, PKCβ and PKCγ are the only Ca^{2+}-dependent ones (Newton, 2001; Newton, 1995; Steinberg, 2008). PKCγ therefore seemed like a reasonable candidate to mediate PTP in P8-10 animals. Although previous studies suggested that PKCγ is not present at calyx of Held synapses in P13-15 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 P8-10 animals.
We co-labeled glutamatergic calyces with an antibody against vesicular glutamate transporter 1 (vGlut1; red) and an anti-PKCγ antibody (green) (Figure 4.4). 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 P8-10 PKCγ ko animals (Figure 4.4, top and middle). These findings establish that PKCγ is indeed present at the calyx of Held in pre-hearing 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 P16-19 mice. We observed intense vGlut1 labeling with a pattern that was consistent with the morphology of adult calyces, but PKCγ was absent (Figure 4.4, 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 pre-hearing animals prompted us to test the role of this isoform in PTP. In P8-10 PKCγ ko mice, PTP is present (54 ± 8%; n=14) at a magnitude that is comparable to that observed in wildtype animals (p=0.65; Figure 4.5A). This indicates that in pre-hearing 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 pre-hearing wildtype 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 wildtype mice, and indicates that PKCγ plays an important role in PTP prior to hearing onset. Remarkably, in P8-10 PKCγ ko mice the mechanism of PTP is very different from that seen in wildtype or in PKCαβ ko mice, and PTP is primarily a result of an increase in the size of the
Figure 4.4

Immunohistochemical localization of PKCγ at the calyx of Held in animals before and after hearing onset. Brain slices containing the MNTB region from wildtype and PKCγ ko animals prior to and after hearing onset were co-labeled with antibodies to PKCγ (green) and an antibody to the presynaptic marker vGlut1 (red). Representative images are shown for P10 and P18 slices from wildtype (WT) and PKCγ ko animals.
Figure 4.4 (continued)
PKCγ mediates PTP in pre-hearing animals at the calyx of Held synapse. (A) PTP in P8-10 wildtype and PKCγ ko animals. (B) Summary of the contributions of RRP and p to synaptic enhancement in P8-10 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 P8-10 wildtype and PKCαβγ triple ko animals. (D) PTP in PKCγ ko animals in the absence and the presence of a PKCβ inhibitor (250 nM). Plots are mean ± SEM.
Figure 4.5 (continued)

A

B

C

D

Figure 4.5 (continued)
RRP (RRP\textsubscript{trainC} 37 ± 13%; n=8) rather than an increase in $p$ ($p_{\text{trainC}}$ 20 ± 11%; Figure 4.5B, Supplemental Figure S5C). Indeed, the contributions of RRP and $p$ are similar to those seen after the onset of hearing in wildtype animals (Figure 4.2H). The elimination of PKCγ from the synapses of pre-hearing 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 Ca\textsuperscript{2+}-dependent isoforms are either genetically eliminated or inhibited. In contrast to PKCαβ ko animals in which PTP was present, PTP is strongly attenuated in P8-10 PKCαβγ triple ko animals (12 ± 9%; n=17; $p<0.01$; Figure 4.5C). A PKCβ inhibitor attenuated the PTP in PKCαγ mice to a similar extent (17 ± 5%; n=12; $p<0.01$; Figure 4.5D). These observations indicate that before hearing onset a combination of PKCβ and PKCγ mediate PTP and that PKCγ plays a prominent role in wildtype 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 Ca\textsuperscript{2+} entry to increase $p$, because increases in Ca\textsuperscript{2+} entry are known to contribute to some forms of short-term synaptic plasticity (Catterall and Few, 2008). Previously we found that increases in Ca\textsuperscript{2+} entry do not account for PKCβ-mediated PTP at the calyx of Held in P11-14 animals (Fioravante et al., 2011), but at that age PKCβ-mediates PTP by increasing the RRP, whereas increasing action-potential-evoked Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} influx. Moreover, at the calyx of Held in pre-hearing animals, it is thought that increases in Ca\textsuperscript{2+} influx contribute to PTP evoked by prolonged stimulation (Habets and Borst, 2006), and may also contribute following 4s at 100 Hz (Korogod et al., 2007).
We therefore tested the hypothesis that PTP in pre-hearing animals is mediated by PKCγ-dependent increases in Ca\(^{2+}\) influx in wildtype animals. We measured presynaptic Ca\(^{2+}\) in the calyx of Held from P8-10 animals as we had done previously for older animals (Fioravante et al., 2011, Supplemental Figure S6). We loaded calyces with Calcium Green-1 dextran and Alexa-594 dextran, and labeled calyces were readily identified (Figure 4.6A, top). Single stimuli evoked rapid Ca\(^{2+}\) transients of 31 ± 9 nM that decayed with a time constant of 66 ±19 ms in wildtype calyces (representative trace shown in Figure 4.6A, bottom). Following tetanic stimulation (4s, 100 Hz) the residual Ca\(^{2+}\) increased to over 100 nM and decayed to resting Ca\(^{2+}\) levels with a time constant of 23 ± 7 s (Figure 4.6C, top). We used the Ca\(^{2+}\) increase evoked by single stimuli as a means of detecting changes in Ca\(^{2+}\) influx. Ca\(^{2+}\) increases evoked by single stimuli were unaltered by tetanic stimulation, indicating that tetanic stimulation does not result in an increase in Ca\(^{2+}\) entry in pre-hearing animals (Figure 4.6C, bottom). We performed similar experiments in PKCγ ko animals in which PTP is mediated by an increase in RRP rather than p. Ca\(^{2+}\) signaling in PKCγ ko animals and wildtype animals was indistinguishable (Figure 4.6B, C). Thus, we find that PKCγ does not produce PTP by increasing action potential evoked Ca\(^{2+}\) entry, and PKCγ-dependent increases in Ca\(^{2+}\) influx do not account for PTP in pre-hearing animals.

**PKCγ suppresses the actions of PKCβ to dictate the mechanism of PTP**

Based on the prominent contribution of p to PTP in wildtype animals prior to the onset of hearing, it seems that PTP is mediated primarily by PKCγ. However, in pre-hearing PKCγ ko animals PTP is produced by an RRP increase mediated by PKCβ. Why does PKCβ mediate PTP in pre-hearing PKCγ ko animals but play such a minor role in P8-10 wildtype animals?

To begin to address this question, we began by using 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 wildtype animals (Figure 4.7, top). Immunofluorescence is eliminated in PKCαβ
Figure 4.6

Tetanic stimulation produced similar presynaptic residual calcium signals in Wildtype and PKCγ ko animals in pre-hearing animals. (A) top: two-photon image of a calyx from a wildtype animal filled with Alexa-594 dextran and Calcium Green-1 dextran. bottom: Ca$^{2+}$ transient evoked by a single stimulus for a wildtype calyx. (B) Same as A, but for a PKCγ ko animal. (C) Plots of residual Ca$^{2+}$ (Ca$_{res}$, top) and Ca$^{2+}$ influx (bottom) in slices from wildtype (filled symbols) and PKCγ ko (open symbols) animals. Tetanic stimulation (4 s, 100 Hz) was at time t=0. Scale bar for (A, B) is 10 µm. Plots are mean ± SEM.
Figure 4.6 (continued)
**Figure 4.7**

**PKCβ is present at the calyx of Held prior to hearing onset.** Brain slices from P8-10 animals containing the MNTB region were co-labeled with antibodies to PKCβ (green) and an antibody to the presynaptic marker vGlut1 (red). Representative images are shown for P10 slices from wildtype (WT), PKCαβ ko, and PKCγ ko animals. Scale bar is 10 μm.
Figure 4.7 (continued)
dko animals (Figure 4.7, middle). In PKCγ knockout animals, PKCβ is still present and there is no obvious increase in expression levels (Figure 4.7, bottom). These findings indicate that in pre-hearing wildtype 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 if this is the case by using AAV to express PKCγ-YFP in globular bushy cells that give rise to calyx of Held synapses. Wildtype animals were used for these experiments. Calyces expressing PKCγ-YFP were readily identified by strong fluorescence (Figure 4.8A). Contributions of RRP and \( p \) to PTP were assessed (Figure 4.8B, C) and synapses expressing PKCγ-YFP showed robust PTP (75 ± 9%; \( n=9 \)) that was mediated primarily by an increase in \( p \) (\( p_{\text{train}} 58 ± 5\% \)), with very little contribution from RRP (11 ± 5%) (Figure 4.8D). 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\( _{\text{train}} 45 ± 10\% \)) (Figure 4.8E), which is typical of hearing wildtype 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 non-expressing 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 4.9A and Supplemental Table S2 for P8-10 animals. The changes in PPR associated with PTP for all groups are summarized in Supplemental Table S2. PTP in pre-hearing 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.
Figure 4.8

**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 postnatal day 19-22 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 wildtype calyx showing PKCγ-YFP fluorescence. (B) Synaptic currents evoked by the first 40 stimuli of 4s, 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 is 10 μm. Bars in (D,E) are mean ± SEM.
Figure 4.8 (continued)
Figure 4.9

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 following tetanic stimulation in wildtype and knockout mice, and in the presence of pharmacological inhibitors. Bar graphs are color coded to indicate whether PKC$\beta$, PKC$\gamma$ or both isoforms were knocked out or pharmacologically inhibited. Green bars indicate summaries of experiments in which PKC$\beta$-YFP was expressed in wildtype animals. (B) A schematic illustration of the differential ways PKC$\beta$ and PKC$\gamma$ contribute to PTP and the functional consequences of their response to a stimulus train. Bars are mean ± SEM.
Figure 4.9 (continued)

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PKC<sub>γ</sub> → p↑

PKC<sub>β</sub> → RRP↑
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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-dependent isoforms of PKC are present at the calyx of Held, PTP is due largely to an increase in \(\rho\) 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 \(\rho\) in pre-hearing PKCγ ko animals are remarkably similar to those observed in wildtype animals after the onset of hearing. The elimination of PKCγ in P8-10 animals essentially transforms the properties of PTP to those of wildtype animals after the onset of hearing, where PKCγ is absent from the calyx of Held. Moreover, the viral expression of PKCγ after the onset of hearing leads to PTP with properties similar to pre-hearing wildtype animals. Thus, it is not simply the age of the animal that determines the properties of PTP; it is the complement of Ca\(^{2+}\)-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 Ca\(^{2+}\)-sensitive isoforms PKC\(\alpha\) and PKC\(\beta\) and the novel isoform PKC\(\delta\) have opposing actions in platelet activation and aggregation (Gilio et al., 2010; Harper and Poole, 2007; Heemskerk et al., 2011; Strehl et al., 2007). It is also known that different classes of PKCs are differentially effective at certain substrates. For example, Ca\(^{2+}\)-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 Ca\(^{2+}\) 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 Ca\(^{2+}\)-dependent isoforms such as PKC\(\beta\) and PKC\(\gamma\) 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 PKC\(\beta\) and PKC\(\gamma\) could prefer different substrates (Nishikawa et al., 1997).

**Possible targets of PKC\(\beta\) and PKC\(\gamma\)**

Further studies are required to determine the molecular targets of PTP and the means by which PKC\(\beta\) and PKC\(\gamma\) produce PTP with different functional properties. We tested and excluded the hypothesis that PKC\(\gamma\) modulates presynaptic Ca\(^{2+}\) entry. This is consistent with
the observation that even at synapses where changes in Ca$^{2+}$ 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 Ca$^{2+}$ influx modulation in short-term plasticity, but our findings differ from those results, because a much different induction protocol was used there to induce a longer lasting form of short term plasticity.

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). At present it is not known if either or both PKCγ and PKCβ enhance transmission by phosphorylating Munc18-1.

**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 pre-hearing wildtype animals or in calyces expressing PKCγ-YFP in hearing wildtype 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 4.9B). The properties of use-dependent plasticity at a synapse are likely tailored to the activity patterns of the presynaptic cell. \textit{In vivo}, before the onset of hearing, neurons in the anteroventral cochlear nucleus (aVCN) fire spontaneous bursts of up to 5 action potentials often at more than 100 Hz (Sonntag et al., 2009; Sonntag et al., 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.

Materials and Methods

Animals

All animal experiments were completed in accordance with guidelines by the Harvard Medical Area Standing Committee on Animals. PKC\( \alpha \beta \) double knockout (ko) mice were obtained through breeding of PKC\( \alpha \) and PKC\( \beta \) knockout animals generated by M. Leitges.
PKCγ ko mice (Abeliovich et al., 1993) were obtained from Jackson Labs. 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 wildtype mice, we crossed PKC het-het or het-wildtype mice to use as wildtype controls. Wildtype 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 4.1 to test for changes in paired-pulse plasticity during PTP. For all other figures, mice of mixed background were used, and age-matched wildtype, 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 the following (in mM): 125 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 0.1 CaCl2, 3 MgCl2, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, pH 7.4 and continuously bubbled with 95% O2/5% CO2. Transverse slices (190-200 μm thick) containing the MNTB were cut from postnatal day (P)8-10 and P16-19 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 CaCl2 and 1 MgCl2. 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 excitatory postsynaptic currents (ESPCs) from MNTB neurons were made with an internal solution containing (in mM): 142 CsF, 5 CsCl, 5 EGTA, 10 HEPES, and 2 QX314-chloride.

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 post-tetanic EPSCs were measured at 0.2 Hz. To measure RRP and \( p \), a second high-frequency train (0.4 s, 100 Hz) was induced 10 s after the first tetanus after wash-in 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 PKC\( \beta \) inhibitor (3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-aniilino-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, Eugene, OR) 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 h at 32 °C after loading. Fluorescence \( \text{Ca}^{2+} \) signals were obtained using a two-photon microscope, and were converted to \( \text{Ca}^{2+} \) by determining the \( R_{\text{max}}/R_{\text{min}} \) ratio (~5) in sealed pipettes. To determine \( R_{\text{max}} \), we washed on 20 \( \mu \)M ionomycin at the end of the experimental session, and the \( R_{\text{max}} \) in ionomycin was used for all \( \text{Ca}^{2+} \) calculations (see Supplemental Figure S6A). The \( R_{\text{train}} \) determined using a high-frequency train did not reach the \( R_{\text{max}} \) value obtained with ionomycin. Calyces with bright green fluorescence at rest were rejected for further study, because they either had elevated resting \( \text{Ca}^{2+} \) levels or were overloaded with \( \text{Ca}^{2+} \) indicator.

Data Analysis
Custom written programs in IgorPro (WaveMetrics) were used to analyze all data. PPR was calculated as \( \text{EPSC}_2/\text{EPSC}_1 \). 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 \( p<0.05 \).

\textit{Immunohistochemistry}

Transverse brainstem slices (190 \( \mu \)m thick) were obtained with a vibratome from P8-10 and P16-19 mice as described above. Slices were fixed in 2\% paraformaldehyde for 1 hr at 4\(^\circ\)C, washed three times in phosphate buffered solution (PBS, Sigma-Aldrich, St. Louis, MO), and then incubated for 1 hour at room temperature in PBS with 0.25\% TritonX-100 (PBST) and 10\% normal goat serum (NGS). Slices were incubated afterwards in primary antibodies (1:500 dilution) in PBST and 10\% NGS overnight at 4\(^\circ\)C. Slices were rinsed in PBS, incubated in secondary antibodies (1:500 dilution), then rinsed in PBS and mounted on Superfrost glass slides (VWR, West Chester, PA). The antibodies used were: anti-vGlut1 guinea pig polyclonal (Synaptic Systems, Göttingen, Germany), anti-PKC\( \gamma \) rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), 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 63x oil objective. Excitation wavelengths were 543 nm for rhodamine (vGlut1) and 488 nm for Alexa Fluor 488 (PKC\( \gamma \)). Emission filters were LP560 for vGlut1 and BP505-530 for PKC\( \gamma \). Stacked optical sections at 1024 x 1024 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’-

TACAAGGCTGGTACCGAGCTCGGATCCGCGGGTCTGGGCCCTGGCGGAGGCGACT -3’;
3’-ACCAGTGCCCTCGAGCATGACAGGCACGGGCACAGGGCTTGT-5’. To generate the AAV vector, PKCγ was inserted between the 2 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 ScaI and Sall.

**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. 14-18 days were allowed for expression prior to slice preparation.

**References**


Chapter 5

Protein kinase C is a calcium sensor for presynaptic short-term plasticity

Diasynou Fioravante*, YunXiang Chu*, Arthur de Jong,
Michael Leitges, Pascal Kaeser, Wade G. Regehr

Submitted.

Author contributions:
*Y.X.C. and D.F. contributed equally to this work by conducting all experiments and analysis.
A.dJ. and P.K. provided technical support for protein purification and conceptual advice. M.L.
provided reagents and technical assistance. Y.X.C., D.F., and W.G.R. wrote the paper.
Abstract:

In presynaptic boutons, calcium (Ca\(^{2+}\)) triggers both neurotransmitter release and short-term synaptic plasticity. Whereas synaptotagmins are known to mediate vesicle fusion through binding of high local Ca\(^{2+}\) to their C2 domains, the proteins that sense smaller global Ca\(^{2+}\) increases to produce short-term plasticity have remained elusive. Here we seek to identify the Ca\(^{2+}\) sensor for post-tetanic potentiation (PTP), a widespread form of short-term plasticity thought to underlie short-term memory. We find that at the functionally mature calyx of Held synapse the Ca\(^{2+}\)-dependent protein kinase C isoforms α and β are necessary for PTP. Expression of PKCβ alone in PKCαβ double knockout mice rescues PTP, and disruption of Ca\(^{2+}\) binding to the PKCβ C2 domain specifically prevents PTP without impairing other PKCβ-dependent forms of synaptic enhancement. We conclude that different C2-domain-containing presynaptic proteins are engaged by different Ca\(^{2+}\) signals, and that Ca\(^{2+}\) increases evoked by tetanic stimulation are sensed by PKCβ to produce PTP.
Introduction

The complex manner in which patterns of action potential (AP) activity are transformed into neurotransmitter release suggests the existence of multiple presynaptic calcium (Ca\(^{2+}\)) sensors (Kaeser and Regehr, 2013). The Ca\(^{2+}\) sensors that trigger synchronous neurotransmitter release have been identified as synaptotagmin-1,-2 and -9 (Sudhof, 2013), but the Ca\(^{2+}\) sensors that regulate short-term use-dependent plasticity remain elusive. For a widespread form of short-term plasticity termed post-tetanic potentiation (PTP), a high-frequency burst of presynaptic APs enhances subsequent AP-evoked release for tens of seconds. PTP requires sustained elevation of Ca\(^{2+}\) produced by prolonged presynaptic activity, and in most cases synaptic enhancement outlives increases in presynaptic Ca\(^{2+}\) (Brager et al., 2003; Fioravante et al., 2011; Fioravante et al., 2012; Fioravante and Regehr, 2011; Habets and Borst, 2005; Habets and Borst, 2007; Korogod et al., 2005; Regehr et al., 1994). Moreover, buffering Ca\(^{2+}\) presynaptically influences PTP dynamics (Delaney and Tank, 1994; Habets and Borst, 2005). Although PTP is a widespread form of plasticity that is thought to contribute to short-term memory (Abbott and Regehr, 2004; Silva et al., 1996), the Ca\(^{2+}\) sensor that mediates this plasticity has not been identified.

Two Ca\(^{2+}\)-dependent isoforms of protein kinase C (PKC\(_{Ca}\); PKC\(\alpha\) and PKC\(\beta\)) were recently shown to be necessary for PTP (Fioravante et al., 2011; Fioravante et al., 2012). These isoforms contain Ca\(^{2+}\)-binding C2 domains (Shao et al., 1996; Sutton and Sprang, 1998), raising the hypothesis that they may function as Ca\(^{2+}\) sensors for PTP. However, it is not clear that the Ca\(^{2+}\) binding properties of the C2 domain of PKC\(_{Ca}\) (dissociation constant, K\(_d\)~1-5 µM) (Kohout et al., 2002) are well-suited to mediate PTP, which is thought to rely, at least in part, on the waning residual Ca\(^{2+}\) after the AP burst (Delaney and Tank, 1994; Fioravante and Regehr, 2011). Moreover, diacylglycerol (DAG) binding to the C1 domain of PKC\(_{Ca}\) (Figure 5.1A) can also regulate the activity of PKC\(_{Ca}\) (Newton, 2010), and it has been proposed that PKC could play a permissive role in PTP rather than function as the Ca\(^{2+}\) sensor (Saitoh et al., 2001).
Indeed, proteins with Ca\(^{2+}\)-binding domains do not necessarily function as Ca\(^{2+}\) sensors for a process; rather, they can have roles that are independent of their Ca\(^{2+}\)-binding properties (e.g., Doc2 in spontaneous neurotransmission, (Groffen et al., 2010; Pang et al., 2011)). Furthermore, Munc13, calmodulin and other Ca\(^{2+}\)-binding proteins have been implicated in short-term plasticity (Mochida et al., 2008; Sakaba and Neher, 2001; Shin et al., 2010; Junge et al., 2004), but it has not been established that Ca\(^{2+}\)-binding to these proteins is required for short-term plasticity. Thus, in order to determine if PKC\(_{Ca}\) isoforms are Ca\(^{2+}\) sensors that mediate PTP, it must be examined whether PTP relies on Ca\(^{2+}\) binding to the PKC\(_{Ca}\) C2 domain.

**Results**

To investigate the function of PKC\(_{Ca}\) isoforms in PTP, we first examined their role at the functionally mature calyx of Held synapse (postnatal day 17-22) (Fedchyshyn and Wang, 2005; Yang et al., 2010) using double knockout mice for PKC\(\alpha\) and \(\beta\) (\(\alpha\beta\) dko). We recorded excitatory postsynaptic currents (EPSCs) from principal neurons in the medial nucleus of the trapezoid body (MNTB) in response to extracellular stimulation. Tetanic stimulation induced PTP in wildtype animals (62 ± 12 %; Figure 5.1B, black) but not in \(\alpha\beta\) dko animals (2.4 ± 1.8 %; Figure 5.1B, purple). Thus, in contrast to the immature calyx of Held where a substantial component of PTP (~20%) is independent of PKC\(_{Ca}\) (Fioravante et al., 2011), PTP at the functionally mature calyx of Held relies entirely on PKC\(_{Ca}\) isoforms.

Given our observation that PKC\(_{Ca}\) isoforms contribute more to PTP with developmental maturation, we tested whether the same holds true for a related form of potentiation that occludes PTP. Phorbol 12,13-dibutyrate (PDBu), a DAG analog, can enhance transmission by activating not only PKC\(_{Ca}\) (Figure 5.1A) but also Ca\(^{2+}\)-insensitive PKC isoforms and other presynaptic proteins (Newton, 2010; Brose and Rosenmund, 2002; Wierda et al., 2007). At immature calyces, ~35% of PDBu-mediated enhancement is independent of PKC\(_{Ca}\) (Fioravante et al., 2011). We found that PDBu enhances release at functionally mature wildtype calyces (at
Expression of PKCβ rescues synaptic potentiation in animals lacking Ca\textsuperscript{2+}-dependent PKCs. Synaptic plasticity was examined at the calyx of Held following tetanic stimulation (B, F) or bath application of the phorbol ester PDBu (C, G) for wildtype (wt, black), PKCαβ dko animals (purple), and PKCαβ dko animals expressing PKCβ\textsuperscript{WT}-YFP (green). A, Domain arrangement of PKC\textsubscript{Ca}. DAG and PDBu bind to the C1 domain and Ca\textsuperscript{2+} binds to the C2 domain. B, C, F, G, Left, Example EPSCs recorded prior to (bold traces) and after (light traces) synaptic enhancement for each experimental condition. Right, EPSCs are plotted as a function of time (mean ± SEM). In F and G, the αβ dko group data from B and C respectively are re-plotted for comparison. D, In this schematic of the auditory brainstem, the ventral cochlear nucleus (VCn) and medial nuclei of the trapezoid body (MNTB) are labeled. An AAV expressing PKCβ\textsuperscript{WT}-YFP was injected in the VCn at postnatal day 4. E, Labeling with an antibody against vGlut1 (red) is shown for a calyx of Held expressing PKCβ\textsuperscript{WT}-YFP (green) in a PKCαβ dko animal at postnatal day 18. Scale bar: 10 µm. H, I, The synaptic mechanism through which PKCβ rescues PTP was examined under conditions that relieve AMPA receptor desensitization and saturation. H, Left, Overlay of EPSCs (10 ms inter-stimulus interval) delivered prior to (bold traces) and 10 s after (light traces) PTP-inducing tetanus. Middle, traces are normalized to the first EPSC to allow comparison of PPR. Right, PPR\textsubscript{POST} (after tetanus) over PPR\textsubscript{PRE} (before tetanus) (mean ± SEM). I, Summary of the readily-releasable pool (RRP) and release probability (p) contributions to PTP (mean ± SEM, also see Supplemental Figure S9). Scale bars in B, C, F and G: 2 nA, 1 ms. Scale bars in G: 2 nA, 5 ms.
Figure 5.1 (continued)
steady state: 97 ± 12 %; Figure 5.1C, black) but not at age-matched αβ dko calyces (at steady state: 3.2 ± 3.4 %; Figure 5.1C, purple). Thus, at the functionally mature calyx of Held, both PTP and PDBu-mediated enhancement rely entirely on PKC<sub>Ca</sub>, suggesting that the contributions of parallel mechanisms to these forms of plasticity diminish with development.

We next assessed whether presynaptic expression of PKCβ in αβ dko animals rescues PTP. PKCβ was chosen because genetic deletion of PKCα had little effect on PTP (Supplemental Figure S7), suggesting that PTP is mediated primarily by PKCβ at the functionally mature calyx of Held. We generated an adeno-associated virus (AAV), which we used to express wildtype PKCβ fused to yellow fluorescent protein (PKCβ<sup>WT</sup>-YFP) in αβ dko animals (Figure 5.1D). Two weeks after injection, we found that virally expressed PKCβ<sup>WT</sup>-YFP localized to glutamatergic terminals positive for the marker vGlut1 (Figure 5.1E). In contrast to non-injected αβ dko animals, we observed reliable PTP in recordings from principal neurons whose presynaptic terminals expressed PKCβ<sup>WT</sup>-YFP (61 ± 7 %; Figure 5.1F, green; compare to non-injected age-matched αβ dko animals, purple). Expression of PKCβ<sup>WT</sup>-YFP did not alter basal synaptic properties (Supplemental Figure S8, S9E). Moreover, PKCβ<sup>WT</sup>-YFP expression supported PDBu-induced potentiation in αβ dko mice (84 ± 11 %; Figure 5.1G, green), which was very similar in amplitude to the potentiation observed in wildtype animals (Figure 5.1C, black; p = 0.43). Thus, expression of PKCβ is sufficient to rescue PTP and PDBu-mediated enhancement in αβ dko animals.

To determine whether rescued PTP and PTP in wildtype animals are mediated by the same synaptic mechanism, we first used paired pulses to calculate the paired-pulse ratios (PPR: EPSC2/EPSC1) before and at the peak of PTP. If PTP reflects an increase in vesicular release probability (p), which is inversely related to PPR, then PPR<sub>POST</sub>/PPR<sub>PRE</sub> should decrease. However, PPR<sub>POST</sub>/PPR<sub>PRE</sub> was unchanged in both wildtype (p = 0.49; Figure 5.1H, black) and αβ dko animals expressing PKCβ<sup>WT</sup>-YFP (p = 0.68; Figure 5.1H, green). This suggests that in both groups PTP is not mediated by an increase in p. We next examined the
contribution of the readily releasable pool (RRP) of vesicles to PTP by evoking EPSCs with AP trains before and at the peak of PTP (Supplemental Figure S9). Analysis of these responses revealed that PTP was mediated by equivalent increases in the RRP in wildtype and rescued groups (RRPWT: 37 ± 9 %, RRPPKCBWT-YFP: 39 ± 12 %, p = 0.88)(Figure 5.1I and Supplemental Figure S9F). Thus, at the functionally mature calyx of Held, the same mechanism mediates PTP in wildtype animals and at synapses in PKCçβ dko animals that express PKCçβWT-YFP.

To determine if PKCç is the Ca²⁺ sensor for PTP, it is necessary to mutate PKCç so as to prevent Ca²⁺ binding. We introduced five aspartate (D) to alanine (A) mutations in the PKCç C2 domain (C2D/A; Figure 5.2A, Supplemental Figure S10). We chose these residues because they have been implicated in Ca²⁺ binding based on structural similarity with other C2 domains (Nalefski and Falke, 1996; Sutton and Sprang, 1998; Ubach et al., 1998). To test whether these mutations prevented Ca²⁺ binding, we purified recombinant C2D/A and wildtype C2 domain (C2WT; Figure 5.2B). We took advantage of the intrinsic fluorescence of tryptophan residues adjacent to the predicted Ca²⁺ binding sites (Supplemental Figure S10), and we examined changes in intrinsic fluorescence of C2WT and C2D/A in response to Ca²⁺ (Figure 5.2C). In the absence of Ca²⁺, C2WT displayed a characteristic intrinsic fluorescence emission spectrum that peaked around 340 nm (Figure 5.2C, dark green). A similar basal emission spectrum was observed for C2D/A (Figure 5.2C, dark blue), suggesting that the D-to-A mutations did not affect domain folding (Junge et al., 2004; Nalefski and Newton, 2001; Pang et al., 2011). In the presence of 1 mM Ca²⁺ (Figure 5.2C, light green), the peak fluorescence intensity of C2WT increased by 17 ± 1.3%, which is indicative of domain rearrangements directly coupled to Ca²⁺ binding. However, no change was observed for C2D/A upon addition of Ca²⁺ (-1.3 ± 2.0%, compare light and dark blue traces in Figure 5.2C), indicating that the D-to-A mutations prevented Ca²⁺ binding.

When activated by either phorbol esters or Ca²⁺, PKC translocates from the cytoplasm to the plasma membrane (Newton, 2010). We utilized this property to test the effects of the D-to-A
Figure 5.2

C2-domain mutations of PKCβ abolish Ca$^{2+}$ binding and Ca$^{2+}$-induced translocation without impairing phorbol ester-induced translocation. **A**, A partial sequence of the PKCβ C2 domain is shown with Ca$^{2+}$-coordinating aspartates in green. These aspartates were mutated to alanines (*blue*) in the C2$^{D/A}$ construct. **B**, Coomassie-stained gel of recombinant wildtype (C2$^{WT}$) and mutant (C2$^{D/A}$) PKCβ C2 domains. **C**, Averaged intrinsic tryptophan fluorescence is shown for C2$^{WT}$ and C2$^{D/A}$. Fluorescence emission spectra were recorded in 0 mM Ca$^{2+}$ (*bold traces*) and 1 mM Ca$^{2+}$ (*light traces*). **D**, Translocation of PKCβ$^{WT}$-YFP (*left*) and PKCβ$^{D/A}$-YFP (*right*) in HEK293 cells was monitored in response to the Ca$^{2+}$ ionophore ionomycin and in response to PDBu. Ca$^{2+}$ increases caused PKCβ$^{WT}$-YFP to translocate, but not PKCβ$^{D/A}$-YFP. Both PKCβ$^{WT}$-YFP and PKCβ$^{D/A}$-YFP translocated in response to PDBu. Scale bar: 10 µm.
Figure 5.2 (continued)

A

B

C

D

PKCβ<sup>wt</sup>-YFP

PKCβ<sup>DA</sup>-YFP

- iono  + iono - iono  + iono - PDBu  + PDBu - PDBu  + PDBu

- 0 Ca
- 1 Ca  

Fluor. (a.u.)

Exc. (nm)

- 0 Ca  
- 1 Ca

C2<sup>WT</sup>

C2<sup>DA</sup>

kDa

45

21

6
mutations on the response of PKCβ to Ca\textsuperscript{2+} increases. We expressed PKCβ\textsuperscript{WT}-YFP or PKCβ\textsuperscript{D/A}-YFP in HEK293 cells and monitored the subcellular distribution of the kinase upon activation. The Ca\textsuperscript{2+} ionophore ionomycin induced translocation of PKCβ\textsuperscript{WT}-YFP (Figure 5.2D, top left), but did not alter the intracellular distribution of PKCβ\textsuperscript{D/A}-YFP (Figure 5.2D, top right). In contrast, PDBu caused both PKCβ\textsuperscript{WT}-YFP and PKCβ\textsuperscript{D/A}-YFP to translocate. This result indicates that Ca\textsuperscript{2+} binding to the PKC C2 domain is necessary for Ca\textsuperscript{2+}-induced, but not phorbol ester-induced, translocation of PKCβ to the plasma membrane (at least at the PDBu concentration required to obtain reliable potentiation in a slice). Moreover, this result suggests that the D-to-A mutations in the C2 domain prevented Ca\textsuperscript{2+} activation of PKCβ without interfering with C1-domain-mediated membrane recruitment of PKCβ.

We next tested whether Ca\textsuperscript{2+} binding to the PKCβ C2 domain is required for PTP. Using AAV to express PKCβ\textsuperscript{D/A}-YFP, we found that PKCβ\textsuperscript{D/A}-YFP localized to vGlut1-positive areas and distributed similarly to PKCβ\textsuperscript{WT}-YFP (Figure 5.3A, compare to Figure 5.1E). Expression of PKCβ\textsuperscript{D/A}-YFP in αβ dko calyces, similar to wildtype PKCβ, did not affect basal synaptic properties (Supplemental Figure S8). However, in stark contrast to wildtype PKCβ, PKCβ\textsuperscript{D/A} failed to rescue PTP (3.6 ± 2.2 %; Figure 5.3B, blue; also see Figure 5.3D, left). The inability of PKCβ\textsuperscript{D/A}-YFP to support PTP could be due to a loss of Ca\textsuperscript{2+} binding to PKCβ; alternatively, the D-to-A mutations may have induced more profound impairments of PKCβ, such as compromised kinase activity. To distinguish between these possibilities, we tested PDBu-induced potentiation in PKCβ\textsuperscript{D/A}-YFP-expressing calyces. Compellingly, PDBu-induced potentiation in PKCβ\textsuperscript{D/A}-YFP-expressing calyces was rescued to wildtype levels (98 ± 23 %; Figure 5.3C, blue; also see Figure 5.3D, right). This indicates that PKCβ\textsuperscript{D/A}-YFP retained its ability to enhance synaptic transmission. We conclude that the inability of PKCβ\textsuperscript{D/A}-YFP to mediate PTP is a direct consequence of its inability to bind Ca\textsuperscript{2+}, and that Ca\textsuperscript{2+} binding to the C2 domain of PKCβ is required for PTP. Therefore, PKCβ is a Ca\textsuperscript{2+} sensor that mediates PTP.
Figure 5.3

PTP requires Ca\(^{2+}\) binding to PKC\(\beta\) but phorbol ester-induced potentiation does not. A, Labeling with an antibody against vGlut1 (red) is shown for a calyx of Held expressing PKC\(\beta^{D/A}\)-YFP (green) in a PKC\(\alpha\beta\) dko animal. B-C, Synaptic plasticity was examined in PKC\(\alpha\beta\) dko animals at calyces of Held expressing Ca\(^{2+}\)-insensitive PKC\(\beta\) (PKC\(\beta^{D/A}\)-YFP, blue traces). Representative traces and time-courses are shown following tetanic stimulation (B) and during bath application of PDBu (C). Scale bars: 1 nA, 1 ms. In B and C, the \(\alpha\beta\) dko group data from Figure 5.1B and 5.1C respectively are re-plotted for comparison. D, Summary plots (mean ± SEM) of the magnitude of synaptic enhancement produced by tetanic stimulation (left) and by PDBu (right).
Figure 5.3 (continued)

A

Anti-γGlut1  PKC\(\beta^{\alpha\alpha}\)-YFP  Merge

10 μm

B  In αβ dko background

C  In αβ dko background

D

wt  PTP

αβ dko

αβ dko + PKC\(\beta^{\alpha\alpha}\)-YFP

αβ dko + PKC\(\gamma^{\alpha\alpha}\)-YFP

PDBu

0 % Increase

0 % Increase
Discussion

To the best of our knowledge, PKCβ is the first Ca\textsuperscript{2+} sensor to be identified specifically for short-term synaptic plasticity. Similar to synaptotagmins, PKCβ requires binding of Ca\textsuperscript{2+} to its C2 domain for its Ca\textsuperscript{2+}-sensing function (Figure 5.3) (Sudhof, 2013). However, PKCβ acts upstream of vesicle fusion (Newton, 2010), does not regulate basal transmission or paired-pulse plasticity (Supplemental Figure S8), and would not be expected to be activated by single stimuli. How is it that PKCβ and synaptotagmins respond to such different activity patterns and, consequently, such different Ca\textsuperscript{2+} signals? It is likely a combination of differences in Ca\textsuperscript{2+} binding properties and subcellular location that underlie these contrasting responses (34).

Synaptotagmin-1 binds Ca\textsuperscript{2+} cooperatively with low affinity and fast kinetics, and is located on synaptic vesicles; therefore, it is poised to detect large, transient Ca\textsuperscript{2+} signals near open voltage-gated Ca\textsuperscript{2+} channels (Sudhof, 2013). In contrast, PKCβ is activated by lower Ca\textsuperscript{2+} levels with lower cooperativity and is cytosolic (Fioravante et al., 2011; Kohout et al., 2002; Nalefski and Newton, 2001; Newton, 2010). Prolonged stimulation is necessary to produce a sufficient buildup of Ca\textsuperscript{2+} to activate PKCβ, which is consistent with the prolonged activity requirement for PTP (Habets and Borst, 2007; Korogod et al., 2005).

PKCβ likely mediates PTP by phosphorylating downstream targets (Shu et al., 2008; Wierda et al., 2007), which could explain how PTP outlives elevations of presynaptic Ca\textsuperscript{2+} (Brager et al., 2003; Fioravante et al., 2012; Fioravante and Regehr, 2011; Regehr et al., 1994). We suggest that PKCβ is a founding member of a new class of Ca\textsuperscript{2+} sensors that function upstream of vesicle fusion to regulate short-term plasticity.

Materials and Methods

**DNA constructs and viruses**

Cloning was performed by Genscript. Viruses were generated by the University of Pennsylvania Vector Core. All constructs were verified by sequencing. Wildtype PKCβ-YFP
(β\textsuperscript{WT}-YFP) was obtained through PCR from Addgene plasmid #14866 (Violín et al., 2003), using the following primers: 5’-GACACAAACAGTCTCGAACTTAATCGAACCAGCAGCAGGTGCACGAGCGGCGGCGTCACGACAGCCCTCCTCACAGATTACTTG-3’; 3’-GGGAAAAAGATCGGATCCTCAGGCGTGCAGCCCTCCTCAGATTACTTG-5’.

To generate an adeno-associated viral vector, PKCβ\textsuperscript{WT}-YFP was inserted into a pENN.AAV.CMV.TurboRFP.RBG cis-plasmid (courtesy of the University of Pennsylvania Vector Core) using SacII and Sall, after removal of the TurboRFP sequence with SpeI and Xhol. Mutant PKCβ-YFP (β\textsuperscript{D/A}-YFP) was generated by replacing the 5 aspartates that coordinate calcium binding (Sutton and Sprang, 1998) with alanines through PCR. For mutagenesis, the following primers were used: 5’- TACCTATGGCCCCCAACGGCTTGTCAGCTCCCTACGTAAAACTGAAACTGAT-3’ and 3’- TAGGGAGCTGACAAGCCGTTGGGGGCCATAGGTACCAGATTTTTTTA-5’; and 5’- AGATCTGGGCTTGGGCCCTGACCAGAGGAATGCCTTTCATGGGTGATCTCTTCGCTTTT-3’ and 3’- ATCCCATGAAGGCAATTCTGCTTGGTCCGGGCGGCAAGCCAGATCTCTTCGCTATGGGTCACGGACAGCT-5’. For subcloning into the pENN cis-plasmid: 5’-TGTGTTGTACACAAGCGCTGCCATGAATTCGTCACGTTCTC-3’ and 3’- TTAACAAAGGAAATCCTTCGAATTCTGATTGGTCAATATTCC-5’. To obtain recombinant C2 domains, the sequence for wildtype or mutant C2 (Torrecillas et al., 2003) (see also Supplemental Figure S10) was inserted into a pGEX-KG vector (Addgene database #2890) using XbaI and Ncol and the following primers: 5’-TCCGGTGGTGGTGGATTCTAGAAGGAACGCCTGGGCGGCATC-3’ and 3’-AAGCTTGAGCTGAGTCACGACCATGGTCATCCTCTGCACGGACAGCC-5’.

Animals

All animal experiments were completed in accordance with guidelines by the Harvard Medical Area Standing Committee on Animals. PKCαβ double knockout (dko) mice were obtained through breeding of PKCα and PKCβ single knockout (ko) animals generated by M. Leitges (Leitges et al., 2002; Leitges et al., 1996). The probability of obtaining an αβ double knockout animal from heterologous (het) crosses is very low (1:16), making viral injection
experiments unfeasible. We therefore bred het-ko animals together to increase the probability of getting desired animals. Similarly, to increase the probability of obtaining wildtype mice, we crossed PKC het-het or het-wildtype mice to use as wildtype controls. Wildtype mice were derived from the same genetic line as αβ dko animals. To prevent genetic drift in the inbred ko lines, we backcrossed them every second generation to C57BL/6J or 129S2. For experiments, animals of both sexes were used and age-matched wildtype, PKCα ko and PKCαβ dko mice from our colony were interleaved.

**Surgery**

P4 pups were stereotactically and unilaterally injected under isofluorane anesthesia with AAVs 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 Wirretrol II capillary micropipettes (Drummond Scientific) pulled to a fine tip (10-20 µm diameter). At this age, the skull is sufficiently soft so it can be penetrated with a 28½-gauge needle without the need for drilling. After the injection, the skin was closed with Gluture (Abbott Laboratories) and pups were allowed to recover on a heating pad prior to returning to the home cage. 14-18 days were allowed for expression prior to slice preparation.

**Preparation of brain slices**

Transverse 190- to 200-µm-thick brainstem slices containing the MNTB were made with a vibratome slicer (VT1000S, Leica) from juvenile (postnatal day 17-22) mice deeply anesthetized with isoflurane. Brains were dissected and sliced at 4 °C in cutting solution consisting of the following (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 0.1 CaCl₂, 3 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O₂/5% CO₂ (pH 7.4). Slices were incubated at 32 °C for 30 min in a bicarbonate-buffered
solution composed of the following (in mM): 125 NaCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, 0.4 ascorbic acid, continuously bubbled with 95% O$_2$/5% CO$_2$ (pH 7.4).

Electrophysiology

Slices were transferred to a recording chamber at room temperature (21-24 °C) in an upright microscope (Olympus) equipped with a 60X objective. During recordings, the standard perfusion solution consisted of the bicarbonate-buffered solution (see above) with 1 μM strychnine and 25 μM bicuculline (R&D Systems) to block inhibition. Slices were superfused at 1-3 ml/min with this external solution. Whole-cell postsynaptic patch-clamp recordings were made from visually identified cells in the MNTB region using glass pipettes of 2-3 MΩ resistance, filled with an internal recording solution of the following (in mM): 20 CsCl, 140 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 EGTA, 5 Na$_2$-phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na. Series resistance (R$_s$) was compensated by up to 70% and the membrane potential was held at -70 mV.

EPSCs were evoked by stimulating presynaptic axons with a custom-made bipolar stimulating electrode midway between the medial border of the MNTB and the midline of the brainstem. For slice recordings from injected animals, principal neurons in the MNTB contralateral to the injection site were selected based on the presence of YFP-expressing presynaptic terminals. A Multiclamp 700B (Axon Instruments/Molecular Devices) amplifier was used. Recordings were digitized at 20 KHz with an ITC-18 A/D converter (Instrutech Corp.) using custom macros (written by M.A. Xu-Friedman) in Igor Pro (Wavemetrics) and filtered at 8 kHz.

The protocol for inducing PTP was as follows: an estimate of baseline synaptic strength was obtained through low-frequency stimulation at 0.2 Hz for 25 s. PTP was induced with a 4-s stimulus train at 100 Hz, followed by low-frequency stimulation to test for PTP. For phorbol ester
experiments, PDBu (1 µM; Tocris) was washed in for 10 min once a stable baseline of at least 3 min was established. Synaptic strength was evaluated by afferent fiber stimuli, repeated every 20 s. During the intertrial intervals, 5 s stretches of postsynaptic current were recorded to assess the frequency and amplitude of mEPSCs. To assess PPR, pulses were delivered at an inter-stimulus interval of 10 ms. For all recordings, the access resistance and leak current were monitored, and experiments were rejected if either of these parameters changed significantly.

Data analysis

Data analysis was performed using routines written in IgorPro (WaveMetrics). PTP magnitude was calculated as the ratio of EPSC amplitude 10 s after the 4-s, 100 Hz train over the average baseline. The magnitude of PDBu-induced potentiation was estimated by averaging the steady-state responses, 430-600 s from wash-in onset. To analyze spontaneous events, mEPSCs were detected using a threshold (average peak to peak noise in the baseline) of the first derivative of the raw current trace, and confirmed visually. Statistical analyses were done using one-way ANOVA tests for multiple group comparisons followed by Tukey post-hoc analysis, or Kruskal-Wallis non-parametric ANOVA for data sets that were not normally distributed. Pairwise comparisons were performed with Student’s t-tests. Level of significance was set at p < 0.05.

To determine the contributions of RRP and p to wildtype and rescued PTP, stimulus trains were used in the presence of kynurenate (1 mM) and CTZ (0.1 mM) to prevent postsynaptic receptor saturation and desensitization. Briefly, the amplitude of the first 40 responses to the stimulus train used to induce PTP and to a stimulus train (400 ms, 100 Hz) 10 seconds later (at the peak of PTP) were measured, and a plot of the cumulative EPSC for each train versus the stimulus number was made. 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 recycling/reserve pool (39).
The size of the RRP can then be determined by a linear fit to the steady-state responses (last 15 EPSCs), which is extrapolated back to the y-axis (Moulder and Mennerick, 2005; Thanawala and Regehr, 2013). \( p \) is then calculated from EPSC1/RRP.

**Immunohistochemistry**

150 \( \mu \)m-thick transverse brainstem slices were prepared as described above from P18-P22 animals injected with AAVs and fixed with 4% paraformaldehyde for 2 h at 4 °C. At the end of fixation, slices were transferred to phosphate buffer (Sigma-Aldrich) and stored at 4 °C until further processing. Slices were then incubated in blocking solution [phosphate buffered solution + 0.25 % Triton X-100 (PBST) + 10 % normal goat serum] for 1 h at room temperature. Slices were incubated with primary antibody [anti-vGlut1 guinea pig polyclonal (Synaptic Systems)] in PBST overnight at 4 °C, followed by incubation with secondary antibody [goat anti-guinea pig Alexa 568-conjugated (Life Technologies)] in PBST for 2 h. Slices were mounted to Superfrost glass slides (VWR) and air-dried for 30 min. Following application of Prolong anti-fade medium (Invitrogen), slices were covered with a top glass coverslip (VWR) and allowed to dry for 24 h prior to imaging. Antibodies were used at 1:500 dilution.

Images were acquired with a Zeiss 510 Meta confocal microscope using a Plan-apochromat 1.4 NA 63X oil lens. Emission filters were BP570-670 nm for the red channel (vGlut1) and BP500-550 for YFP (PKCβ). Single optical sections at 1024x1024 (average of 3 scans) were obtained sequentially for the different channels. Color channels were split and merged in ImageJ to obtain the composite images in RGB.

**Protein purification**

N-terminal GST fusion proteins of PKCβ C2\(^{WT}\) and C2\(^{D/A}\) were expressed in *Escherichia coli* BL21 cells. For purification, cells were resuspended in PBS supplemented with 500 \( \mu \)M EDTA, 0.5 mg/mL lysozyme (Amresco) and protease inhibitor cocktail (Easypack, Roche), and
sonicated. After centrifugation at 11200 RPM for 30 min, the soluble fraction was collected and incubated with glutathione sepharose 4B beads (GE healthcare) for 1 h at 4°C. Samples were cleared from nucleic acid contaminants with benzonase (40 U/mL, Sigma) for 3 h at RT, and subsequently eluted from the beads with solution containing 100 mM Tris, 10 mM CaCl₂, 5 mM Glutathione (pH 7.4) for 1 h at 4°C. GST was cleaved with thrombin-agarose (100 µL resin/mg protein, Sigma) for 24 h at 4°C, and samples were dialyzed to solution containing 40 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.5 mM sodium EGTA. GST was removed from the samples using glutathione sepharose 4B beads. Ten µl of purified protein was run on a 12 % SDS gel and Coomassie-stained to check for purity (Figure 5.2B).

**Intrinsic Tryptophan Fluorescence Assay**

Intrinsic tryptophan fluorescence of purified C²WT and C²D/A was monitored in dialysis buffer (see above). Emission spectra were recorded from 325 to 425 nm on a Spectramax M5 microplate reader (Molecular Devices). Excitation was set at 295 nm and peak intrinsic fluorescence change (ΔF) upon addition of 1 mM free Ca²⁺ was estimated at 341 nm. To correct for the effect of volume increase on fluorescence readings upon addition of Ca²⁺-containing buffer, ΔF in buffer-alone controls was subtracted from fluorescence values in buffer+Ca²⁺ groups. Experiments were repeated with 2 independently purified batches of protein, for a total of 7 times. Similar results were obtained every time.

**Protein translocation assay**

HEK293 cells plated on glass coverslips were transfected with PKCβWT-YFP or βD/A-YFP expression vectors using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, coverslips were transferred to the imaging chamber of a custom-build 2-photon laser scanning microscope system and superfused with buffer (138 mM NaCl, 1.5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, pH 7.4) at 2 ml/min. YFP was excited
at 840 nm with a Ti-Sapphire laser through a 60x, 1.1 NA water-immersion Olympus lens. A
500-550 BP emission filter was used. 512x512 frame scans were acquired at a rate of 1 line/4
ms, every 30 s. To stimulate translocation, the superfusion solution was switched to one
containing 1 µM PDBu or 10 µM ionomycin (R&D Systems) for 15 min. Experiment was
repeated 3 times for β<sup>WT</sup>-YFP and twice for β<sup>D/A</sup>-YFP, with similar results. Acquired images were
exported to ImageJ and brightness/contrast was adjusted equally for all images within an
experiment for display purposes.

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Chapter 6

Conclusion

The studies in this work provide insight into the molecular mechanisms of two forms of short-term synaptic plasticity: post-tetanic potentiation and glycine-induced synaptic enhancement. Using electrophysiological and molecular genetic approaches, we demonstrate that Ca\textsuperscript{2+}-dependent isoforms of PKC (PKC\textsubscript{Ca}) function as the Ca\textsuperscript{2+} sensors for PTP. We further show that while different PKC\textsubscript{Ca} isoforms mediate PTP, they do so by differentially increasing the release probability or the size of the readily-releasable pool of vesicles. Thus, PKC\textsubscript{Ca} isoform specificity is crucial for determining the mechanism of PTP, which could have important functional consequences for high-frequency firing at the calyx of Held synapse.

The roles of PKC in PTP

PKC as a calcium sensor for PTP

The involvement of Ca\textsuperscript{2+}-dependent isoforms of PKC in PTP is compatible with the long-held hypothesis that a build-up of residual Ca\textsuperscript{2+} from repetitive firing activates a Ca\textsuperscript{2+} sensor protein to increase synaptic efficacy (Katz and Miledi, 1968) (Zucker and Regehr, 2002). These classical isoforms contain Ca\textsuperscript{2+}-binding C2 domains (Newton, 2010), but it was not known if the Ca\textsuperscript{2+} binding properties of PKC allow for its function as a Ca\textsuperscript{2+} sensor for PTP. To overcome the limitations of putative pharmacological tools, we adopted a molecular genetic approach using PKC\textalpha, PKC\textbeta, and PKC\textgamma knockout mice to show that PKC\textsubscript{Ca} are necessary for the potentiation of evoked synaptic responses. Further genetic manipulations and rescue experiments definitively show that PKC\textbeta functions as the Ca\textsuperscript{2+} sensor for PTP. Not all proteins that have C2 domains function as Ca\textsuperscript{2+} sensors, as they can have roles independent of their Ca\textsuperscript{2+}-binding properties (Groffen et al., 2010; Pang et al., 2011). Other Ca\textsuperscript{2+}-binding proteins, such as...
Munc13 and calmodulin, have been implicated in short-term plasticity (Junge et al., 2004; Mochida et al., 2008; Sakaba and Neher, 2001; Shin et al., 2010), but it has not been established that these proteins require Ca$^{2+}$ binding to modulate changes in synaptic strength. To the best of our knowledge, PKC$\beta$ is the first Ca$^{2+}$ sensor identified specifically for short-term synaptic plasticity.

**PKC isoforms differentially mediate PTP**

Our results establish that Ca$^{2+}$-dependent PKC isoforms mediate most of the PTP at the calyx of Held, with PKC$\beta$ and PKC$\gamma$ playing more prominent roles than PKC$\alpha$. We show using immunohistochemistry that PKC$_{Ca}$ isoforms are expressed at the calyx of Held throughout development, but that the expression of specific isoforms varies relative to the onset of hearing. The identity of the expressed isoform further determines the presynaptic mechanism of PTP. Specifically, PKC$\beta$ mediates PTP by increasing the size of release-ready vesicles after hearing onset, and PKC$\gamma$ is not expressed at this stage in development. PKC$\gamma$ is only detected in pre-hearing calyces and mediates PTP by predominantly increasing the release probability. However, PKC$\beta$ is still expressed early on, suggesting that PKC$\gamma$ somehow suppresses the activities of the $\beta$ isoform.

Our findings raise the question, how can different PKC isoforms that are structurally similar perform specialized functions? These isoform-specific mechanisms could arise when PKC proteins are differentially expressed throughout the CNS, or in a developmentally regulated manner. In neurons that express multiple PKC isoforms, these proteins can have different subcellular localizations. There are several physiological examples where different PKC isoforms perform specialized functions within the same cell. For instance, in platelet activation and aggregation, PKC$\alpha\beta$ and PKC$\delta$ exhibit opposing actions (Gilio et al., 2010; Harper and Poole, 2007; Heemskerk et al., 2011; Strehl et al., 2007). Similarly, at *Aplysia* sensory-motor synapses a Ca$^{2+}$-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 Ca\(^{2+}\)-dependent PKC isoform (Sossin, 2007; Zhao et al., 2006). PKC isoforms can also exert separate functions by activating distinct substrates. For example, while classical PKCs prefer to phosphorylate serine/threonine residues that are N-terminal to the phosphorylation site, novel PKCs prefer to phosphorylate hydrophobic residues (Nishikawa et al., 1997; Sossin, 2007). Different PKC isoforms have also been shown to differentially regulate L-type Ca\(^{2+}\) channels by phosphorylating different residues (Yang and Tsien, 1993). Hence, it is possible that different Ca\(^{2+}\)-dependent PKCs could phosphorylate different sites on the same substrate to produce PTP through different presynaptic mechanisms.

*Increases in mEPSC frequency and evoked synaptic responses*

At many synapses including the calyx of Held, high-frequency stimulation increases the frequency of mEPSCs, and this process has also been shown to depend on presynaptic residual Ca\(^{2+}\) (Delaney and Tank, 1994; Eliot et al., 1994; Habets and Borst, 2005; He et al., 2009; Korogod et al., 2005). Similarly, activation of presynaptic ionotropic receptors also potentiates spontaneous release in a Ca\(^{2+}\)-dependent manner (Turecek and Trussell, 2001). However, it is unclear if changes in spontaneous and evoked transmission are mediated by the same molecular mechanism. In PTP, the timecourse of the enhancement in mEPSC frequency post-tetanus is faster than the increase in evoked responses (τ ~ 12 s and 45 s respectively) (Korogod et al., 2005), and inhibition or elimination of Ca\(^{2+}\)-dependent PKCs do not affect tetanus-induced changes in minis frequency (Korogod et al., 2007; Xue and Wu, 2010). These results suggest that potentiation of evoked versus activity-dependent spontaneous release are regulated independently. Thus, synapses throughout the CNS may contain a different Ca\(^{2+}\) sensor other than PKCs that mediates activity-dependent spontaneous release. Candidate proteins that may act as high-affinity Ca\(^{2+}\) sensors include Doc2 proteins (Groffen et al., 2010) and synaptotagmin-7 (Bacaj et al., 2013; Wen et al., 2010).
**PKC in glycine-induced synaptic enhancement**

Glycine-induced synaptic potentiation is similar to PTP in many ways. Both forms of short-term plasticity require an increase in presynaptic $\text{Ca}^{2+}$ concentration (Kim et al., 2005; Korogod et al., 2007; Turecek and Trussell, 2001), and our findings show that both are mediated by $\text{Ca}^{2+}$-dependent isoforms of PKC. In animals after hearing onset, PKC increases glycine-induced evoked release by predominantly enhancing the effective size of the readily-releasable pool of vesicles. It is likely that PKC$_{\text{Ca}}$, once activated by residual $\text{Ca}^{2+}$, phosphorylate the same substrate (possibly Munc18-1) (Wierda et al., 2007) to produce both forms of plasticity.

Several notable differences exist between glycine-induced potentiation and PTP. First, PKC$\alpha$ and PKC$\beta$ appear to have different degrees of contribution to these two forms of short-term plasticity. In PTP, PKC$\beta$ predominantly mediates PTP over PKC$\alpha$, but both isoforms contribute comparably to glycine-induced enhancement. The $\text{Ca}^{2+}$ signals underlying both forms of plasticity are also different. Presynaptic residual $\text{Ca}^{2+}$ induced by tetanic stimulation in PTP reaches $\sim$150-200 nM. Glycine activation of presynaptic ionotropic receptors leads to the opening of VGCCs and a sustained elevation of residual $\text{Ca}^{2+}$ of $\sim$50 nM, but with larger local $\text{Ca}^{2+}$ increases closer to $\text{Ca}^{2+}$ channels. While we have convincingly shown that PKC$\beta$ acts as a $\text{Ca}^{2+}$ sensor for PTP, similar studies need to be done to determine if PKC$\alpha$ and PKC$\beta$ act as sensors for glycine-induced synaptic potentiation.

Several other forms of plasticity related to glycine-induced enhancement have been studied at other CNS synapses. For example, at cortical (Christie et al., 2011; Corlew et al., 2007; Sjostrom et al., 2003), hippocampal (Vogt and Regehr, 2001), and cerebellar synapses (Glitsch and Marty, 1999), somatic depolarization can induce depolarization of presynaptic terminals to increase neurotransmitter release. Glycine-induced modulation of evoked release can also be mimicked by small presynaptic depolarization through direct whole-cell recordings.
of the calyceal terminal (Awatramani et al., 2005). Based on these observations, it is possible that Ca\(^{2+}\)-dependent PKCs contribute to synaptic plasticity arising from somatic depolarization.

**Activation of PKC by residual calcium**

The residual Ca\(^{2+}\) increase produced by tetanic stimulation and glycine activation of presynaptic ionotropic receptors is only several hundred nanomolars. If Ca\(^{2+}\)-dependent PKCs function as the Ca\(^{2+}\) sensors for these forms of synaptic plasticity, can they respond to such small Ca\(^{2+}\) increases? The Ca\(^{2+}\)-binding affinities for PKCs in a cuvette without lipid membranes are ~40 \(\mu M\) (Kohout et al., 2002), which is much higher than the observed residual Ca\(^{2+}\) concentrations. However, PKC isoforms display cooperative Ca\(^{2+}\) binding in the presence of plasma membrane phospholipids, and the Ca\(^{2+}\)-binding affinities decrease down to 0.1-5 \(\mu M\) (Corbalan-Garcia et al., 1999; Corbin et al., 2007; Guerrero-Valero et al., 2007; Kohout et al., 2002). It is also possible that factors in the intracellular milieu raise the binding affinity of PKCs for Ca\(^{2+}\), as is the case for calmodulin (Xia and Storm, 2005). Therefore, PKC isoforms could be sufficiently sensitive to detect residual Ca\(^{2+}\). Another possibility is that PKC\(\alpha\) and PKC\(\beta\) are located sufficiently close to P-type Ca\(^{2+}\) channels such that during Ca\(^{2+}\) channel opening they are exposed to high local Ca\(^{2+}\) increases within a microdomain that are much higher than the average presynaptic Ca\(^{2+}\) levels.

**Substrates of PKC for short-term plasticity**

The molecular substrates of PKC in short-term plasticity still need to be definitively determined. Because PKC\(\beta\) and PKC\(\gamma\) mediate PTP through different presynaptic mechanisms at the calyx of Held, it is likely that the isoforms phosphorylate different downstream targets to increase pool size or release probability. For both PTP and glycine-induced potentiation, we tested and excluded the hypothesis that PKCs modulate presynaptic Ca\(^{2+}\) entry to produce short-term plasticity. Currently, Munc18-1 is the leading substrate of Ca\(^{2+}\)-dependent PKCs for
mediating short-term plasticity. Munc18-1 is a promising candidate because it contains multiple PKC phosphorylation sites (Barclay et al., 2003; Fujita et al., 1996), and it has been implicated in regulating changes in pool size and release probability (Nili et al., 2006; Toonen et al., 2006). Previous studies have shown that, in cultured hippocampal cells, PKC phosphorylation of Munc18-1 is necessary for a form of tetanus-induced plasticity and for synaptic potentiation produced by phorbol esters (Wierda et al., 2007). Most recently, viral expression of a mutant form of Munc18-1 where two PKC phosphorylation sites are inactivated significantly reduced the magnitude of PTP at the calyx of Held (Genc et al., 2014). These observations suggest that PKCβ and PKCγ could both enhance transmission by phosphorylating Munc18-1, perhaps by phosphorylating different sites. One or both of these isoforms could also regulate transmission by phosphorylating other targets in the presynaptic bouton including SNAP25 (Gonelle-Gispert et al., 2002; Houeland et al., 2007; Nagy et al., 2002; Zamponi et al., 1997).

**Functional role of short-term plasticity in the auditory brainstem circuit**

Several lines of evidence suggest that short-term plasticity plays a crucial role in auditory processing. Sustained sound-driven discharge of the calyx of Held can reach ≥800 Hz, while spontaneous firing rates in silence can range from 1-100 Hz (Kopp-Scheinpflug et al., 2011; Sonntag et al., 2009; Sonntag et al., 2011). *In vitro* and *in vivo* recordings have revealed that the response entrainment (the ratio of spikes generated per presynaptic stimulus) of calyceal stimulation is ~50% when high frequency trains are delivered (Kopp-Scheinpflug et al., 2011; Lorteije et al., 2009). This means that increases in synaptic strength due to PTP or glycine-induced potentiation could help prevent spike failures during high-frequency activity, thus increasing spike fidelity in auditory processing.

Our results demonstrated that forms of short-term plasticity, such as PTP, can be induced by either an increase in p or RRP. The difference in the presynaptic mechanism can be important from a functional perspective. Although the calyx of Held typically fires prolonged
high-frequency trains of action potentials, the firing patterns differ before and after hearing onset. Prior to the opening of the ear canal, calyces fire short bursts of action potentials at <100 Hz (Sonntag et al., 2009). This developmental age coincides with the time when PTP is predominantly mediated by changes in $p$, which results in more rapidly depressing evoked responses. Because prolonged firing activity ensues after hearing onset, such a mechanism of PTP may not be effective for maintaining signal transmission during longer high-frequency firing. Thus, increasing RRP during short-term plasticity in older animals could help to enhance release during sustained activity.

**Comparison of calyx findings to other CNS synapses**

The calyx of Held appears to be a highly specialized synapse due to the size and morphology of its large presynaptic terminal. Because the calyx and MNTB principal cells allow for simultaneous pre- and postsynaptic recordings, this structure has become a model synapse for the study of synaptic transmission. The calyx has approximately 300-400 active zones (Satzler et al., 2002; Smith et al., 1998; Taschenberger et al., 2002) compared to only one or a few active zones at conventional bouton-like synapses in the hippocampus, cerebellum, and cortex. These synapses in the mammalian brain are typically smaller (diameter <1 μm), and have small EPSCs with lower release probability. Despite its large size and quantal output, the calyx acts similarly to smaller fast synapses of the CNS. Electron microscopy studies show that the individual active zones of the calyx are morphologically similar to those of conventional nerve terminals (Lenn and Reese, 1966; Smith et al., 1998). Similar to other excitatory CNS synapses, the calyx releases glutamate with presynaptic stimulation, contains spherical clear-core vesicles (Satzler et al., 2002), and have multiple types of voltage-gated $\text{Ca}^{2+}$ channels that mediate neurotransmitter release (Iwasaki et al., 2000; Wu et al., 1999). In many respects, the calyx functionally resembles other conventional fast synapses, suggesting that experimental results observed at the calyx may have a general significance in the CNS.
Concluding remarks

The work in this thesis led to the discovery and confirmation that PKC_{Ca} isoforms are necessary for two forms of short-term synaptic plasticity. These findings elucidated the roles of Ca^{2+}-dependent PKC isoforms in PTP throughout development, and revealed how similar isoforms of the same protein can produce PTP through different presynaptic mechanisms. Importantly, we showed that the specific identity of the Ca^{2+}-dependent PKC isoform that mediates PTP controls the mechanism and functional consequences of PTP. The molecular mechanisms of short-term plasticity at the calyx may be extended to that of more conventional synapses, as bouton-like terminals can also change their synaptic properties after use-dependent activity.

There are several important questions that remain to be investigated. What are the downstream substrates of PKC in mediating short-term synaptic plasticity? What are the functional roles of PTP in the auditory brainstem? Investigating the role of downstream effectors of PKC_{Ca} in PTP will allow us to fine-tune our manipulations of the PTP molecular cascade, and thus abolish PTP without perturbing more global pathways associated with PKC during development. These results will pave the way for future behavioral studies where short-term plasticity can be spatially and temporally altered during development.
References


Appendix

Supplemental Figures and Tables

Supplementary Figure S1

Genetic deletion of PKCαβ does not affect basal properties of spontaneous transmission.

(A-B), Plots of basal miniature EPSC amplitude (A) and frequency (B) for wildtype (black), PKCα-/- (green), PKCβ-/- (red), and PKCα-/-β-/- (purple) groups.
Supplementary Figure S2

Minimal contribution of receptor desensitization and saturation to PTP. Plots of normalized EPSC amplitude as a function of time, before and after tetanic stimulation (inverted triangles), for wildtype (black) and double knockout (purple) groups. Blocking AMPA receptor saturation and desensitization with kynurenate (KYN) and cyclothiazide (CTZ), respectively (filled symbols), left PTP largely unaffected, suggesting that the impairment of PTP in double knockout animals is not due to these postsynaptic factors.
Supplementary Figure S3

Inhibiting myosin light chain kinase does not significantly affect basal properties of synaptic transmission in slices from wildtype and PKCαβ double knockout mice. Basal values for $f_0$, $\Sigma EPSC_0$, and slope are plotted for wildtype (black) and double knockout (purple) groups, following treatment with vehicle (open bars) or ML9 (filled bars).
Supplemental Figure S4

Basal properties of synaptic transmission for different knockout animals and in the presence of different PKC inhibitors for pre-hearing (A-D) and hearing (E-H) animals. (A, E) EPSC amplitude measured in standard extracellular solution at a holding potential of -60 mV. (B, F) The basal paired-pulse ratio (Δt = 20 ms) measured in all animal groups in standard extracellular solution. (C, D, G, H) Experiments performed in the presence of CTZ and kynurenate, as in Figure 2, were used to determine RRP₀ (C, G) and p₀ (D, H). Pre-hearing animals were P8-10 and hearing animals were P16-19, with the exception of the wildtype + PKCy-YFP (P19-21). N=8-21 for each condition, bars are mean ± SEM, and there was no significant differences using one-way ANOVA tests in any of the parameters measured for each age group.
Supplemental Figure S5

Estimating the contribution of RRP and $p$ to PTP in calyces from pre-hearing animals.

Representative experiments are shown that were used to assess the contributions of RRP and $p$ to PTP in P8-10 animals for PKCαβ knockout animals (A), wildtype animals in the presence of a PKCβ inhibitor (B), and PKCy knockout animals (C). Experiments were performed in the presence of CTZ and kynurenate to prevent AMPA receptor saturation and desensitization. Synaptic currents evoked by the first 40 stimuli of 4s, 100 Hz train (top, left traces) and by a 40 pulse 100 Hz train (bottom, left traces) 10 s after tetanic stimulation (at the peak of PTP) are shown. Graphs on the right show cumulative EPSCs plotted as a function of stimulus number. The linear fit (gray lines) to the last 15 points was back-extrapolated to the y-axis to calculate the $\Sigma$EPSC, which provides a measure of the RRP$\text{}_{\text{train}}$. Cumulative EPSCs for the first 40 stimuli of the tetanic train (closed circles) and for the second 40-pulse, 100 Hz train (open circles) are shown for representative cells from a PKCαβ knockout animals (A, right), wildtype animals in the presence of a PKCβ inhibitor (B, right), and PKCy knockout animals (C, right).
Supplemental Figure S5 (continued)

A  PKC\(\alpha\beta\) ko

B  wildtype + PKC\(\beta\) inhibitor

C  PKC\(\gamma\) ko
Supplemental Figure S6

Presynaptic calcium measurements at the calyx of Held from pre-hearing animals.

Calyces were bulk loaded with Calcium Green-1 dextran and Alexa 594. R is equal to the ratio of green to red fluorescence. (A) Calibration parameters are shown for calyces from P8-10 wildtype (wt) and PKCγ ko animals. \( R_{\text{rest}} \) is the ratio of a calyx measured in the absence of stimulation. \( R_{\text{train}} \) was average ratio in a calyx (from 3.90 to 3.95 s after the onset of stimulation) measured during a 4 s, 100 Hz train and \( R_{\text{max}} \) ionomycin was measured in calyces following the bath application of ionomycin. Values are normalized to the maximum fluorescence \( (R_{\text{max}} \text{pipette}) \), which was obtained with a sealed pipette containing 10 mM CaCl\(_2\). \( R_{\text{min}} \) was determined in a similar manner, but using a solution with 10 mM EGTA substituted for CaCl\(_2\).

(B) Comparison of the resting \( \text{Ca}^{2+} \) concentration, the amplitude of \( \text{Ca}^{2+} \) transients evoked by a single stimulus \( (\Delta \text{Ca}_1) \), the decay time constant of single \( \text{Ca}^{2+} \) transients \( (\tau_1) \), and the decay time constant \( (\tau) \) of \( \text{Ca}_{\text{res}} \) following tetanic stimulation for wt and PKCγ ko animals. (C) Representative example showing \( \text{Ca}^{2+} \) measurements from the P9 calyx from a wildtype animal. The calyx was stimulated at 0.2 Hz before and after tetanic stimulation (4 s, 100 Hz, ▼). Individual \( \text{Ca}^{2+} \) transient \( (\text{left}) \) were measured for 512 ms with a 4.5 s gap between measurements, and for display purposes the plot does not reflect the gap between \( \text{Ca}^{2+} \) measurements. Plot of the \( \text{Ca}_{\text{res}} \) \( (\text{top, right}) \) and the normalized amplitude of the \( \text{Ca}^{2+} \) transient \( (\text{bottom, right}) \) are shown. (D) Similar to C but for a calyx from a P10 PKCγ ko animal.
Supplemental Table S1

**Magnitude of PTP.** The number of cells (n) is listed next to each animal group for P8-10 animals (bottom) and P16-19 animals (top). Experiments were conducted in the absence of cyclothiazide and kynurenate. All values reported are mean percent changes ± SEM.

<table>
<thead>
<tr>
<th>Animal group (n)</th>
<th>PTP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P16-19</strong></td>
<td></td>
</tr>
<tr>
<td>Wildtype (10)</td>
<td>57 ± 11</td>
</tr>
<tr>
<td>PKCαβ ko (12)</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>Wildtype + PKCβ inhibitor (7)</td>
<td>12 ± 1</td>
</tr>
<tr>
<td><strong>P8-10</strong></td>
<td></td>
</tr>
<tr>
<td>Wildtype (12)</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>PKCαβ ko (13)</td>
<td>40 ± 9</td>
</tr>
<tr>
<td>PKCγ ko (14)</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>PKCαβγ ko (17)</td>
<td>12 ± 9</td>
</tr>
<tr>
<td>Wildtype + PKCβ inhibitor (10)</td>
<td>59 ± 15</td>
</tr>
<tr>
<td>PKCαγ ko (10)</td>
<td>61 ± 11</td>
</tr>
<tr>
<td>PKCαγ ko + PKCβ inhibitor (12)</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>PKCαβ ko + PKCβ inhibitor (3)</td>
<td>54 ± 15</td>
</tr>
<tr>
<td>PKCαβ ko + pan-PKC inhibitor (11)</td>
<td>14 ± 3</td>
</tr>
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</table>
Supplemental Table S2

Contributions of RRP and $p$ to PTP quantified using three different methods. Table summarizes the magnitude of PTP and the contributions of pool size (RRP) and release probability ($p$) for all animal groups. RRP and $p$ data were calculated using the cumulative EPSC method (RRP$_{train}$, $p_{train}$), the corrected EPSC method (RRP$_{trainC}$, $p_{trainC}$) and the Elmqvist and Quastel method (RRP$_{EQ}$, $p_{EQ}$) for P8-10 animals (bottom) and P16-19 animals (top). The number of cells (n) is listed next to each animal group. These are different experiments than those summarized in Table S1, and all experiments were conducted in the presence of cyclothiazide and kynurenate. All values reported are mean percent changes ± SEM.

<table>
<thead>
<tr>
<th>Animal group (n)</th>
<th>PTP</th>
<th>RRP$_{train}$</th>
<th>$p_{train}$</th>
<th>RRP$_{trainC}$</th>
<th>$p_{trainC}$</th>
<th>RRP$_{EQ}$</th>
<th>$p_{EQ}$</th>
<th>PPR</th>
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<tr>
<td><strong>P16-19</strong></td>
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<td></td>
</tr>
<tr>
<td>Wildtype (8)</td>
<td>52 ± 9</td>
<td>40 ± 9</td>
<td>9 ± 5</td>
<td>34 ± 9</td>
<td>15 ± 6</td>
<td>30 ± 7</td>
<td>18 ± 5</td>
<td>-14 ± 5</td>
</tr>
<tr>
<td>PKCαβ ko (12)</td>
<td>19 ± 6</td>
<td>7 ± 5</td>
<td>17 ± 6</td>
<td>4 ± 4</td>
<td>21 ± 7</td>
<td>9 ± 5</td>
<td>17 ± 8</td>
<td>-20 ± 17</td>
</tr>
<tr>
<td>Wildtype + PKCγ-YFP (9)</td>
<td>75 ± 9</td>
<td>22 ± 6</td>
<td>63 ± 11</td>
<td>11 ± 5</td>
<td>58 ± 5</td>
<td>2 ± 4</td>
<td>74 ± 13</td>
<td>-35 ± 3</td>
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<tr>
<td>Wildtype + no PKCγ-YFP (3)</td>
<td>60 ± 7</td>
<td>52 ± 12</td>
<td>6 ± 7</td>
<td>45 ± 10</td>
<td>11 ± 7</td>
<td>25 ± 6</td>
<td>20 ± 5</td>
<td>-18 ± 1</td>
</tr>
<tr>
<td><strong>P8-10</strong></td>
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<tr>
<td>Wildtype (10)</td>
<td>67 ± 16</td>
<td>21 ± 6</td>
<td>37 ± 11</td>
<td>18 ± 6</td>
<td>42 ± 12</td>
<td>11 ± 5</td>
<td>52 ± 15</td>
<td>-42 ± 8</td>
</tr>
<tr>
<td>PKCαβ ko (9)</td>
<td>42 ± 11</td>
<td>-0.4 ± 4</td>
<td>42 ± 7</td>
<td>-2 ± 4</td>
<td>45 ± 9</td>
<td>2 ± 3</td>
<td>45 ± 10</td>
<td>-35 ± 6</td>
</tr>
<tr>
<td>PKCγ ko (8)</td>
<td>64 ± 11</td>
<td>36 ± 12</td>
<td>21 ± 10</td>
<td>37 ± 13</td>
<td>20 ± 11</td>
<td>38 ± 13</td>
<td>17 ± 12</td>
<td>-12 ± 7</td>
</tr>
<tr>
<td>PKCαβγ (4)</td>
<td>7 ± 12</td>
<td>5 ± 7</td>
<td>6 ± 17</td>
<td>7 ± 10</td>
<td>5 ± 18</td>
<td>5 ± 6</td>
<td>4 ± 16</td>
<td>-6 ± 2</td>
</tr>
<tr>
<td>Wildtype + PKCαβ inhibitor (14)</td>
<td>56 ± 17</td>
<td>9 ± 5</td>
<td>44 ± 16</td>
<td>8 ± 5</td>
<td>47 ± 16</td>
<td>9 ± 4</td>
<td>44 ± 15</td>
<td>-34 ± 5</td>
</tr>
</tbody>
</table>
Supplemental Figure S7

At the functionally mature calyx of Held, PKCα does not contribute to PTP but plays a small role in phorbol ester-induced potentiation. PTP (A) and PDBu-induced potentiation (B) are plotted as a function of time (mean ± SEM), for wildtype (black) and PKCα ko (red) groups. C-D, Summary plots (mean ± SEM) of peak PTP (10 s after tetanus; C) and steady-state PDBu-induced potentiation (430-600 s in PDBu; D) for indicated groups.
Supplemental Figure S8

**PKCα isoforms do not regulate basal synaptic properties.** Box-plots of basal synaptic properties for wildtype (*black*), PKCαβ dko (*purple*), and PKCαβ dko groups expressing either wildtype PKCβ (*βWT-YFP; green*) or D-to-A mutant PKCβ (*βD/A-YFP; blue*). **A**, Basal EPSC amplitude; **B**, Basal PPR; **C**, Basal miniature EPSC (mEPSC) amplitude; **D**, Basal mEPSC frequency. Medians and interquartile ranges (Q3-Q1) are shown. Whiskers extend to max and min values for each group. Box-plots were used to illustrate the full data range of each group; additionally, the data sets in C were not normally distributed.
Supplemental Figure S9

Determining the contributions of RRP and $p$ in wildtype and rescued PTP at the functionally mature calyx of Held. Synaptic mechanisms of PTP were examined using stimulus trains in the presence of kynurenate and CTZ. A-C, Example synaptic currents evoked by the first 40 stimuli of a 4s, 100 Hz train (dark traces) and by a 40-pulse 100-Hz train (light traces) at the peak of PTP are shown for wildtype group (A) and PKC$\alpha$$\beta$ dko groups expressing wildtype PKC$\beta$ ($\beta^{WT}$-YFP; C). B-D, The change in RRP size and $p$ for the examples shown in A and C were determined using the train method (see Materials and Methods for methodology). E, Box-plots of basal RRP size (RRP1; left) and basal $p$ ($p_1$; right), estimated from the 1st AP train for wildtype group (black) and $\alpha$$\beta$ dko group expressing $\beta^{WT}$-YFP (green). Medians and interquartile ranges (Q3-Q1) are shown. Whiskers extend to max and min values for each group. Box-plots were used to illustrate the full data range of each group. F, Cumulative histograms of changes in RRP (RRP2/RRP1) and $p$ ($p_2/p_1$) with PTP.
Supplemental Figure S9 (continued)
Supplemental Figure S10. Protein sequence alignment for PKCβ C2^{WT} and C2^{D/A}.
**Supplemental Table S3.** Top of the table shows the summary of results for PTP and phorbol ester (PDBu) wash-in experiments for all animal groups used for Chapter 5. Bottom of the table depicts the summary of basal properties of synaptic transmission for similar animal groups.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean ± SEM</th>
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</thead>
<tbody>
<tr>
<td><strong>PTP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>16</td>
<td>62 ± 12 %</td>
</tr>
<tr>
<td>α ko</td>
<td>7</td>
<td>67 ± 12 %</td>
</tr>
<tr>
<td>αβ dko</td>
<td>15</td>
<td>2.4 ± 1.8 %</td>
</tr>
<tr>
<td>αβ dko + PKCβ^WT^-YFP</td>
<td>24</td>
<td>61 ± 7 %</td>
</tr>
<tr>
<td>αβ dko + PKCβ^D/A^-YFP</td>
<td>16</td>
<td>3.6 ± 2.2 %</td>
</tr>
<tr>
<td><strong>overall p &lt; 0.001</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt vs. α ko: p = 0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt vs. αβ dko: p &lt; 0.01</td>
<td></td>
<td></td>
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<tr>
<td>wt vs. αβ dko + PKCβ^WT^-YFP: p = 0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt vs. αβ dko + PKCβ^D/A^-YFP: p &lt; 0.01</td>
<td></td>
<td></td>
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<tr>
<td>αβ dko vs. αβ dko + PKCβ^WT^-YFP: p &lt; 0.01</td>
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<td></td>
</tr>
<tr>
<td>αβ dko vs. αβ dko + PKCβ^D/A^-YFP: p = 0.91</td>
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<tr>
<td><strong>PDBu</strong></td>
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</tr>
<tr>
<td>wt</td>
<td>8</td>
<td>97 ± 12 %</td>
</tr>
<tr>
<td>α ko</td>
<td>9</td>
<td>60 ± 8 %</td>
</tr>
<tr>
<td>αβ dko</td>
<td>8</td>
<td>3.2 ± 3.4 %</td>
</tr>
<tr>
<td>αβ dko + PKCβ^WT^-YFP</td>
<td>11</td>
<td>84 ± 11 %</td>
</tr>
<tr>
<td>αβ dko + PKCβ^D/A^-YFP</td>
<td>7</td>
<td>98 ± 23 %</td>
</tr>
<tr>
<td><strong>overall p &lt; 0.001</strong></td>
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</tr>
<tr>
<td>wt vs. α ko: p = 0.04</td>
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<tr>
<td>wt vs. αβ dko: p &lt; 0.01</td>
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<tr>
<td>wt vs. αβ dko + PKCβ^WT^-YFP: p = 0.43</td>
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<td>wt vs. αβ dko + PKCβ^D/A^-YFP: p = 0.99</td>
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<td>αβ dko vs. αβ dko + PKCβ^WT^-YFP: p &lt; 0.01</td>
<td></td>
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<tr>
<td>αβ dko vs. αβ dko + PKCβ^D/A^-YFP: p &lt; 0.01</td>
<td></td>
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<tr>
<td><strong>PPR ratio</strong></td>
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<td></td>
</tr>
<tr>
<td>wt</td>
<td>9</td>
<td>1.12 ± 0.19</td>
</tr>
<tr>
<td>αβ dko + PKCβ^WT^-YFP</td>
<td>9</td>
<td>0.99 ± 0.04</td>
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<td><strong>p = 0.49</strong></td>
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<tr>
<td><strong>RRP ratio</strong></td>
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<tr>
<td>wt</td>
<td>9</td>
<td>37 ± 9 %</td>
</tr>
<tr>
<td>αβ dko + PKCβ^WT^-YFP</td>
<td>9</td>
<td>39 ± 12 %</td>
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<td><strong>p = 0.88</strong></td>
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<td><strong>Release probability (p) ratio</strong></td>
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<tr>
<td>wt</td>
<td>9</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>αβ dko + PKCβ^WT^-YFP</td>
<td>9</td>
<td>1.1 ± 0.06</td>
</tr>
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<td><strong>p = 0.78</strong></td>
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**Supplemental Table S4.** Summary of results for basal synaptic properties for various animal groups used in experiments from Chapter 5.

<table>
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<th>N</th>
<th>Mean ± SEM</th>
<th>Median ± IQR</th>
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<td><strong>Basal EPSC (pA)</strong></td>
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<tr>
<td>wt</td>
<td>17</td>
<td>5127 ± 698</td>
<td>4233 ± 3096</td>
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<tr>
<td>qβ dko</td>
<td>14</td>
<td>6388 ± 282</td>
<td>6187 ± 859</td>
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<tr>
<td>qβ dko + PKCB&lt;sup&gt;WT&lt;/sup&gt; -YFP</td>
<td>32</td>
<td>4757 ± 381</td>
<td>4417 ± 2078</td>
</tr>
<tr>
<td>qβ dko + PKCB&lt;sup&gt;D/A&lt;/sup&gt; -YFP</td>
<td>16</td>
<td>4528 ± 533</td>
<td>4330 ± 2087</td>
</tr>
<tr>
<td>p = 0.09</td>
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<td></td>
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<tr>
<td><strong>Basal PPR</strong></td>
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<tr>
<td>wt</td>
<td>17</td>
<td>0.90 ± 0.03</td>
<td>0.87 ± 0.10</td>
</tr>
<tr>
<td>qβ dko</td>
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<td>0.91 ± 0.02</td>
<td>0.90 ± 0.10</td>
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<td>0.89 ± 0.02</td>
<td>0.93 ± 0.09</td>
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<td>qβ dko + PKCB&lt;sup&gt;D/A&lt;/sup&gt; -YFP</td>
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<td>0.95 ± 0.02</td>
<td>0.94 ± 0.10</td>
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<td><strong>Basal mEPSC amplitude (pA)</strong></td>
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<tr>
<td>wt</td>
<td>8</td>
<td>23.5 ± 8.0</td>
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<td>qβ dko</td>
<td>8</td>
<td>28.4 ± 3.1</td>
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<tr>
<td>qβ dko + PKCB&lt;sup&gt;WT&lt;/sup&gt; -YFP</td>
<td>12</td>
<td>24.9 ± 6.4</td>
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<td>qβ dko + PKCB&lt;sup&gt;D/A&lt;/sup&gt; -YFP</td>
<td>6</td>
<td>21.3 ± 2.3</td>
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<td>p = 0.13</td>
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<td>wt</td>
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<td>4.0 ± 1.5</td>
<td>2.8 ± 2.4</td>
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<tr>
<td>qβ dko</td>
<td>8</td>
<td>4.5 ± 0.8</td>
<td>4.1 ± 2.2</td>
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<tr>
<td>qβ dko + PKCB&lt;sup&gt;WT&lt;/sup&gt; -YFP</td>
<td>12</td>
<td>5.0 ± 1.4</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>qβ dko + PKCB&lt;sup&gt;D/A&lt;/sup&gt; -YFP</td>
<td>6</td>
<td>3.1 ± 0.6</td>
<td>2.7 ± 2.0</td>
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<tr>
<td>p = 0.78</td>
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<td><strong>Basal RRP size (pA)</strong></td>
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<td>wt</td>
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<td>15428 ± 2925</td>
<td>13877 ± 7565</td>
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<td>qβ dko + PKCB&lt;sup&gt;WT&lt;/sup&gt; -YFP</td>
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<td>11711 ± 2542</td>
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<td>wt</td>
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<td>0.27 ± 0.07</td>
<td>0.19 ± 0.19</td>
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<td>qβ dko + PKCB&lt;sup&gt;WT&lt;/sup&gt; -YFP</td>
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<td>0.29 ± 0.02</td>
<td>0.29 ± 0.05</td>
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<td>p = 0.80</td>
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