MODULATION OF $GABA_A$ RECEPTOR FUNCTION BY PKA AND PKC PROTEIN PHOSPHORYLATION

By

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To Xiaofei and my parents, for supporting me all the time.

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CHAPTER I

INTRODUCTION

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GABA is important for inhibitory signaling in the CNS

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS In GABAergic neurons, glutamate is decarboxylated to form GABA by glutamic acid decarboxylase (GAD). Once synthesized, GABA is packaged into presynaptic vesicles and then released into the synaptic cleft where it binds to postsynaptic receptors and is taken up by GABA transporters (GATs). Although GABA can be transported rapidly by GATs into presynaptic terminals or into neighboring glial cells, where it is metabolized by GABA-transaminase (GABA-T), there is some GABA spillover from synaptic to extrasynaptic sites. Three classes of GABA receptors, termed $GABA_A$, $GABA_B$ and $GABA_C$ receptors, mediate GABAergic transmission. Among these receptors, $GABA_A$ and $GABA_C$ receptors are ionotropic GABA receptors and belong to the superfamily of ligand-gated ion channels (LGICs), while $GABA_B$ receptors are metabotropic G-protein-coupled receptors (Bormann, 2000).

In adult brain, $GABA_A$ receptor channels are activated by GABA to selectively conduct chloride ion influx through its pore, resulting in neuronal

hyperpolarization. This causes an inhibitory effect on neurotransmission by decreasing the chance of evoking action potentials. However, early in development, GABAergic synaptic transmission is excitatory. The nature of GABAergic transmission depends on the intra- and extracellular concentration of Cl^-, which sets the reversal potential for GABAergic currents. The shift from excitatory to inhibitory transmission is coupled to a developmental induction of the expression of the neuronal (Cl^-)-extruding K^+/Cl^- co-transporter (KCC2), which cause a significant drop of intracelluar Cl^- concentration (Ganguly et al., 2001;Rivera et al., 1999).

Brain function is based on an exquisite balance between excitatory and inhibitory neurotransmission. Aside from the natural agonist GABA, $GABA_A$ receptors are also regulated by many allosteric modulators including benzodiazepines (BDZs), barbiturates, anesthetics, neurosteroids, anti-convulsants and ethanol (Macdonald and Olsen, 1994;Hevers and Luddens, 1998). GABAergic signaling is essential to set the inhibitory tone of neurons in the brain by controlling spike timing and sculpting neuronal rhythms. Impaired GABA signaling has been implicated in a wide variety of CNS disorders including epilepsy, anxiety, cognitive disorders, insomnia, and drug addiction (Johnston, 2005;Mohler, 2006;Moult, 2009). Therefore, many receptor modulators may be targeted as potential therapeutic drugs.

$GABA_A$ receptors mediate phasic and tonic inhibition in CNS

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the adult mammalian CNS. Two types of inhibitory neurotransmission, phasic and tonic

inhibition, are mediated by $GABA_A$ receptors (Farrant et al., 2005) (Figure 1.1). Phasic inhibition is produced by rapid, synchronous activation of $GABA_A$ receptors at

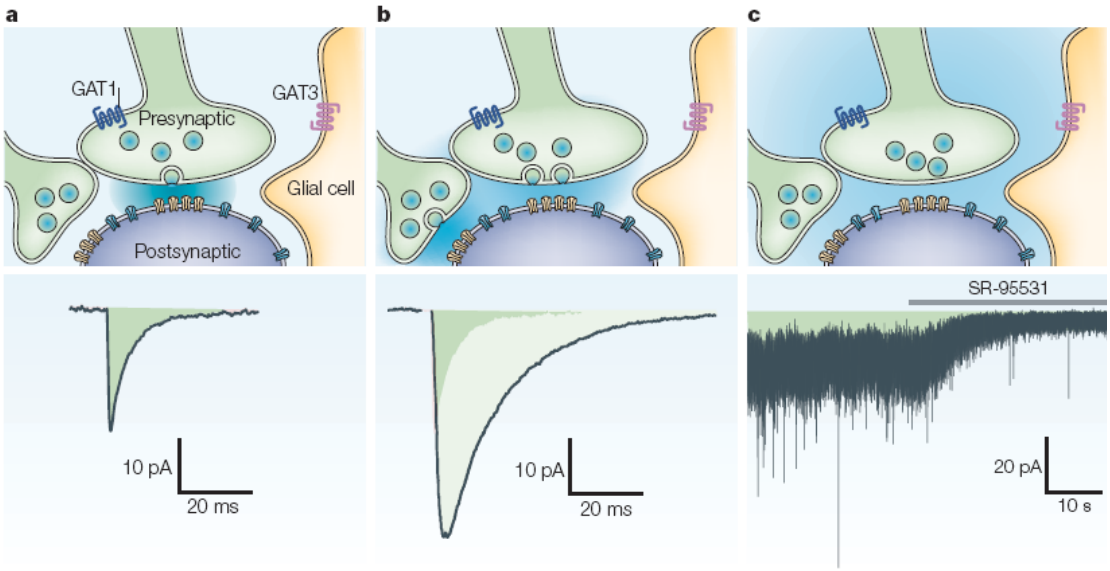

Figure 1.1. Modes of $GABA_A$ receptor activation.

A, The release of a single vesicle from a presynaptic terminal activates only those $GABA_A$ receptors that are clustered in the postsynaptic membrane immediately beneath the release site (yellow). The diffuse blue shading indicates the spread of released GABA. The current record shows an averaged waveform of miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of the sodium channel blocker tetrodotoxin. The area beneath the record is shaded to indicate the charge transfer. After synaptic release, GABA is quickly up-taken by GABA transporters (GATs). **B,** Action potential-dependent release of multiple vesicles or evoked release from several terminals promotes GABA 'spillover' and activates both synaptic receptors and perisynaptic or extrasynaptic receptors (blue). The current record shows the larger and much slower averaged waveform of IPSCs evoked by electrical stimulation. The area of the mIPSC is superimposed for comparison. **C,** A Low concentrations of ambient GABA, which persist despite the activity of the neuronal and glial GABA transporters (GAT1 and GAT3), tonically activates high-affinity extrasynaptic receptors. The trace shows the 'noisy' tonic current that results from stochastic opening of these high-affinity $GABA_A$ receptors, with superimposed phasic currents. A high concentration (10 μM) of the $GABA_A$ antagonist gabazine (SR-95531) blocks the phasic IPSCs and tonic channel activity, causing a change in the 'holding' current and a reduction in current variance. The shaded area beneath the current record before SR-95531 application represents the charge carried by tonically active $GABA_A$ receptors. The current records are from whole-cell patch-clamp recordings of granule cells in acute cerebellar slices from adult mice. The recordings were made with symmetrical chloride concentrations at a holding voltage of –70mV and a temperature of 25°C. pA

synapses by action-potential-dependent release of high concentrations of GABA from presynaptic terminals; in contrast, tonic inhibition is produced by persistent activation of $GABA_A$ receptors outside of synapses at low ambient GABA concentrations that is due to spillover from the synapses or release from glia. Phasic and tonic inhibition play different, but important, roles in regulating neuronal and network excitability. Phasic inhibition is important for timing-based signaling, setting the temporal window for synaptic integration and synchronizing networks of neurons. Tonic inhibition plays an important role in maintaining baseline inhibitory tone and neuronal membrane input resistance.

Phasic and tonic currents have been shown to have different pharmacological and current kinetic properties. Specific receptor populations are expressed and localized in different brain regions and have different subcellular localizations, which allow phasic and tonic currents mediated by different receptor subtype to have different sensitivity to GABA and other modulators like BDZs and neurosteroids. The majority of native $GABA_A$ receptor isoforms are thought to be composed of $\alpha\beta\gamma$ or $\alpha\beta\delta$ subunit combinations with distinct functional and pharmacological properties. $\alpha\beta\delta$ receptors are commonly found in peri- and extrasynaptic locations and contribute to tonic inhibition with high GABA sensitivity. Synaptic $GABA_A$ receptor currents are mostly mediated by $\alpha\beta\gamma$ receptors as γ subunits contribute to synaptic targeting of $GABA_A$ receptors by associated proteins such as $GABA_A$ receptor-associated protein (GABARAP) (Wang et al., 1999) and gephryin (Kneussel and Betz, 2000). However, some γ subunit-containing receptors, like $\alpha5\beta3\gamma2$ receptors in hippocampal CA1

pyramidal neurons, are also found in extrasynaptic regions and are involved in conducting tonic currents in those brain areas (Caraiscos et al., 2004). For tonic currents mediated by γ subunit-containing receptors, it is also possible that synaptic γ subunit-containing receptors can respond to both local synaptically released GABA and to ambient extracellular GABA.

Expression and regional CNS localization of $GABA_A$ receptor subunit subtypes

$GABA_A$ receptors belong to the cys-loop superfamily of ligand-gated ion channels (LGICs) that also includes nicotinic acetylcholine (nACh) receptors, 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors, glycine receptors and $GABA_C$ receptors .Being heteropentameric LGICs, $GABA_A$ receptors are assembled from seven subunit families: α1-6, β1-3, γ1-3, δ, ε, θ and π (Macdonald and Olsen, 1994;Hedblom and Kirkness, 1997;Davies et al., 1997;Bonnert et al., 1999;Mehta and Ticku, 1999). There is a high degree of sequence identity (70-80%) within each subunit family. The sequence identity among different subunit families is around 30-40%. About 20-30% sequence homology exists among all $GABA_A$ receptor subunit and other Cys-loop receptor families (Burt and Kamatchi, 1991;Macdonald and Olsen, 1994).

The expression pattern of $GABA_A$ receptor subunits has been studied in adult rat brain using immunohistochemical staining (Pirker et al, 2000). Among α subunit subtypes, the α1 subtype was widely expressed, while the other α subtypes were more confined to certain brain areas. The α2 subtype was preferentially located in forebrain

areas and the cerebellum. The α3 subtype was found in the olfactory bulb, septal and basal forebrain areas, hippocampus, hypothalamus, and amygdala. The α4 subtype was most prominent in thalamus and dentate gyrus, and then in striatum and cortex. The α5 subtype expression was highest in hippocampus, the olfactory bulb and hypothalamus. And the α6 subtype was only present in cerebellum and cochlear nucleus. All three β subtypes were widely distributed in the brain, notably in the cerebral cortex. And β2 subtype was found distributed similarly as α1 subtypes in many interneurons throughout the brain. Among all the γ subtypes, the γ2 subtype was the most highly and widely expressed one in the whole brain except in thalamus. Finally, the δ subunit was found in cerebellum, thalamus, dentate gyrus, striatum and cortex often co-distributed with α4 or α6 subunit.

Although α1β2γ2 receptors are thought to be the dominant receptor isoform in the CNS, α4 subunit-containing receptors are relatively abundant in brain regions involved in both partial and generalized epilepsies, including cortex, hippocampus, and thalamus. Compared to α1 and most other α subunits, α4 and α6 subunit-containing receptors have unique sensitivity to specific allosteric modulators such as diazepam and zinc. Deletion of α4 subunits in engineered mice substantially reduced extrasynaptic, tonic inhibition in thalamus and dentate gyrus, as well as significantly modified frequency and kinetics of mIPSCs in dentate granule cells (DGCs) (Chandra et al., 2006;Liang et al., 2008). The α4 subunits were often assembled with γ or δ subunits in combination with β2/3 subunits as well as without γ and δ subunits. The α4β3δ isoform is predominantly localized peri- and extrasynaptically (Nusser and

Mody, 2002;Wei et al., 2003;Belelli et al., 2005;Cope et al., 2005), while the α4βγ2 isoform has been shown to have both synaptic and extrasynaptic localization in specific brain regions (Hsu et al., 2003;Peng et al., 2004;Liang et al., 2006;Chandra et al., 2006).

In addition to the relative abundance of α4 subunits in brain regions involved in both partial and generalized epilepsies, another interesting feature of α4 subunit-containing receptors is the substantial plasticity of α4 subunit expression in a variety of experimental and pathophysiological situations. The α4 subunit has been shown to be up-regulated in multiple animal models of chronic epilepsy, and the increase in expression was reported to be accompanied by up-regulation of γ2 subunits in some epileptic animals and in δ subunit KO mice (Peng et al., 2002;Cagetti et al., 2003;Peng et al., 2004). The sub-cellular localization of α4 subunits has been shown to be regulated and α4 subunit-containing receptors have been shown to move from extra-synaptic to synaptic locations in the hippocampus (Liang et al., 2006). Due to this intriguing plasticity of α4 subunits, it is important to investigate the intrinsic properties and modulation of α4 subunit-containing receptors, such as α4βγ2 and α4βδ $GABA_A$ receptors. Understanding the differences between α4βγ2 and α4βδ receptors and the differences between α4 subunit-containing and other α subunit-containing receptors will help clarify the functional deficits such as increased seizure susceptibility reported in some pathological situations.

Modulation of ligand-gated ion channels by phosphorylation

Neuronal activity is mediated by changes in electrical activity, which depends on the movement of ions across cell membranes through ion channels. In fact, the patterns of neuronal electrical activity are not static and are subject to dynamic regulation by a variety of external influences. Therefore, modulation of the properties of membrane ion channels is of fundamental importance for the regulation of neuronal electrical activity and of higher neural functions. Protein phosphorylation is the dominant means, among all potential molecular mechanisms, to modulate the properties of ion channels.

In respect of the fact that most ion channels are regulated by protein kinases and phosphatases, protein phosphorylation plays a central role in a wide variety of cellular, metabolic, and signaling processes (Levitan 1994, 1999). Meanwhile, most ion channels can be phosphorylated by several different protein kinases and regulated in different protein kinase signaling pathways, each influencing channel activity in a unique way. Thus, mechanisms of modulation by phosphorylation exhibit a striking diversity that ranges from changes in subunit assembly and receptor aggregation at the synapse to shifts in the voltage-dependence and kinetics of channel activities. The technology of patch-clamp even allowed the identification of particular amino acid residues in ion channel proteins that participate in specific modulatory changes in channel biophysical properties by using engineering mutagenesis.

The research of nACh receptors is the earliest studies revealing regulation of ion channel properties by protein kinases and phosphatases. In early experiments

using Torpedo nACh receptors to study their modulation by phosphorylation, the receptors were found to be a substrate for protein kinase A (PKA), protein kinase C (PKC) and protein tyrosine kinase (PTK). Furthermore, the rate of current desensitization is affected by the status of phosphorylation on nACh receptors in the presence of agonist. Intriguingly, the phosphorylation by PKA also facilitates the assembly of receptors in the plasma membrane and stabilizes receptor subunits. In addition, tyrosine phosphorylation promotes accumulation of nicotinic receptors in arrays resembling postsynaptic receptor aggregation at the vertebrate neuromuscular junction (Ross et al., 1987,1991;Qu et al., 1990). In neuronal nACh receptor channels, which consist of different α and β subunits but having similar overall structure and consensus phosphorylation sequences with their Torpedo and muscle counterparts, similar phenomena were observed. The conserved phosphorylation sites are potent to regulate the amount of functional receptors on plasma membrane and the receptor desensitization rate. These observations suggest the different types of nACh receptor are modulated by a common mechanism of protein phosphorylation..

The other LGICs examined to date also have been shown either to be regulated by protein phosphorylation or to contain consensus sequences for phosphorylation by protein kinases. The two ionotropic glutamate receptors, N-methyl-D-aspartic acid receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs), are also regulated by protein phosphorylation. PKA, PKC, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and various tyrosine kinases are among key protein kinases that regulate NMDAR function. PKC phosphorylation

of serine 890 disrupted surface clusters of the NR1 subunits whereas PKC/PKA phosphorylation of serine 896 and 897 had no effect (Tingley et al., 1997). Phosphorylation of the PKC-sensitive serine sites in the NR1 subunit also prevented calmodulin binding to the NR1 subunit and thereby inhibited inactivation of NMDARs by calmodulin (Hisatsune et al., 1997). Phosphorylation of NR2A or NR2B at either tyrosine or serine sites usually positively regulated NMDAR-mediated currents (Wang and Salter, 1994; Rostas et al., 1996; Liao et al., 2001) and altered NMDAR glutamatergic transmission by regulating receptor trafficking and interactions with various synaptic scaffold/signaling proteins in response to changing inputs (Hall and Soderling, 1997;Dunah et al., 2004).

Like NMDARs, AMPARs are regulated by protein kinases and phosphatases. Phosphorylation of GluR1 subunits on PKA (serine 845) and PKC/CaMKII (serine 831) sites generally potentiated AMPAR currents (Roche et al., 1996; Derkach et al., 1999; Banke et al., 2000), which could contribute to augmentation of the AMPAR-dependent form of synaptic plasticity such as long-term potentiation (LTP). PKA, PKC, and CaMKII phosphorylation of GluR1 and GluR4 also drove AMPARs into synapses (Estaban et al., 2003). AMPARs interact with various intracellular proteins through their C-termini to organize into macromolecular complexes. The C-terminus of the GluR1 subunit interacts with a PDZ domain-containing protein SAP97 (Leonard et al., 1998; Tavalin et al., 2002) and A-kinase-anchoring proteins 79/150 (AKAP79/150) (Snyder et al., 2005), which targets PKA to GluR1 for serine 845 phosphorylation (Leonard et al., 1998; College et al., 2000). In the course of long-

term depression (LTD), SAP97/AKAP/PKA complexes recruit and activate protein phosphatase 2B to dephosphorylate serine 845, leading to AMPAR internalization and the expression of LTD (College et al., 2000; Tavalin et al., 2002). Through a binding motif overlapped with the phosphorylation sites on tyrosine 876 and serine 880, the C-terminus of the GluR2 subunit interacts with multiple scaffold proteins including AMPA receptor binding protein (ABP)/GRIP, ATPase N-ethylmaleimide-sensitive fusion protein or N-ethylmaleimide-sensitive factor (NSF), α- and β-soluble NSF attachment proteins (SNAPs), and protein interacting with C-kinase 1 (PICK1) (Kim and Sheng, 2004). Phosphorylation of GluR2 on tyrosine 876 by tyrosine kinases or serine 880 by PKC decreased the affinity of GluR2 to GRIP (Lau and Huganir, 1995; Carvalho et al., 1999; Chung et al., 2000), which triggered GluR2 internalization and thus reduced surface GluR2 expression. This process is believed to mediate the expression of hippocampal LTD (Seidenman et al., 2003).

Although both $GABA_A$ and glycine receptors are chloride ion channels and mediate inhibitory transmission in the CNS, glycine receptors, which play a prominent inhibitory role in the spinal cord and brainstem, are less well characterized than $GABA_A$ receptors and the other LGIC receptors discussed above (with regard to phosphorylation). Glycine receptors also contain consensus phosphorylation sites and are substrates for several protein kinases. PKA and PKC have both been shown to potentiate glycine receptor currents in response to activation of Gs or Gq coupled pathways in spinal trigeminal neurons and in substantia nigra neurons, respectively (Song and Huang, 1990;Nabekura et al., 1996b). However, single channel recording

indicated that the probability of channel opening or mean open time was altered by activation of PKA or PKC, respectively, in two different studies, accounting for the increase of whole cell currents by the kinases. Controversially, activation of α2-adrenoceptors and its downstream Gi coupled pathway in substantia nigra neurons and in rat sacral dorsal commissural neurons has been reported to increase glycinergic input that is believed to be mediated by inhibition of PKA (Nabekura et al., 1996a;Nabekura et al., 1999), but this is controversial since PKA was activated directly or indirectly by different methods with involvement of different G-protein coupled pathways in those studies. Thus, in substantia nigra neurons, glycine receptors are positively regulated by PKC and negatively regulated by PKA. Regulation of glycine receptor function by PKC is accomplished by activation of $5HT_2$ receptors in rat sacral dorsal horn neurons (Xu et al., 1996) and activation of αl-adrenoceptors in substantia nigra neurons (Nabekura et al., 1996b), which all indicate crosstalk between different receptors in signal transduction.

In summary, complex modulation by protein phosphorylation is generally observed in most ligand-gated ion channels. The molecular mechanisms responsible for the diversity of functional effects are not clear. Nevertheless, it suggests that neurons can finely tune their synapses by altering expression of receptor subunit subtypes and various protein kinases. Changes in receptor desensitization or open-channel probability would have profound effects on the efficacy of synaptic transmission. In addition, regulation of receptor density at the postsynaptic membrane through an alteration in the aggregation of receptors or the local assembly of the

subunits could modify postsynaptic responses. Finally, the ability of other signaling pathways at the synapse to modulate phosphorylation of the receptors allows these properties to be dynamically regulated, resulting in short- or long-term changes in synaptic transmission. To significantly advance our understanding of molecular mechanisms of neuronal plasticity, the next direction is to employ more and more novel approaches and new technology to investigate the ways in which channel modulation contributes to the regulation of cellular physiology in neurons.

Phosphorylation sites of $GABA_A$ receptors

As a member of the cys-loop receptor family, each $GABA_A$ receptor subunit contains a large extracellular N-terminal domain that has a conserved disulfide cysteine loop and one or more glycosylation sites (Figure 1.2). The extracellular N-terminal domain is followed by four membrane-spanning domains connected by either short segments (TM1-TM2 and TM2-TM3) or a long cytoplasmic domain (TM3-TM4). This large intracellular domain, which links the third and fourth transmembrane domains, contains several functional domains such as the sequences interacting with cytoplasmic proteins that are involved in regulation of receptor such as receptor clustering and subcellular localization. There are also consensus sequences for phosphorylation by multiple kinases in this major intracellular loop,

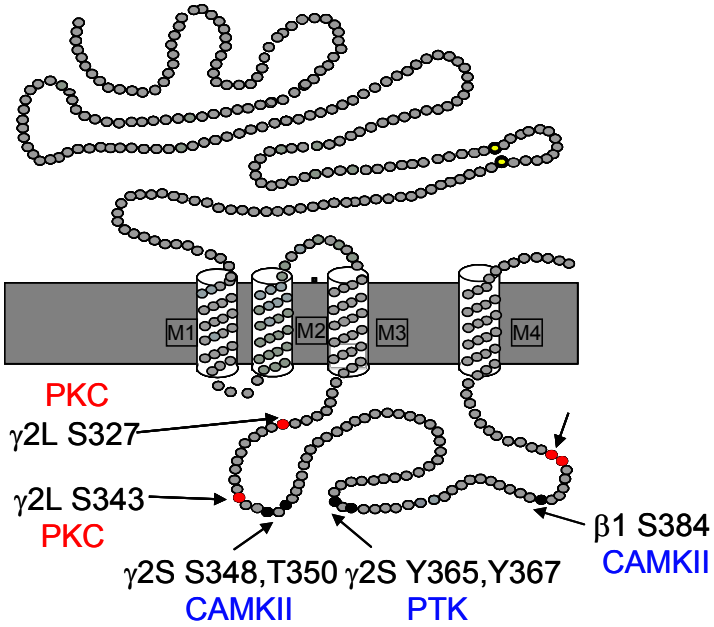

Figure 1.2. Phosphorylation sites in $GABA_A$ receptor subunit.

A schematic presentation of a $GABA_A$ receptor subunit is presented. In the large extracellular N-terminal domain, there is a disulfide bond between two cysteines (yellow) in the conserved cysteine loop. The large intracellular TM3-TM4 loop contains many consensus sequences for phosphorylation by multiple kinases, including PKA, PKC, CaMKII, PTK and cGMP-protein kinase (PKG). Four serines in β3 and γ2L subunits are indicated by red circles.

including PKA, PKC, CaMKII, PTK and cGMP-protein kinase (PKG). Extensive studies using GST-fusion proteins have led to characterization of some major targets for different kinases. Among all the investigated subunits, $GABA_A$ receptor β and γ subunits have been identified as substrates for various protein kinases (Moss et al., 1992;McDonald and Moss, 1997).

To examine the potential phosphorylation sites in mapped consensus sequences, point mutation by site-directed mutagenesis was commonly used to determine if replacing serine or threonine residues could eliminate phosphorylation. For example, in *in vitro* experiments using GST-fusion proteins encoding only the soluble TM3-TM4 intracellular loop of each individual receptor subunit, the

conserved β subunit serines (S409 in β1,β3 and S410 in β2 subunits) were shown to be phosphorylated by several serine/threonine protein kinases including PKA, PKC, PKG and CaMKII (McDonald and Moss, 1994;McDonald and Moss, 1997;Moss et al., 1992). Besides the conserved β subunit serines (S409 of β1,β3 and S410 of β2), additional phosphorylation sites for different protein kinases were identified in the β and γ subunit major intracellular loops (Moss et al., 1992;Krishek et al., 1994;McDonald et al., 1998;Brandon et al., 2000;Brandon et al., 2001). Serine 408 in β3 subunits is also a substrate for PKA and PKC and β1 subunit serine 384 is specifically phosphorylated by CaMKII. The two alternative splice variants γ2L and γ2S, which differ by 8 amino acids (LLRMFSFK), both contain a PKC phosphorylation site at serine 327. However, the γ2L subunit fusion protein could be phosphorylated to a higher stoichiometry due to phosphorylation of serine 343 within the 8 amino acid insertion. The γ2 subunits also contain two CaMKII substrates at serine 348 and threonine 350 and two substrates for PTKs at tyrosine 365 and 367 (residue number in γ2S).

Development of antibodies recognizing specific $GABA_A$ receptor subunits allowed study of phosphorylation of $GABA_A$ receptors in the brain. The phosphorylation patterns of subunits *in vitro* were usually verified *in vivo*. However, some interesting differences were observed. For example, although all three β subunits are substrates of PKA *in vitro*, in recombinant expression systems like HEK cells or in native cortical neurons *in vivo*, β2 subunits were not phosphorylated by PKA, because the adaptor protein AKAP79/150, critical for PKA signaling in

different contexts, selectively targets β1 and β3 subunits but not β2 subunits (Brandon et al., 2003). Thus, for those phosphorylation sites that are substrates of multiple kinases, they are likely phosphorylated by only one protein kinase in a specific cell type. For example, S409 of β1,3 subunits was mainly phosphorylated by PKC rather than by PKA or CaMKII in cortical and striatal neurons (Brandon et al., 1999;Brandon et al., 2003). To be the predominant protein kinase that phosphorylates a given site, the kinase might have a higher affinity for the phosphorylation site or be anchored by some adaptor proteins to facilitate protein-protein interaction.

Dynamic modulation of $GABA_A$ receptors by multiple protein kinases

Diverse effects of protein kinase activation/inhibition were observed in different studies focusing on specific properties of $GABA_A$ receptors. Phosphorylation is involved in the dynamic modulation of $GABA_A$ receptors including subunit expression, current kinetics, pharmacological properties, postsynaptic clustering and, receptor recycling. The effects of activation or inhibition of a variety of protein kinases on $GABA_A$ receptor function have been examined in a range of neuronal preparations. In particular, the effects of PKA and PKC have been studied extensively. In many studies focusing on the synaptic activities of $GABA_A$ receptors, the results are complex and often contradictory, with effects ranging from potentiation to inhibition of GABA-evoked currents (Brandon et al., 2000b; Smart et al., 2001). The variability of reported results reflects the complexity of the modulatory mechanisms of protein phosphorylation and indicates that several

different pathways for receptor modulation might be stimulated simultaneously to different extents and to cause the varying results in different systems. For example, peak current amplitudes can be altered by changes in both ion channel function and surface expression. Receptor function might be modulated by direct phosphorylation of $GABA_A$ subunits, while the modulation of receptor density on the cell surface might be affected by cellular machineries like protein internalization or recycling. In some circumstances, the mechanism can be determined by using proper approaches. Using detailed analysis of phosphorylation modulation of receptor function, the diverse modulatory actions of protein phosphorylation can be understood, and thus, better predictions can be made of the consequence of activation of individual protein kinases under specific *in vivo* conditions.

With neuronal preparations, some of the differences observed in modulation of $GABA_A$ receptor function by protein kinases could be due to extensive receptor heterogeneity in the native environment. Thus, the functional effects of $GABA_A$ receptor phosphorylation have been characterized extensively in heterologous cells, which allow investigation of $GABA_A$ receptors with a relatively uniform composition and also allow determination of direct correlations between the phosphorylation of individual residues and channel function using a mutation strategy. Even though the use of recombinant receptors simplified the studies, modulatory effects by protein kinases were still diverse and complex. Several studies reported that PKA activation decreased $GABA_A$ receptor current (Gyenes et al., 1994;Harrison and Lambert, 1989;Moss et al., 1992;Porter et al., 1990;Chen et al., 1990;Poisbeau et al.,

1999;Capogna et al., 1995;Robello et al., 1993), whereas others reported that PKA enhanced $GABA_A$ receptor current (Angelotti et al., 1993;Feigenspan and Bormann, 1994;Kapur and Macdonald, 1996;Cheun and Yeh, 1992;Cheun and Yeh, 1996;Kano and Konnerth, 1992;Nusser et al., 1999). Activation of PKC by phorbol esters resulted in down-modulation of $GABA_A$ receptors (Kellenberger et al., 1992;Krishek et al., 1994;Leidenheimer et al., 1992;Sigel et al., 1991). However the active catalytic domain of PKC (PKM) enhanced GABA-stimulated currents in L929 cells (Lin et al., 1994;Lin et al., 1996). The γ subunit was found to be specifically phosphorylated by PKC but not by PKA. The γ2L subunit contains one more PKC phosphorylation site, S343 in the alternative spliced 8 amino acid insert, than the γ2S subunit, with one common PKC site S327 within the major intracellular loop that provides a basis for differential regulation of γ2L and γ2S subunit-containing receptors. Indeed, ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the additional PKC site S343 in γ2L subunits. And the postsynaptic clustering of cytoplasmic loops of γ2L, but not γ2S, subunits could be facilitated by PKC at rat spinal cord inhibitory synapses. Finally, phosphorylation of two Tyr residues (Y365/367) in the γ2-subunit by SRC tyrosine kinase enhanced the function of α1β1γ2 and α1β3γ2 $GABA_A$ receptors (Wafford et al., 1992; Moss et al., 1995).

Different effects of PKA activation on peak currents have been proposed to be due to different subunit compositions of $GABA_A$ receptor isoforms employed in different studies. In HEK-293 cells, receptors containing one of the three β subunits (β1-3) in combination with α1 and γ2S subunits were modulated differently by PKA.

Phosphorylation of the β1 subunit Ser409 by PKA resulted in a time-dependent decrease of GABA-induced currents. The β3 subunit differs from the other β subunits in having two adjacent Ser at positions 408 and 409, which, when both are phosphorylated by PKA, caused enhanced GABA-activated current. No functional effects of PKA activation were seen for receptors containing the β2 subunit due to a lack of PKA-dependent phosphorylation of this subunit in HEK-293 cells (McDonald et al., 1998;Brandon et al., 2003). However, specific effects associated with different receptor subunits could not explain everything. In some studies using the same combination of recombinant $GABA_A$ receptor subunits, contradictory effects of PKA on $GABA_A$ receptor currents have been reported (Moss et al., 1992;Angelotti et al., 1993;Hinkle and Macdonald, 2003), suggesting complicated and very plastic mechanisms of PKA modulation in different experimental system. For the conflicting effects of PKC, the presence of different PKC isozymes might provide a basis for the variable effects of PKC activation on $GABA_A$ receptor currents. However, specific PKC isozymes were not identified in those studies with different methods to activate PKC. The fact that both β and γ2 subunits are substrates of PKC could also contribute to diversity of the PKC effect due to different types of crosstalk between two subunits.

Involvement of protein phosphorylation in epilepsy

Epileptogenesis is a complex result of both genetic and acquired factors, expressed as an imbalance between activating systems of neuronal depolarization (excitatory amino acid release with postsynaptic NMDA receptor activation,

spreading depolarization following abnormal progression) and the inhibiting systems of neuronal hyperpolarization (GABAergic synapses). It is widely accepted that a deficiency of GABAergic neurotransmission could contribute to the development of epileptic seizures. The positive allosteric modulators of $GABA_A$ receptors, such as the benzodiazepines, have been used widely to treat epilepsy (Macdonald et al., 2004;Foster and Kemp, 2006).

Abnormalities of protein phosphorylation have been reported in some animal models of epilepsy. For example, the prolonged seizures of status epilepticus (SE) caused decreased phosphorylation of β3 subunits by PKC associated with a deficit in receptor stability on the cell surface (Terunuma et al., 2008), although most previous papers reported elevated PKC activation with both seizures and SE. The explanation for this discrepancy most likely lies in the experimental details. For example, PKC responses to the same event may yield different responses among cell types. Kainate-induced seizures have been reported to reduce PKC mRNA levels in hippocampus but to increase mRNA levels in dentate gyrus (Guglielmetti et al. 1997). There have also been reports of elevated cAMP levels as well as PKA activity in the hippocampus of experimental animal models with epilepsy (Tehrani and Barnes, Jr., 1995;Yechikhov et al., 2001) and repeated injections of an initially subconvulsant dose of cAMP into the rat amygdala has been shown to produce seizures (Yokoyama et al., 1989).

These data suggest that protein phosphorylation by multiple protein kinases may be involved in the pathogenesis of acquired epilepsies, although the relationship between phosphorylation and seizures might not be unidirectional and differ with

various protein kinases. Generally, cellular proteins, such as ion channels or enzymes, are all under constant modulation and regulation by processes that drive their phosphorylation and dephosphorylation. Kinases will phosphorylate proteins by transferring a phosphate group from a molecule of ATP, while phosphatases will remove the phosphate group, thereby dephosphorylating the protein in question. The presence or absence of a phosphate group on a critical amino acid within the protein will alter the configuration of the protein and may have functional consequences to either enhance or impair the activity of the channel or enzyme. Depending on which amino acids are within the target of phosphorylation, the functional impact on the cell could be excitatory or inhibitory. Giving the various results of functional modulation of $GABA_A$ receptors from inhibition to potentiation, we can expect that phosphorylation of $GABA_A$ receptors could have opposite consequences in epileptogenesis. It is possible that impaired activity of $GABA_A$ receptors after protein phosphorylation underlies development or progression of SE; alternatively, prolonged excitation associated with SE may trigger autoregulatory processes, such as preventing excessive excitation by enhancement of GABAergic inhibition by protein phosphorylation. In addition, protein kinases might be temporarily activated by reactions activated during seizures which might reverse to its previous level following seizure termination. Therefore, protein kinase-mediated inhibition of $GABA_A$ receptors could contribute to a mechanism to cause temporary loss of function of ion channels only during seizures with normal ion channel function during interseizure intervals. Thus, further study of protein phosphorylation levels before, during and

after seizures and determination of its role in epileptogenesis may be helpful in developing therapies for epilepsy.

Rationale for experimental design of the thesis

Phosphorylation has been of intense interest for several decades. So far, the effects of phosphorylation by multiple protein kinases of the subunits of the most prevalent α1βγ2 $GABA_A$ receptor have been studied the most. Following identification of specific phosphorylation sites, numerous reports of protein kinase modulation of $GABA_A$ receptor subunits have been made and reveal the complexity of modulation of several properties of $GABA_A$ receptors by multiple protein kinases. The complexity of modulation by protein phosphorylation provides mechanisms for neurons to finely tune their GABAergic inhibition in specific brain areas with different receptor composition in response to various protein kinases. Given the extensive heterogeneity of $GABA_A$ receptors in the native environment and the differential pattern of phosphorylation and modulatory effects associated with different subunits such as β(1-3) subunit subtypes and two γ2(S and L) subunit variants, it has been extremely difficult to explain results obtained using native neuronal preparation composed of a mixture of several subunit subtypes. Therefore, in this dissertation, I studied individual recombinant $GABA_A$ receptors of known subunit composition in a heterologous expression system and compared the modulatory effects of several protein kinases on specific $GABA_A$ receptor isoforms.

It should be noted that most studies of phosphorylation have focused on the effects on synaptic inhibition, using mainly the synaptic α1βγ2 $GABA_A$ receptor isoform. However, other receptor isoforms with more restricted expression areas such as α4 subunit-containing receptors and δ subunit-containing receptors outside synapses have not been studied before. Thus, the first goal of this dissertation was to determine the modulatory effects of two protein kinases, PKA and PKC, on the macroscopic current properties of α4β3γ2 and α4β3δ receptors, which each have very different current properties and localization. As expected, there were differential effects of phosphorylation on α4β3γ2 and α4β3δ $GABA_A$ receptor isoforms, implicating different consequence for synaptic and extrasynaptic events in response to protein phosphorylation. Meanwhile, there is evidence for plasticity of α4 subunits coassembled with γ2 or δ subunits under pathological conditions. Thus, characterization of the differential modulation of these two different receptor isoforms will help also to understand the pathogenesis of diseases such as epilepsy that are due to modification of GABAergic inhibition.

Another novel investigation of this dissertation was to focus on properties of macroscopic currents that have not been studied previously as synaptic phenomena. Activated by saturating concentration of agonist, macroscopic currents were evoked to replicate IPSPs and the effects of phosphorylation on their non-equilibrium properties, including peak amplitude, desensitization and deactivation, were characterized. These non-equilibrium properties are subject to modification by subtle changes in receptor activation or gating, and thus provide the opportunity for regulation by protein kinase

modulation (Jones and Westbrook, 1995, 1996; Haas and Macdonald, 1999; Overstreet et al., 2000; Bianchi and Macdonald, 2002). In contrast, the equilibrium properties of macroscopic currents reflect a stabilized activity that is only attained by sustained exposure of $GABA_A$ receptors to low GABA concentrations in the extrasynaptic space in contrast to the brief, synaptic exposure that occurs following release of GABA during synaptic transmission. The equilibrium properties of $GABA_A$ receptors are also important for GABAergic inhibitory transmission, since they affect the activity levels of neuronal networks that include inhibitory neurons.

It has been clearly demonstrated that protein kinases have diverse effects that are both dependent and independent of receptor phosphorylation, which may explain why some of the effects have not been replicated by mutations that mimic phosphorylation at conserved sites. The last aim of this dissertation is to explore phosphorylation-dependent effects specifically mediated by receptors composed of different subunits, as well as phosphorylation-independent effects that are usually specifically associated with different protein kinases. Using site-directed mutagenesis, I was able to determine the specific effects of modifying the PKA and PKC phosphorylation sites in β or γ subunits to mimic phosphorylation. There are some effects produced by PKA and PKC activation that are demonstrated to be dependent of direct phosphorylation of those specific sites, but many effects of PKA and PKC activation have been shown not to be due simply to direct phosphorylation of the receptor. The results suggested that the direct phosphorylation of β or γ subunits only contributed partial effects by protein kinases. There were other mechanisms beyond

the phosphorylation of those sites that also contributed to PKA- or PKC-specific effects, such as phosphorylation of related proteins involved $GABA_A$ receptor regulatory mechanism. Since most protein kinases, including PKA and PKC, can be recruited to macromolecular protein complexes bound to the receptor by different adaptor proteins, crosstalk between $GABA_A$ receptors and other cellular proteins in the complex might be involved in those protein kinase-specific effects. In the future, it will be beneficial to determine which proteins are in the receptor-protein complex and to characterize their roles in transducing the effects of phosphorylation to clarify mechanisms underlying the specific modulatory actions of different protein kinases.

Based on my preliminary data, I decided to investigate modulation of the current properties of α4β3δ and α4β3γ2L $GABA_A$ receptor isoforms by different protein kinases:

1. Comparison of PKA modulation of α4β3δ and α4β3γ2L $GABA_A$ receptor isoform currents

   Spontaneous current: Chapter II

   GABA-activated tonic current: Chapter II

   Total tonic current: Chapter II

   Phasic current: Chapter III

2. Determine the roles of different subunits in mediating PKC modulation of α4β3γ2L currents kinetics: Chapter IV

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CHAPTER II

MODULATION OF SPONTANEOUS AND GABA-EVOKED TONIC α4β3δ AND α4β3γ2L $GABA_A$ RECEPTOR CURRENTS BY PROTEIN KINASE A

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Abstract

Protein kinase A (PKA) has been reported to regulate synaptic αβγ $GABA_A$ receptor currents, but whether or not PKA regulates $GABA_A$ receptor peri- and extrasynaptic tonic currents is unknown. $GABA_A$ receptors containing α4 subunits are important in mediating tonic inhibition and exist as both α4βδ and α4βγ receptors in the brain. To mimic GABA-independent and GABA-dependent tonic currents, we transfected HEK 293T cells with α4β3δ or α4β3γ2L subunits and recorded spontaneous currents in the absence of applied GABA and steady-state currents in the presence of 1 μM GABA. Both α4β3δ and α4β3γ2L receptors displayed spontaneous currents, but PKA-activation increased spontaneous α4β3δ currents substantially more than spontaneous α4β3γ2L currents. The increase in spontaneous α4β3δ currents was due to an increase in single channel open frequency. In contrast, PKA-activation did not alter steady state, tonic currents recorded in the presence of 1 μM GABA. We concluded that PKA had a GABA concentration-dependent effect on α4β3δ and α4β3γ2L currents. In the absence of GABA, spontaneous α4β3δ, and to a lesser

extent α4β3γ2L currents, could provide a basal, tonic current that could be regulated by PKA. With increasing concentrations of extracellular GABA, however, tonic α4β3δ and α4β3γ2L currents would become more GABA-dependent and less PKA-sensitive. Thus, in brain regions with fluctuating extracellular GABA levels, the dynamic range of GABA-activated tonic currents would be set by PKA, and the increase in tonic current produced by increasing GABA would be reduced by PKA mediated phosphorylation. When ambient GABA reaches micromolar concentrations, PKA would have no effect on steady-state tonic currents.

Introduction

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the adult mammalian CNS and mediate both phasic and tonic inhibition (Farrant and Nusser 2005). Phasic inhibition is produced primarily by rapid, synchronous activation of post-synaptic αβγ receptors with brief pre-synaptic release of high concentrations of GABA, and in contrast, tonic inhibition is produced by persistent activation of peri- and extra-synaptic αβδ receptors at low concentrations of ambient GABA (Essrich et al. 1998; Nusser et al. 1998; Semyanov et al. 2004) as well as some αβγ receptors that are located outside of synapses (Caraiscos et al. 2004; Zhang et al. 2007). Tonic currents play critical roles in regulating neuronal excitability. Time-averaged net charge transferred by peri- or extrasynaptic $GABA_A$ receptors can be larger than time-averaged charge transferred by phasic currents conducted by synaptic receptors. In addition to GABA-activated tonic currents, there is also

evidence that some $GABA_A$ receptor channels conduct persistent spontaneous currents in the absence of GABA (McCartney et al. 2007). In the presence of ambient, extracellular GABA in brain areas where those receptors are expressed, total tonic inhibitory currents could be composed of varying combinations of both GABA-activated current and spontaneous current. In the current study, we studied both types of tonic current, spontaneous currents in the absence of applied GABA and steady-state currents in the presence of a low (1 μM) concentration of GABA. The increase in tonic current produced by applied GABA from the spontaneous baseline current was a measure of GABA-activated tonic current, which reflected the change of tonic currents during fluctuation of extracellular GABA concentration.

Phosphorylation by PKA has been shown to have complicated effects on the kinetic properties of phasic currents, but the effects of PKA on tonic currents have not been reported. In the current study, we examined the effects of PKA on spontaneous and GABA-evoked steady state currents that contribute to tonic inhibition. Tonic inhibition has been shown to be mediated by a number of different $GABA_A$ receptors including those that contain α4 subunits, which are expressed primarily at extra-synaptic sites in thalamus and dentate gyrus (Sun et al. 2004; Jia et al. 2005; Sun et al. 2007). Substantially reduced tonic inhibition was found in both brain areas of α4 subunit knockout mice (Chandra et al. 2006; Liang et al. 2008), suggesting an important role for α4 subunit-containing receptors in mediating tonic inhibition. α4 subunits were found to be co-assembled with β2/3 subunits and often with γ or δ subunits to produce α4βγ2 and α4βδ receptors. These two receptors have been shown

to have distinct physiological and pharmacological properties (Brown et al. 2002; You and Dunn 2007). Comparison of the modulatory effects of PKA on α4β3δ and α4β3γ2L receptor currents may help clarify the pathogenesis of neurological diseases, since α4 subunits were up-regulated in several animal models of chronic epilepsy and alcohol withdrawal syndrome (Brooks-Kayal et al. 1998; Cagetti et al. 2003; Peng et al. 2004; Zhang et al. 2007), and γ2 and δ subunits also showed plasticity in expression and might be co-regulated with α4 subunits in these animal models.

Methods and Materials

Transient transfection of $GABA_A$ receptor subunits into HEK 293T cells

The cDNAs encoding rat α4, β3, γ2L and δ $GABA_A$ receptor subunit subtypes were subcloned into the expression vector pCMVNeo. A 60 mm cell dish of HEK 293T cells was co-transfected with 1 or 2 μg of each subunit plasmid with a 1:1:1 cDNA ratio and 1 μg of the pHook-1 cDNA (Invitrogen, Carlsbad, CA) by FuGene6 transfection reagent (Roche) using a ratio of 3 μl of reagent per 1 μg of cDNA. pHook-1 was used as a marker of positively transfected cells, which were selected 24 hrs after transfection by magnetic hapten-coated beads (Greenfield, Jr. et al. 1997).

Electrophysiological recordings

Whole cell and single channel recordings were performed at room temperature. Signals were processed by using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) in voltage-clamp mode. Currents were obtained from HEK 293T cells transfected with α4β3γ2L and α4β3δ $GABA_A$ receptor subunits.

Patch pipettes (resistance 1-1.5 MΩ) used for whole-cell recording were pulled from thin-walled borosilicate capillary glass (World Precision Instruments, Sarasota, FL) on a P-2000 laser electrode puller (Sutter Instrument Co., San Rafael, CA) and filled with an internal solution containing 153 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES pH 7.3, with 4 mM ATP added on the day of recording. The pipette potential was held at -20 mV during recordings. Lifted cells were subjected to continuous background perfusion of the external solution consisting of 142 mM NaCl, 8 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4.

Single-channel currents were recorded using a patch-clamp technique in the cell-attached configuration (Hamill et al. 1981). The bath solution contained (mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose and 10 Hepes at pH 7.4. The pipette solution contained (mM): 120 NaCl, 5 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 10 glucose, 10 Hepes at pH 7.4. In addition, the pipette solution contained the indicated concentration of GABA when needed. The pipette potential was held at +80 mV. Glass electrodes were pulled from thick-walled borosilicate capillary glass (World Precision Instruments, Inc.) on a P-2000 Quartz Micropipette Puller (Sutter Instruments, Novato, CA) and fire-polished to a resistance of 10-20 MΩ on an MF-830 Micro Forge (Narishige, Tokyo, Japan) before use.

GABA application and kinase modulation

GABA (Sigma, St. Louis, MO) was prepared as 1 mM stock in distilled water and kept frozen. The stock solution of GABA was diluted to the final concentration of 1 μM prior to experiments. Activators and inhibitors of PKA were purchased from

Calbiochem (San Diego, CA). PKA was activated by application of 8Br-cAMP (1 mM; 8-Bromoadenosine-3',5'-cyclic monophosphate, sodium salt) or blocked by specific PKA inhibitory peptide (PKI; 100 μg/ml).

In the cell-attached configuration for single channel recording, GABA was applied in the pipette solution while 8Br-cAMP was applied in bath solution. For lifted whole-cell recording, GABA and kinase modulators were delivered through a four parallel glass square barrel to lifted HEK 293T cells using a modified SF-77B Perfusion Fast-Step application system (Warner Instrument Corp., Hamden, CT) (Hinkle et al. 2003). The drug application was initiated by an analog pulse triggered by the pClamp 9 software (Molecular Devices, Union City, CA) that caused the motor of the Warner Fast-Step to reposition the multi-barrel array from one barrel to another (e.g., external solution to GABA). By using this modified application system, we consistently obtained rise times (10–90%) less than 800 μsec with open-tip electrodes stepped from standard bath solution to bath solution. Alternatively, PKA was activated or inhibited by internal application of 8Br-cAMP or PKI in the whole-cell recording pipette solution.

Prolonged application (28 sec) of GABA (1 μM) to lifted whole cells was used to assess steady-state currents. Between every two applications of GABA, external solution was applied for at least 3 min to ensure maximal recovery from the GABA exposure. We also added pre-application steps to the protocol for modulator experiments to enable us to pre-apply a membrane-permeable modulator such as 8Br-cAMP. In the pre-application experiments, GABA was first applied to lifted cells

expressing α4β3γ2L or α4β3δ $GABA_A$ receptors as a pre-control. After washout of GABA, the PKA activator 8Br-cAMP (1 mM) was applied for 4 min and then the lifted cell was stepped back into external solution for a brief washout (3 sec) of any residual external 8Br-cAMP before it was subjected to a second GABA application. This second GABA-activated current, recorded immediately after an 8Br-cAMP pre-application, was defined as the experimental current. After the second GABA application, the cell was washed in external solution for more than 6 min before it was subjected to the final GABA application to evoke a post-control current. Due to the variability of currents among cells, the experimental currents were normalized to their matched pre-control currents so that differences made by pre-application were shown as a ratio of the control current.

Data analysis

Macroscopic whole-cell currents were low-pass filtered at 2 kHz, digitized at 10 kHz, and analyzed using pClamp9 software. Single channel currents were also low-pass filtered at 2 kHz and digitized at 20 kHz. Single channel currents were analyzed using TAC (Bruxton, Seattle, WA) software. Open and closed times were analyzed by using the "50% threshold criterion". All events were carefully checked visually before being accepted. Open and closed time histograms as well as amplitude histograms were generated using TACFit (Bruxton). The single-channel amplitude (*i*) was calculated by fitting all-point histograms with single or multiple Gaussian curves. The difference between the fitted "closed" and "open" peaks was taken as *i*. Duration histograms were fitted by a maximum likelihood method with

exponential components in the form: $\sum A_i \tau_i^{-1} \exp[-t/\tau_i]$, where A_i and τ_i are the relative area and the time constant of the *i*th component, respectively, and *t* is the time. Mean open or close time was calculated as follow: $\sum A_i \tau_i$ (Fisher and Macdonald 1997; Hernandez et al. 2008). The number of components required to fit the interval histograms was increased until an additional component did not significantly improve the fit with 95% confidence interval (Fisher and Macdonald 1997).

Single channel openings occurred as bursts of one or more openings or clusters of bursts. Bursts were defined as one or more consecutive openings that were separated by closed times that were shorter than a specified critical duration (t_{crit}) prior to and following the openings (Twyman et al. 1990). A t_{crit} duration of 5 ms was used in the current study. Clusters were defined as a series of bursts preceded and followed by closed intervals longer than a specific critical duration ($t_{cluster}$). A $t_{cluster}$ of 10 ms was used in this study. All numerical and bar graph data were expressed as mean ± standard error. Statistical significance was compared between the kinase modulator group and the control solution group. Statistical analyses were performed using Graph Pad Prism 4. Student's paired or unpaired *t* tests were used to compare pairs of data, or appropriate ANOVA analyses were used for comparing three or more groups. Statistical significance was taken as: $*p<0.05$, $**p<0.01$, or $***p<0.001$.

Results

Spontaneous currents were recorded from α4β3δ and α4β3γ2L receptors.

To characterize α4β3δ and α4β3γ2L currents, we co-transfected HEK 293T cells with α4, β3 and δ or γ2L subunits and recorded from control untransfected (Figure 2.1 A1) and transfected lifted whole cells (Figure 2.1 A2,3). In the absence of GABA, we recorded significantly larger holding currents from cells transfected with α4β3δ or α4β3γ2L subunits than from untransfected cells (Figure 2.1 B). When untransfected cells were voltage clamped to -20 mV, holding currents averaged 33.4 ± 8.6 pA (n = 8) (Figure 2.1 B, left open bar). In contrast, holding currents in cells voltage clamped to the same membrane potential expressing α4β3δ or α4β3γ2L receptors were significantly larger (Figure 2.1 B, middle and right open bars), averaging 81.8 ± 10.8 pA (n = 22) and 154.4 ± 25.3 pA (n = 20), respectively ($p < 0.01$ α4β3δ transfected vs. untransfected cells; $p < 0.001$ α4β3γ2L transfected vs. untransfected cells). The larger holding currents in cells expressing α4β3δ or α4β3γ2L receptors were caused by spontaneous receptor channel openings since they were reversibly blocked by applying the non-competitive $GABA_A$ receptor antagonist picrotoxin (PTX) (100 μM) (Figure 2.1 A2, 3). The holding currents of untransfected cells, however, were not altered by PTX (Figure 2.1 A1) (0.3 ± 2.7 pA, n = 8) (Figure 2.1 B, left slashed bar). PTX-sensitive spontaneous α4β3δ and α4β3γ2L currents averaged 54.1 ± 9.3 pA (n = 22) and 120.0 ± 18.6 pA (n = 20), respectively (Figure 2.1 B, middle and right slashed bars), which were significantly larger than those of untransfected cells. After PTX application, the remaining holding currents averaged

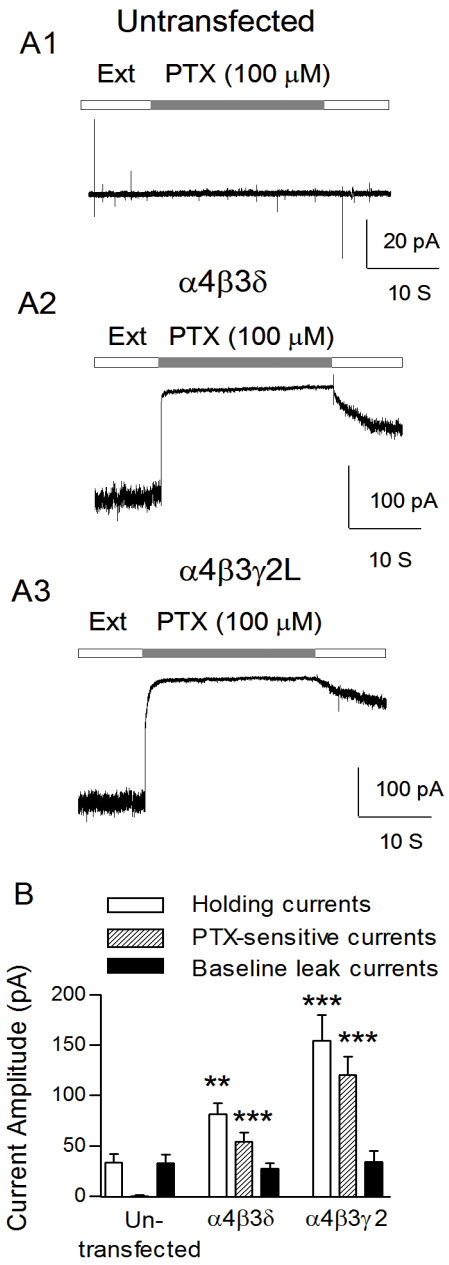

Figure 2.1. α4β3δ and α4β3γ2L receptors exhibit agonist-independent spontaneous currents.

A, Holding and spontaneous currents were recorded from untransfected cells (A1) and cells transfected with α4β3δ (A2) or α4β3γ2L subunits (A3). With all three transfection conditions, cells had baseline holding currents in external bath control (Ext) in the absence of applied GABA, but only cells transfected with α4β3δ or α4β3γ2L subunits had spontaneous currents that could be blocked by the $GABA_A$ receptor antagonist PTX (100 μM). **B,** The amplitude of total holding currents (open bars), picrotoxin-sensitive spontaneous currents (slashed bars) and baseline, picrotoxin-insensitive, leak currents (solid bars) were recorded from untransfected cells and from cells transfected with α4β3δ or α4β3γ2L $GABA_A$ subunits. Currents were significantly different from control current at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

27.7 ± 5.1 pA (n = 22) and 34.3 ± 10.6 pA (n = 20) for cells expressing α4β3δ and α4β3γ2L receptors, respectively (Figure 2.1 B, middle and right solid bars), respectively, which were not different from those recorded from untransfected cells after PTX application (33.1 ± 8.5 pA, n = 8) (Figure 2.1 B, left solid bar). The most accurate assessment of spontaneous current is to determine the difference between the holding currents recorded before and after PTX application (PTX sensitive spontaneous current). With or without transfection, cells had consistent baseline leak currents (Figure 2.1 B, solid bars). Thus, when it was not practical to apply PTX repeatedly, we used the total holding current as an indicator of spontaneous current, recognizing that a small, ~30 pA baseline leak current should be subtracted to estimate the true spontaneous current mediated by $GABA_A$ receptors.

PKA-activation enhanced spontaneous α4β3δ currents more extensively than spontaneous α4β3γ2L currents.

To determine if spontaneous α4β3δ and α4β3γ2L currents were subject to PKA modulation, we applied the PKA activator, 8Br-cAMP (1 mM), for 4 min to cells expressing each receptor isoform. 8Br-cAMP slowly increased spontaneous α4β3δ currents (Figure 2.2 A) to a maximal extent in about 3 min, but had only a small effect on spontaneous α4β3γ2L currents (Figure 2.2 B). We confirmed that the 8Br-cAMP-induced current was due to increased $GABA_A$ receptor channel activity by applying PTX (100 μM), which reversibly blocked both basal and PKA-enhanced spontaneous α4β3δ and α4β3γ2L receptor currents (Figure 2.2 A, B).

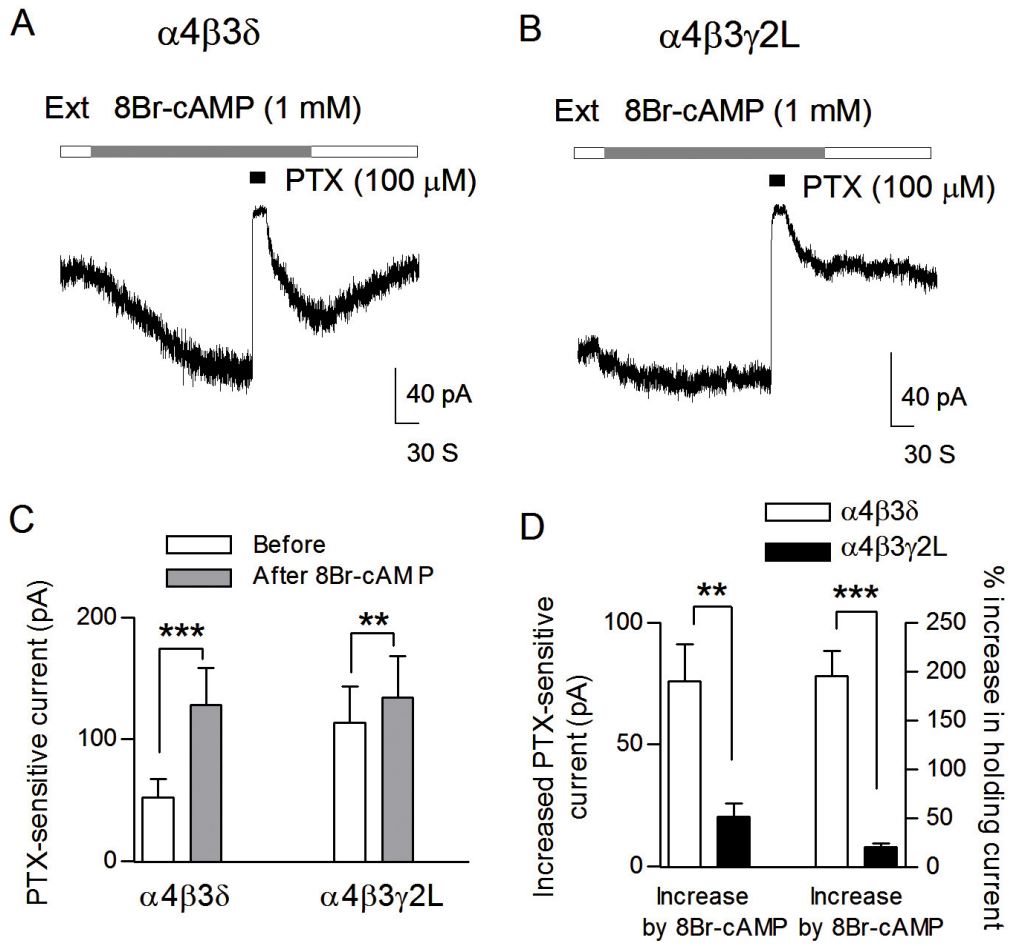

Figure 2.2. The PKA activator, 8Br-cAMP, increased spontaneous α4β3δ, but not α4β3γ2L, receptor currents.

A and B, Spontaneous whole cell α4β3δ (A) and α4β3γ2L (B) currents in external bath control (Ext) were increased with application of the membrane permeable PKA activator, 8Br-cAMP (1 mM). The spontaneous and 8Br-cAMP-activated α4β3δ and α4β3γ2L currents were reversibly blocked by 100 μM PTX. **C,** Amplitudes of PTX-sensitive spontaneous α4β3δ (left) and α4β3γ2L (right) currents before (open bar) and at the end of a 4 min 8Br-cAMP (1 mM) application (gray bar) were compared. **D,** The enhancement of PTX-sensitive spontaneous current amplitudes and the percentage increase of holding currents after 4 min 8Br-cAMP applications were plotted for α4β3δ (open bars) and α4β3γ2L (solid bars) currents. Currents were significantly different from control current at $^{**}p < 0.01$ or $^{***}p < 0.001$.

Before application of 8Br-cAMP, basal PTX-sensitive spontaneous α4β3δ and α4β3γ2L currents averaged 52.2 ± 15.3 pA (n = 10) and 113.4 ± 29.8 (n = 10) pA, respectively (Figure 2.2 C, open bars). After 8Br-cAMP treatment, PTX-sensitive spontaneous α4β3δ and α4β3γ2L currents increased to 128.0 ± 30.3 pA (n = 10) and 134.0 ± 34.0 (n = 10) pA, respectively (Figure 2.2 C, gray bars). The increase of PTX-sensitive spontaneous α4β3δ currents produced by 8Br-cAMP averaged 75.8 ± 15.2 pA (n = 10), which was a 195.5 ± 26.1% increase compared to the basal PTX-sensitive spontaneous currents (Figure 2.2 D, open bars). The 8Br-cAMP-enhanced PTX-sensitive spontaneous α4β3γ2L currents was very small and averaged only 20.6 ± 5.3 pA (n = 10), which was a 20.3 ± 4.1% increase compared to the basal PTX-sensitive spontaneous currents (Figure 2.2 D, filled bars) and was significantly smaller than the increase of PTX-sensitive spontaneous α4β3δ currents produced by 8Br-cAMP ($p < 0.001$).

To determine if the changes in spontaneous currents associated with application of extracellular 8Br-cAMP were due to activation of intracellular PKA, we added 8Br-cAMP (1 mM) or PKA kinase inhibitor (PKI, 100 μg/ml) to the intra-pipette solution to constitutively activate or inhibit PKA and recorded holding currents as measures of spontaneous currents. With intracellular 8Br-cAMP, α4β3δ receptor holding currents (Figure 2.3 A1, solid bar) were significantly larger (177.3 ± 57.8 pA, n = 7) than those obtained with control intra-pipette solution (87.3 ± 9.9 pA, n = 30, $p < 0.01$) (Figure 2.3 A1, open bar) or with PKI (100 μg/ml) in the recording

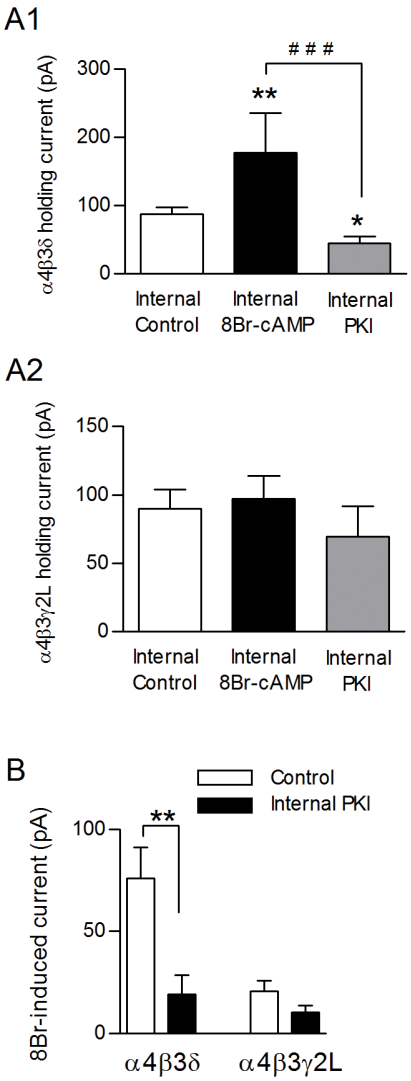

Figure 2.3. 8Br-cAMP-induced increase of spontaneous α4β3δ and α4β3γ2L currents was due to activation of PKA.

A, Amplitudes of α4β3δ (A1) and α4β3γ2L (A2) holding currents with intra-pipette control solution (Control, open bar) and with intra-pipette solution containing the PKA activator 8Br-cAMP (1 mM, solid bar) or the PKA inhibitor PKI (100 μg/ml, grey bar) were compared. **B,** Spontaneous α4β3δ (left) and α4β3γ2L (right) currents after a 4 min application of extracellular 8Br-cAMP (1 mM) were recorded from cells with pipettes filled with control solution (Control, open bar) or containing (Internal PKI, solid bar). Currents were significantly different from control current at * $p<0.05$, ** $p<0.01$, or *** $p<0.001$. ### $p < 0.001$ internal 8r-cAMP vs. internal PKI.

pipette (44.7 ± 10.0 pA, n = 18, $p < 0.001$) (Figure 2.3 A1, gray bar). Since intracellular and extracellular application of 8Br-cAMP caused the same effect on α4β3δ holding currents, it is likely that the effect of extracellular 8Br-cAMP was due to activation of intracellular PKA rather than to a direct, nonspecific extracellular effect on the receptors. α4β3δ holding currents recorded with intracellular PKI (Figure 2.3 A1, gray bar) were also significantly smaller than α4β3δ holding currents recorded with control intra-pipette solution (Figure 2.3 A1, open bar) , suggesting some basal PKA-activation of spontaneous α4β3δ currents.

With α4β3γ2L receptors, holding currents with internal 8Br-cAMP (97.1 ± 17.0 pA, n = 3) (Figure 2.3 A2, solid bar) were slightly, but not significantly, larger than holding currents with intracellular PKI (69.2 ± 22.6 pA, n = 17) (Figure 2.3 A2, gray bar), and neither current was significantly different from α4β3γ2L holding currents with control intra-pipette solution (89.8 ± 14.3 pA, n = 7) (Figure 2.3 A2, open bar). The inclusion of a constant baseline leak current (~30 pA) in each condition and a smaller effect of PKA on spontaneous α4β3γ2L currents may have contributed to the failure to detect a significant difference in holding currents.

To exclude the possibility that the 8Br-cAMP-induced increase in spontaneous α4β3δ current was non-specific, we applied extracellular 8Br-cAMP (1 mM) and recorded spontaneous α4β3δ currents from cells with PKI (100 μg/ml) in the intra-pipette solution. The average increase of spontaneous α4β3δ currents after a 4 min incubation in 8Br-cAMP decreased from 75.8 ± 15.2 pA (n = 10) in cells with control intra-pipette solution (Figure 2.3 B, open bar) to 19.1 ± 9.3 pA (n = 4, $p < 0.01$) in

cells with internal PKI (Figure 2.3 B, solid bar), indicating that inhibition of intracellular PKA significantly blocked the effect of 8Br-cAMP applied extracellularly. Intracellular application of PKI also reduced the 8Br-cAMP-induced spontaneous α4β3γ2L current from 20.6 ± 5.3 pA (n = 10) (Figure 2.3 B, open bar) to 10.4 ± 3.2 pA (n = 6, p > 0.05) (Figure 2.3 B, solid bar), but this was not a significant difference. These results demonstrated that both α4β3δ and α4β3γ2L receptor channels opened spontaneously, but that PKA-activation was much more effective in increasing spontaneous α4β3δ than α4β3γ2L receptor currents.

PKA-activation did not alter total tonic α4β3δ and α4β3γ2L currents in the presence of a low concentration of GABA.

Tonic current usually reflects activation of $GABA_A$ receptors by a low, ambient concentration of GABA, while for receptors with spontaneous openings like α4β3δ and α4β3γ2L receptors, tonic current could be contributed both by spontaneous current and current activated by ambient GABA. As extracellular GABA can be detected at micromolar concentrations in several brain regions such as hippocampus where tonic inhibition is present (Lerma et al. 1986; Nusser and Mody 2002), we recorded total tonic α4β3δ and α4β3γ2L currents as well as their holding currents in the presence of 1 μM GABA with and without PKA-activation. To determine if total tonic currents were also regulated by PKA phosphorylation, the PKA activator 8Br-cAMP (1 mM) was pre-applied for 4 min to α4β3δ and α4β3γ2L receptors (see methods section). Before and immediately after 8Br-cAMP pre-application, GABA (1 μM) was applied for 28 sec to the cells to reach a steady-state, total tonic current.

After termination of the GABA application, the cells were washed extensively (> 6 min) with external solution to ensure maximal reversal of any 8Br-cAMP effect.

For each 8Br-cAMP pre-application experiment, we recorded three GABA-activated currents: pre-control currents recorded prior to 8Br-cAMP pre-application (Figure 2.4, green traces), currents recorded immediately after 8Br-cAMP pre-application (Figure 2.4, black traces) and post-control currents recorded after wash (Figure 2.4, gray traces). With activation of PKA, alterations of both transient and steady-state α4β3δ and α4β3γ2L currents were observed. PKA-activation affected $GABA_A$ receptor currents in minutes, altering the transient properties of α4β3δ currents more than those of α4β3γ2L currents. Specifically, fast phases of desensitization were accelerated, and current peak amplitudes were increased. Enhancement of α1β1,3γ2L $GABA_A$ receptor current desensitization by PKA has also been reported (Hinkle and Macdonald 2003), suggesting that a common mechanism of PKA modulation to change the current kinetic properties of different $GABA_A$ receptor isoforms. The alteration in time course of these currents indicate that PKA-activation modified the kinetic properties of α4β3δ and α4β3γ2L receptors; however, the time course of currents activated by low concentrations of GABA has less physiological relevance than their steady-state current levels, and thus, only steady-state currents were assessed. The steady-state current at the end of a 28 sec application of GABA (1 μM) was a measure of total tonic current (Figure 2.3 A, B; grey arrows). The GABA-activated increase in current from holding baseline at the end of application to the steady-state, tonic current was also measured as

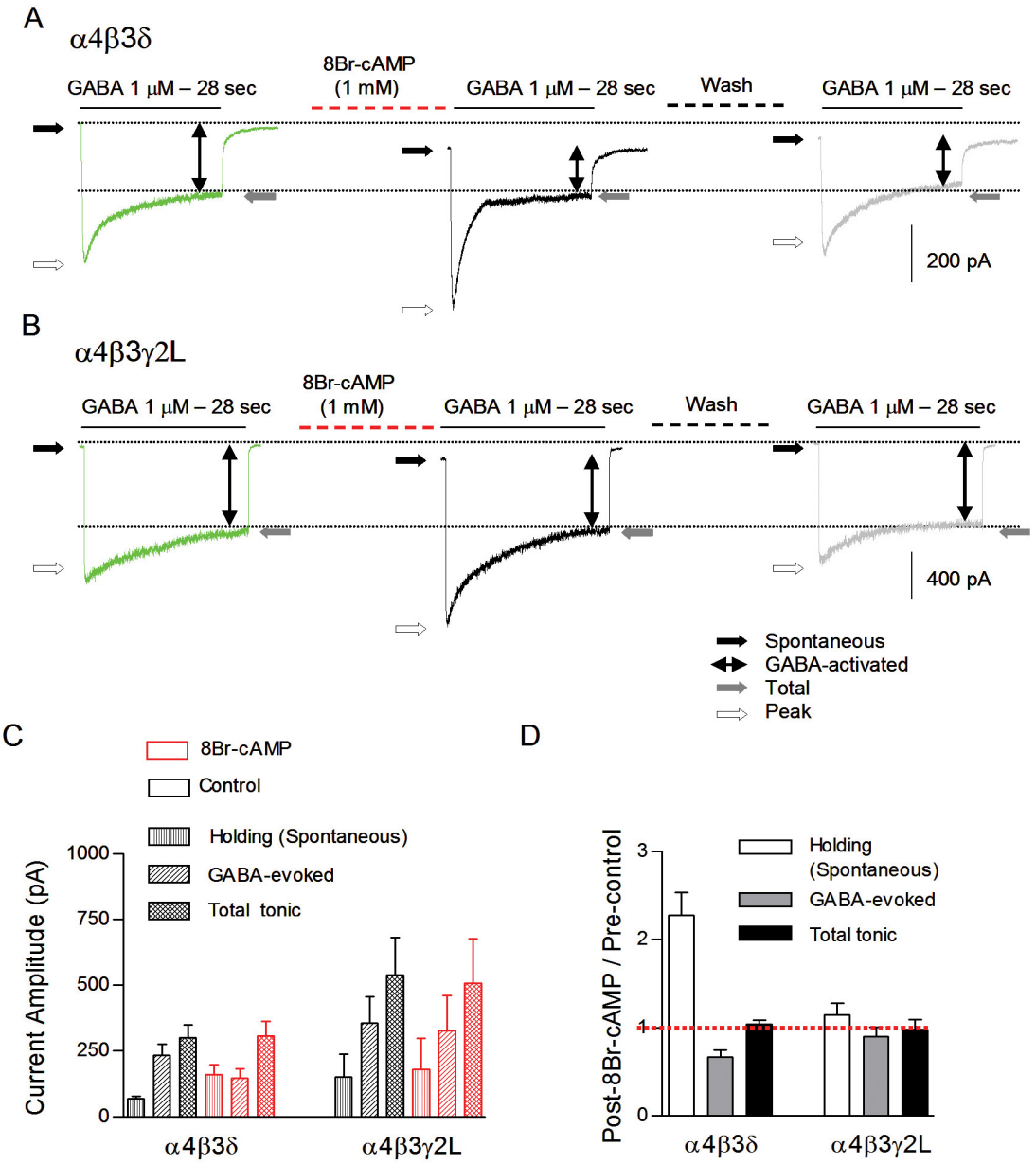

Figure 2.4. PKA-activation did not alter α4β3δ or α4β3γ2L total tonic currents, but did increase spontaneous and decrease GABA-activated tonic α4β3δ, and to a lesser extent α4β3γ2L, currents.

A and B, Pre-application of 8Br-cAMP (1 mM) had different effects on spontaneous currents and GABA-evoked tonic and total tonic currents recorded from α4β3δ (A) and α4β3γ2L (B) receptors. Control currents (Pre-control, left green), currents immediately after 8Br-cAMP pre-application (8Br-cAMP, middle black) and currents after wash (Post-control, right gray) evoked by 1 μM GABA were recorded from the same cell. **C,** The amplitudes of spontaneous (vertical striped bars), GABA-evoked tonic (slashed bars) and total tonic (stippled bars) α4β3δ (left) and α4β3γ2L (right) pre-control (white) currents were compared to currents recorded following pre-application in 8Br-cAMP (red). **D,** Amplitudes of spontaneous, GABA-evoked tonic and total tonic currents recorded from α4β3δ and α4β3γ2L receptors were normalized to pre-control amplitudes.

GABA-activated tonic currents (Figure 2.4 A, B; two-headed arrows). Due to the lack of full recovery of currents with repeated application of picrotoxin, we measured the total holding currents (Figure 2.4 A, B; black arrows) as a measure of spontaneous currents before GABA application. Although we can measure the sum of spontaneous and the additional GABA-activated currents, it is not possible to determine the relative contributions of these two currents to the total tonic current at a given ambient GABA concentration since it is very likely that GABA binding to receptors would prevent spontaneous openings.

To easily visualize the changes in steady-state currents, we drew two lines to align the three currents with the pre-control baseline holding current (upper) and the pre-control steady-state, total tonic current (lower). Holding currents, indicated by the black arrows, were reversibly increased by PKA-activation to a larger extent for α4β3δ (Figure 2.4 A) than for α4β3γ2L currents (Figure 2.4 B). In contrast to the effect of PKA-activation on holding currents, steady-state, total tonic currents, indicated by the grey arrows, were unchanged by PKA-activation for both α4β3δ and α4β3γ2L receptors (Figure 2.4 A, B). As a result, the magnitude of the increase in GABA-activated tonic current, indicated by the vertical two-headed arrows, was decreased by PKA-activation for α4β3δ receptors (Figure 2.4 A) more than for α4β3γ2L receptors (Figure 2.4 B).

To quantify the effects of PKA-activation on basal spontaneous currents before GABA application, the increase of current evoked by 1 μM GABA and steady-state, tonic currents at the end of the GABA application, we compared the amplitudes

of holding, GABA-activated and steady-state, tonic currents of pre-control currents (Figure 2.4 C, white) and of currents following 8Br-cAMP treatment (Figure 2.4 C, red) for α4β3δ (Figure 2.4 C, left) and α4β3γ2L (Figure 2.4 C, right) receptor isoforms. The amplitudes of steady-state, total tonic α4β3δ currents were not altered by 8Br-cAMP pre-application (300.5 ± 47.7 vs. 307.6 ± 53.6 pA, n = 11) (Figure 2.4 C, cross hatched bars). However, α4β3δ holding currents were increased by 8Br-cAMP (67.9 ± 9.6 vs. 160.5 ± 37.0 pA, n = 11) (Figure 2.4 C, vertical striped bars). Since the holding currents, but not steady-state currents, were increased by 8Br-cAMP, this resulted in smaller GABA-activated tonic α4β3δ currents (232.7 ± 42.1 vs. 147.1 ± 34.5 pA, n = 11) (Figure 2.4 C, slashed bars). Compared to pre-control currents, α4β3δ holding currents were increased by 127.4 ± 26.2% (n = 11) (Figure 2.4 D, open bar), and total tonic α4β3δ currents were unaltered (+3.9 ± 4.8%, n = 11) (Figure 2.4 D, black bar) after 8Br-cAMP pre-application. As a result, the magnitude of α4β3δ GABA-activated currents was decreased by 33.2 ± 7.8% (n = 11) after 8Br-cAMP pre-application (Figure 2.4 D, gray bar).

The amplitudes of steady-state, total tonic α4β3γ2 currents were not altered by 8Br-cAMP pre-application (538.8 ± 142.1 vs. 505.6 ± 172.0 pA, n = 5) (Figure 2.4 C, cross hatched bars). Similarly, α4β3γ2 holding currents (150.7 ± 85.9 vs. 179.0 ± 119.3 pA, n = 5) (Figure 2.4 C, vertical striped bars) and GABA-activated tonic α4β3γ2 currents (356.5 ± 99.2 vs. 326.7 ± 134.2 pA, n = 5) (Figure 2.4 C, slashed bars) were not significantly altered by 8Br-cAMP pre-application. Compared to the effects on α4β3δ currents, PKA had much smaller effects on the holding (+14.6 ± 13.2%, n =

5) (Figure 2.4 D, open bar) and GABA-activated (-10.2 ± 10.9%, n = 5) (Figure 2.4 D, grey bar) α4β3γ2L currents and also did not alter total tonic α4β3γ2L currents (-2.7 ± 10.9%, n = 5) (Figure 2.4 D, black bar). Our results suggest that PKA-activation enhanced spontaneous α4β3δ currents but reduced the magnitude of GABA-activated tonic α4β3δ currents, had no significant effects on spontaneous and GABA-activated tonic α4β3γ2L currents and had no effect on total tonic α4β3δ and α4β3γ2L currents in the presence of micromolar ambient GABA.

PKA-activation enhanced spontaneous single channel α4β3δ currents by increasing open frequency.

To determine the mechanisms underlying the enhancement of spontaneous α4β3δ currents by PKA-activation, we obtained cell-attached patch clamp recordings in the absence of applied GABA from cells transfected with α4β3δ subunits and compared the properties of spontaneous single channel openings obtained with and without activation of PKA. In the cell-attached patch clamp configuration at an intra-pipette potential of +80 mV, α4β3δ $GABA_A$ receptors displayed spontaneous single channel openings with an amplitude of 1.02 pA (n = 5, Table 1). The spontaneous α4β3δ single channel currents occurred as frequent isolated single openings and brief bursts of a few openings, but no prolonged clusters of bursts were evident (Figure 2.5 A). When 1 mM 8Br-cAMP was added to the bathing solution, the amplitude of spontaneous single channel α4β3δ currents was not altered (1.03 pA, n = 5, $p > 0.05$, Table 1). However in addition to the brief bursts of openings, frequent prolonged (1 to 2 sec) clusters of bursts were apparent (Figure 2.5 B). Thus, PKA-activation

caused the single channel opening pattern to change due to modulation of spontaneous channel openings, rather than altering single channel conductance.

To characterize the effect of PKA-activation on the kinetic properties of spontaneous α4β3δ single channel currents, we first measured the durations of

Table 1. Kinetic properties of α4β3δ $GABA_A$ receptors.

	Spontaneous		GABA 1μM	
	Control (n = 5)	+8Br-cAMP (n = 5)	Control (n = 4)	+8Br-cAMP (n = 4)
Current amplitude (pA)	1.02 ± 0.05	1.03 ± 0.04	1.07 ± 0.08	1.03 ± 0.04
Open duration (ms)	5.82 ± 0.59	3.98 ± 0.32*	3.99 ± 0.48	2.58 ± 0.23*
Closed duration (ms)	661.4 ± 86.1	158.3 ± 1.3***	401.5 ± 53.9	271.6 ± 66.1
Open probability	0.006 ± 0.002	0.027 ± 0.005**	0.011 ± 0.001	0.017 ±0.005
Burst duration (ms)	7.01 ± 0.97	6.75 ± 1.24	6.07 ± 1.27	6.94 ± 1.55
Burst frequency (S^{-1})	0.99 ± 0.18	4.58 ± 0.62***	1.34 ± 0.07	2.96 ± 0.40**
Po	0.25 ± 0.02	0.63 ± 0.03***	0.72 ± 0.05	0.85 ± 0.06
τ_{o1} (ms)	0.81 ± 0.18	0.69 ± 0.07	0.66 ± 0.08	0.43 ± 0.10
Weight τ_{o1} (%)	14 ± 4	21 ± 4	21 ± 3	17 ± 5
τ_{o2} (ms)	6.85 ± 1.05	3.45 ± 0.31*	3.40 ± 0.46	2.14 ± 0.18
Weight τ_{o2} (%)	75 ± 8	70 ± 3	69 ± 1	77 ± 4
τ_{o3} (ms)	13.63 ± 1.58	11.22 ± 1.09	7.87 ± 0.56	4.81 ± 0.24**
Weight τ_{o3} (%)	11 ± 4	9 ± 2	10 ± 2	6 ± 2
Mean open duration (ms)	6.36 ± 0.65	3.89 ± 0.27**	3.88 ± 0.43	2.44 ± 0.10*

Values represent mean ± S.E.M. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ (unpaired t-test) relative to control conditions, respectively.

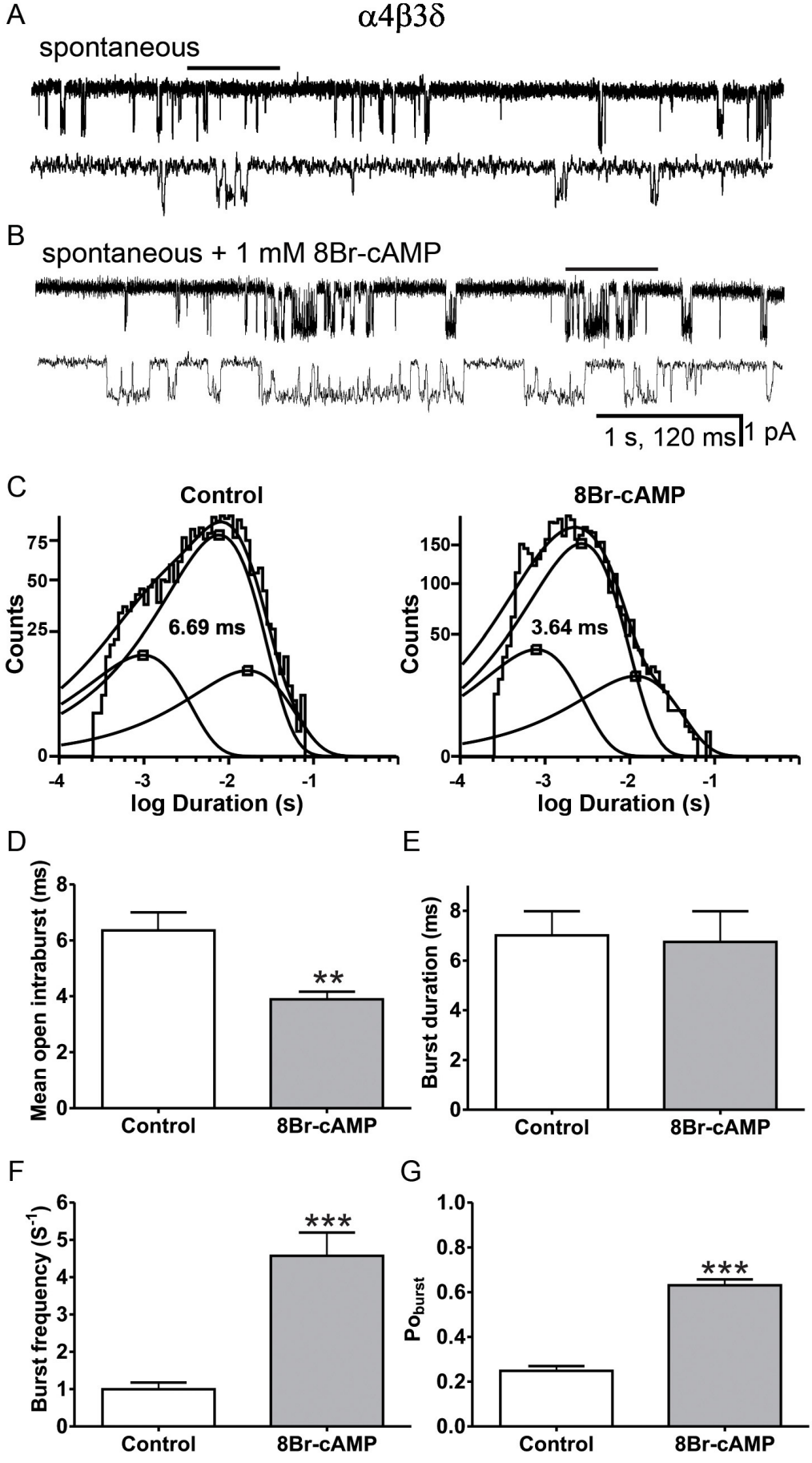
A
α4β3δ
spontaneous
B
spontaneous + 1 mM 8Br-cAMP
1 s, 120 ms
1 pA
C
Control
8Br-cAMP
Counts
6.69 ms
3.64 ms
log Duration (s)
D
Mean open intraburst (ms)
**
Control
8Br-cAMP
E
Burst duration (ms)
F
Burst frequency (S-1)
***
G
POburst

Figure 2.5. PKA-activation increased spontaneous, single channel α4β3δ receptor current by increasing channel open frequency.

A and B, Spontaneous single channel current traces recorded from cells expressing α4β3δ receptors in the absence (A) and presence (B) of 1 mM 8Br-cAMP. The portion of the single channel current trace below the filled bar was expanded below. **C, D, E, F and G,** Single channel burst analysis was performed for spontaneous currents recorded without (white) and with (grey) 1 mM 8Br-cAMP application. Representative open duration histograms obtained with and without 8Br-cAMP were fitted to three exponential functions with mean open durations of 6.69 ms and 3.64 ms, respectively (C). Comparisons of single channel mean open duration (D), burst duration (E), burst frequency (F) and open probability (F) were made between control currents (white) and currents recorded in the presence of 1 mM 8Br-cAMP (grey). Values represent mean ± S.E.M. * and *** indicate $p < 0.05$ and $p < 0.001$ (unpaired t-test) relative to control conditions, respectively.

openings and closings and determined the open probability of single channel α4β3δ currents in the absence and presence of 8Br-cAMP. In the absence of 8Br-cAMP, the mean durations of openings and closings of spontaneous α4β3δ receptor single channel currents were 5.82 ms and 661.4 ms (n = 5, Table 1), respectively, and the open probability of spontaneous openings was 0.006. In the presence of 8Br-cAMP, both mean open and closed durations were significantly decreased (open duration 3.98 ms, $p = 0.025$; closed duration 158.3 ms, $p = 0.0006$; n = 5, Table 1). However, the mean open (32 % reduction) and closed (76 % reduction) durations were reduced to different extents, resulting in a spontaneous α4β3δ channel open probability that was significantly increased to 0.027 (n = 5, $p = 0.0023$, Table 1). The increased overall open probability with shorter mean open duration and even larger reduction of mean close duration strongly suggests that 8Br-cAMP substantially increased channel opening frequency to produce an overall increase of open probability.

We also compared the single channel kinetic properties of spontaneous α4β3δ currents evoked in the absence and presence of 1 mM 8Br-cAMP. Representative

open duration histograms from both control and experimental conditions were fitted best by three exponential probability density functions (Figure 2.5 C). The mean open duration time constants and their respective mean distribution weights are summarized in Table 1. Interestingly, spontaneous α4β3δ currents recorded with application of 1 mM 8Br-cAMP displayed an approximately twofold reduction in the second time constant (τo_2 = 3.45 ms, $p < 0.05$) when compared with that from control currents recorded without 8Br-cAMP (τo_2 = 6.85 ms), but there were no differences in the respective weights of the time duration functions (Table 1). Thus, with PKA-activation spontaneous α4β3δ single channel currents displayed a significant decrease in the mean open duration (control = 6.36 ms; 8Br-cAMP = 3.89 ms, $p < 0.01$) (Figure 2.5 D; Table 1). Although enhancement of spontaneous α4β3δ currents by 8Br-cAMP did not affect mean burst duration (control = 7.01 ms; 8Br-cAMP = 6.75 ms) (Figure 2.5 E; Table 1), we found significant increases in both burst frequency (control = 0.99 s^{-1}; 8Br-cAMP = 4.58 s^{-1}, $p < 0.001$) and open probability (Po) (control = 0.25; 8Br-cAMP = 0.63, $p < 0.001$) (Figure 2.5 F, G; Table 1). Furthermore, these effects shifted the channel to a higher activity level by producing an approximately fivefold increase in burst frequency and a threefold increase in open probability compared with control conditions, respectively. Thus in the presence of 8Br-cAMP, unbound α4β3δ receptors open in bursts with shorter mean open duration, higher burst frequency and higher open probability with no changes in burst duration.

PKA had minimal effects on α4β3δ receptor single channel open probabilities in the presence of bath applied (ambient) GABA.

To determine how PKA-activation affects steady-state, tonic currents in the presence of low concentrations of GABA, we determined the effect of 8Br-cAMP on α4β3δ receptor single channel currents in the presence of 1 μM GABA, an ambient GABA concentration detected in many brain areas. We added GABA to the intra-pipette solution in the cell-attached configuration and recorded α4β3δ receptor single channel currents in the absence (Figure 2.6 A) and presence (Figure 2.6 B) of 8Br-cAMP. The amplitudes of α4β3δ single channel currents with 1 μM GABA with (1.03 pA, n = 4, $p > 0.05$) and without (1.07 pA, n = 4, $p > 0.05$) PKA-activation were not different from those of spontaneous currents (1.02 pA, n = 5) (Table 1). However, compared to spontaneous single channel currents, openings in the presence of 1 μM GABA contained more bursts of openings and many clusters of bursts (<1 s) and only rarely occurred as isolated, brief openings (Figure 2.6 A). It is worth noting that α4β2δ single channel currents also displayed one main conductance level in the presence of GABA similar to α4β3δ receptors, but without discrete clusters of openings (Akk et al. 2004; Keramidas and Harrison 2008). This difference in single channel open properties could be due to the expression of different β subunits, and more clusters of openings may result in larger whole cell currents conducted by β3 subunit-containing receptors. The clusters of openings of α4β3δ receptors were consistently evoked by 1 μM GABA in the presence of 1 mM 8Br-cAMP (Figure 2.6 B). Application of 8Br-cAMP resulted only in small effects on single channel mean

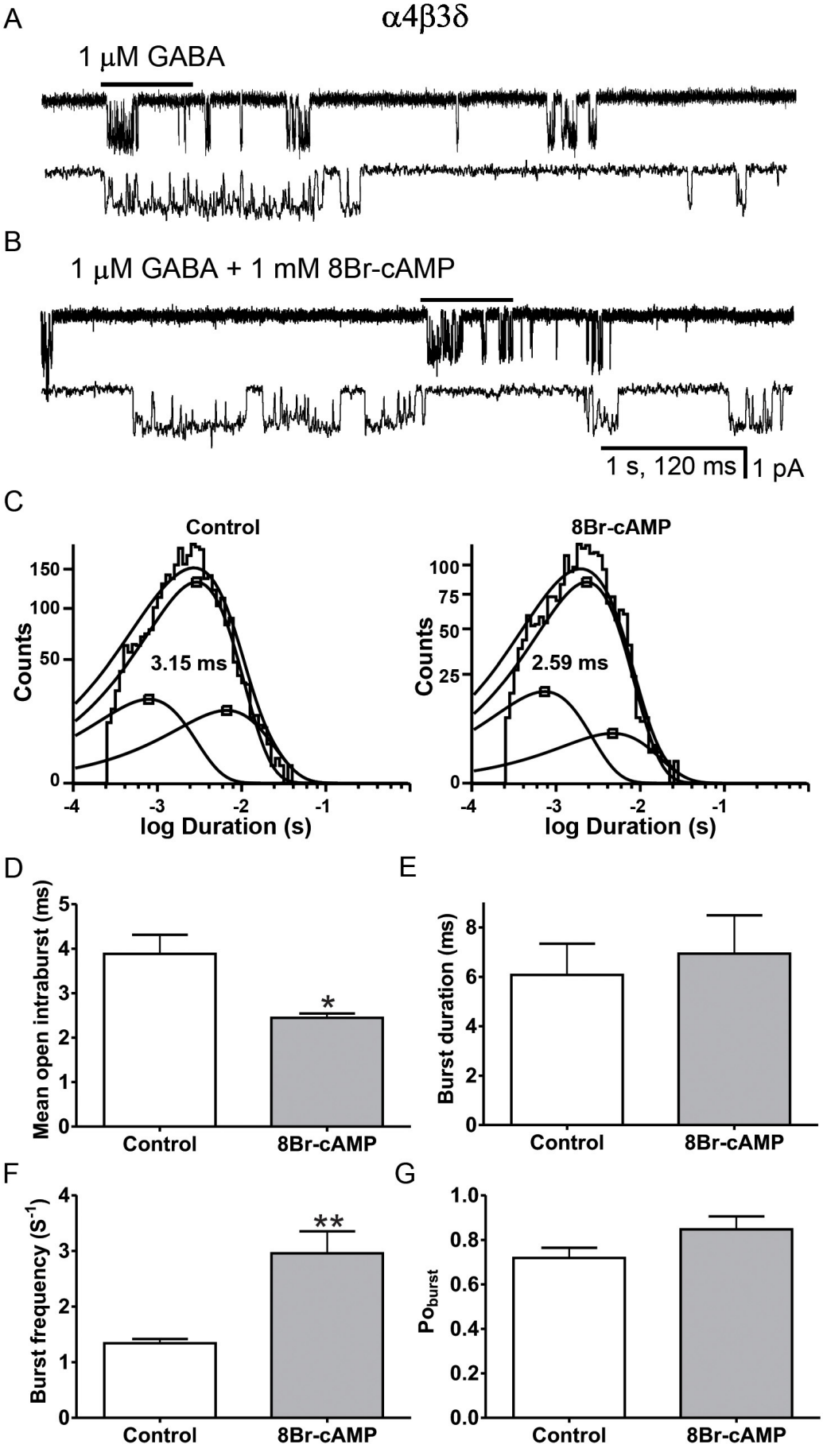
A
α4β3δ
1 μM GABA
B
1 μM GABA + 1 mM 8Br-cAMP
1 s, 120 ms
1 pA
C
Control
8Br-cAMP
Counts
3.15 ms
2.59 ms
log Duration (s)
D
Mean open intraburst (ms)
Control
8Br-cAMP
E
Burst duration (ms)
F
Burst frequency (S-1)
G
POburst

Figure 2.6. PKA-activation did not significantly increase single channel opening of α4β3δ receptors in the presence of ambient GABA.

A and B, Single channel currents activated by 1 μM GABA were recorded from cells expressing α4β3δ receptors in the absence (A) and presence (B) of 1 mM 8Br-cAMP. The portion of the single channel current trace below the filled bar was expanded below. **C, D, E, F and G,** Single channel burst analysis was performed for currents evoked by 1 μM GABA without (white) and with (grey) 1 mM 8Br-cAMP application. Representative open duration histograms obtained with and without 8Br-cAMP were fitted to three exponential functions with mean open durations of 3.15 ms and 2.59 ms, respectively (C). Comparisons of single channel mean open duration (D), burst duration (E), burst frequency (F) and open probability (F) were made between control currents (white) and currents recorded in the presence of 1 mM 8Br-cAMP (grey). Values represent mean ± S.E.M. * and ** indicate $p < 0.05$ and $p < 0.01$ (unpaired t-test) relative to control conditions, respectively.

open and closed durations. The mean open duration in the presence of 1 μM GABA and 8Br-cAMP (2.58 ms, n = 4, Table 1) was decreased by 35% compared to control mean open duration (3.99 ms, n = 4, p = 0.036, Table 1). The mean closed duration in the presence of 1 μM GABA and 8Br-cAMP (271.6 ms, n = 4, Table 1), however, was not different from the mean close duration in the absence of 8Br-cAMP (401.5 ms, n = 4, p = 0.1786, Table 1). As a result, the open probabilities of α4β3δ receptor single channel currents in presence of 1 μM GABA with (0.017, n = 4) and without (0.011, n = 4, p = 0.21) PKA-activation were not significantly different (Table 1).

In addition, kinetic analysis was performed for both spontaneous and GABA(1 μM)-evoked α4β3δ currents in the absence and presence of 1 mM 8Br-cAMP. As with spontaneous currents, α4β3δ currents evoked by 1 μM GABA with and without 8Br-cAMP displayed open duration distributions that were fitted best by three exponential probability density functions as shown in the representative open duration histograms (Figure 2.6 C). In contrast to spontaneous currents, in the presence or absence of 8Br-cAMP, α4β3δ currents evoked by 1 μM GABA had a decrease in the

largest time constant (τo_3 = 4.81 ms, $p < 0.01$) when compared with that from control currents recorded without 8Br-cAMP (τo_3 = 7.87 ms), but with no effects in their distribution weights within bursts (Table 1). Nonetheless, the mean open durations were slightly reduced when compared to bursts recorded in the absence of 1 mM 8Br-cAMP (control = 3.88 ms; 8Br-cAMP = 2.44 ms, $p < 0.05$) (Figure 2.6 D, Table 1). On the other hand, burst duration (Figure 2.6 E) and open probability were not affected (Figure 2.6 G) (Table 1). Thus, 8Br-cAMP application produced about a twofold increase in burst frequency (Figure 2.6 F) (control = 1.34 s^{-1}; 8Br-cAMP = 2.96 s^{-1}, $p < 0.01$). The small changes produced by application of 8Br-cAMP on the open probabilities of α4β3δ receptor single channel currents in the presence of 1 μM GABA was consistent with the observation that PKA-activation had no significant effect on macroscopic total tonic currents recorded in the presence of 1 μM GABA.

Discussion

In this study we demonstrated that both α4β3δ and α4β3γ2L receptors conduct spontaneous currents in addition to GABA-activated phasic and tonic currents. PKA-activation increased spontaneous α4β3δ currents, did not alter total tonic α4β3δ currents, and thus decreased GABA-activated tonic α4β3δ currents. In contrast, PKA only had minimal effects on spontaneous, GABA-activated tonic and total tonic α4β3γ2L currents, suggesting that PKA differentially modulates α4β3δ and α4β3γ2L receptor currents, and that the extent of modulation is affected by the concentration of GABA. When ambient GABA is maintained at low, sub micromolar levels,

spontaneous α4β3δ current would provide a background tonic current that could be regulated by PKA, but when ambient GABA is at micromolar levels, total tonic α4β3δ current would not be altered by PKA. As a result, we conclude that PKA phosphorylation could play a key role in determining the magnitude of spontaneous (agonist-independent) α4β3δ currents and also could set the dynamic range of GABA-activated (agonist-dependent) tonic currents in response to changes in ambient GABA level. Furthermore, using the single channel recording technique, we determined that increased single channel burst frequency was responsible for the alterations in current caused by activation of PKA.

α4β3δ and α4β3γ2L receptor channels open spontaneously.

Although spontaneous $GABA_A$ receptor channel openings have been reported previously, the specific $GABA_A$ receptor isoform(s) that contribute to the spontaneous currents was not clearly due to $GABA_A$ receptor heterogeneity and the relatively small magnitude of spontaneous currents. Homomeric β1 and β3 receptors and dimeric α4β1 receptors exhibited spontaneous currents; however, these receptors were reported to be insensitive to GABA (Sigel et al. 1989; Khrestchatisky et al. 1989; Krishek et al. 1996; Wooltorton et al. 1997). More recently, αβε receptors were reported to generate spontaneous currents (Neelands et al. 1999; Maksay et al. 2003; Wagner et al. 2005), and there have also been reports of spontaneous currents recorded from different αβδ/γ receptor isoforms, including α6β2δ, α6β2γ2, α1β3γ2 and α4β2δ receptors, in different cell types (Knoflach et al. 1996; Bianchi and Macdonald 2001; Hadley and Amin 2007). In our recombinant expression system,

we recorded significant spontaneous currents from both α4β3δ and α4β3γ2L receptors. These currents were not likely contributed by formation of homomeric β3 subunit receptors or dimeric α4β3 receptors because transfection of β3 subunit alone or of α4 and β3 subunits did not produce detectable spontaneous currents. Although this result seemed contradictory to the previous founding of spontaneous currents of homomeric β and dimeric α4β receptors expressed in Xenopus oocytes, it could be due to low expression or surface targeting of those two receptors in our expression systems. In brain areas expressing either of the two α4 subunit-containing receptors, spontaneous currents mediated by α4β3δ and α4β3γ2L receptors could play an important role in setting resting neuronal inhibitory tone.

Single channel recordings from α4β3δ receptor channels demonstrated that spontaneous single channel currents were composed of low frequency, isolated, brief openings. Thus spontaneously opening channels have a low open probability, and therefore, conduct relatively small currents. Nonetheless, spontaneous currents could provide a significant fraction of a neuron's resting input conductance and have a substantial influence on neuronal excitability since spontaneous currents are continuous and independent of neurotransmitter release.

For receptors with spontaneous openings, spontaneous current could also contribute to total tonic current in the presence of low concentration of ambient GABA; however, the relative contributions of these two currents to the total tonic current could vary at different ambient GABA levels if GABA binding to receptors prevents spontaneous openings. At low, nanomolar GABA concentrations it is

possible that there is a mixture of unbound, spontaneously opening channels and GABA-bound GABA-activated receptors that contribute to total tonic current, but at micromolar GABA concentrations, which are high ambient GABA concentrations that occur in the extracellular space, it is likely that most if not all steady-state current is GABA-evoked.

PKA increased α4β3δ channel open frequency in the absence of GABA but not in the presence of GABA.

We demonstrated that spontaneous α4β3δ currents were enhanced by PKA, which would provide a regulatory mechanism that would be particularly important in maintaining the basal inhibitory tone in the context of very low extracellular GABA concentrations. In contrast, we demonstrated that at micromolar GABA concentrations, total tonic α4β3δ currents were not altered by PKA. To understand the mechanism of this modulation by PKA, we acquired single channel currents in the absence and presence of 1 μM GABA. The results obtained for modulation of macroscopic and single channel α4β3δ receptor currents by PKA were consistent. The increased spontaneous whole cell current produced by PKA-activation was associated with no alteration of single channel conductance, a moderate decrease in single channel mean open duration, a large decrease in single channel mean closed duration and an increase in single channel open probability. Thus, we concluded that the decrease in mean closed duration with PKA was consistent with an increased open frequency, although we could not exclude the possibility of more than one channel on the patch so that mean close time cannot be used as a clear measure of open frequency.

The increase in open frequency was associated with a PKA-induced change from spontaneous, brief isolated single channel openings to more frequent bursts and clusters of openings. Kinetic analysis of spontaneous single channel currents in the absence and presence of 8Br-cAMP (1 mM) indicated an increase of burst frequency and a higher open probability induced by PKA, which could cause increased spontaneous current. However, in the presence of 1 μM GABA, no significant increase in channel open frequency or open probability was observed, consistent with the finding that 1 μM GABA minimized the PKA effect on the total tonic α4β3δ currents.

There have been numerous reports that modulators of $GABA_A$ receptors regulated receptor function by altering the efficacy or potency of GABA (Bianchi and Macdonald 2003; Zheleznova et al. 2008). In our study, PKA phosphorylation clearly increased the efficacy of α4β3δ spontaneous openings, which were independent of GABA-binding. The absence of alteration of tonic current by PKA in the presence of GABA does not necessarily suggest that $GABA_A$ receptor gating was unaffected by PKA-activation. Failure to have a PKA effect could also be explained by saturation of α4β3δ receptors at 1 μM of GABA, so that any changes produced by PKA on receptor affinity would not occur at a saturating concentration of GABA. More data obtained with sub-micromolar concentrations of GABA would help address this issue

The extent of PKA modulation of tonic α4β3δ currents should differ at different concentrations of ambient GABA.

Extracellular neurotransmitter concentrations always vary temporally and spatially, and therefore, the relative proportion of spontaneous and GABA-evoked tonic currents should change with changes in regional ambient GABA level. In the absence of GABA, tonic current would be composed only of GABA-independent, spontaneous α4β3δ current that would be regulated by PKA. In contrast, in the presence of 1 μM GABA, total tonic α4β3δ currents were not altered by PKA. However, the magnitude of the increase in α4β3δ current produced by GABA was reduced by the "floor effect" produced by the PKA-induced increase in spontaneous current. These results suggest that PKA phosphorylation has complex effects on spontaneous and GABA-activated α4β3δ currents, which would vary in type and extent with different extracellular ambient GABA concentrations. Without PKA-activation, α4β3δ steady-state currents were primarily dependent on GABA-activation because their spontaneous currents are low, resulting in a large dynamic range for GABA-activated tonic current. With PKA-activation, however, spontaneous α4β3δ currents were elevated and the magnitude of GABA-activated tonic currents was reduced, resulting in a smaller GABA-activated tonic current dynamic range. In other words, PKA-activation can drive the channels to activities that are close to those produced by 1 μM GABA and therefore addition of 1 μM GABA in the presence of PKA-activation results in only small additional current. Therefore, given a high spontaneous basal current upon PKA-activation, during an increase of extracellular

ambient GABA concentration that might occur with a local or regional physiological or pathological decrease in local neuronal excitability, the increase in tonic α4β3δ current produced by GABA would be restricted to a small range, and thus, the response to a change in extracellular GABA would be reduced. We suggest that PKA could help maintain tonic inhibition mediated by α4β3δ receptors at a stable level to avoid large changes in neuronal excitability due to changes of extracellular GABA concentration. Thus, this result suggests that some antiepileptic drugs such as tiagabine and vigabatrin, which potentiate GABAergic tonic currents by increasing extracellular GABA (Suzdak and Jansen1995; Overstreet and Westbrook2001), would be less effective in conditions with higher PKA-activation.

The difference in PKA-modulation of tonic α4β3δ currents at different ambient GABA levels could also have physiological significance since there are regional and developmental variation in extracellular GABA concentrations (Hamori and Somogyi 1983; Engel et al. 1998; Frahm and Draguhn 2001). Various mechanisms can affect ambient GABA concentrations by regulating the release and uptake of extracellular GABA by neurons and glia. For example, GABA uptake in the hippocampus is developmentally regulated and cell type-specific (Draguhn and Heinemann 1996; Semyanov et al. 2003). In mature guinea pig hippocampus, interneurons expressed $GABA_A$ receptor-mediated tonic currents in response to extracellular GABA, while pyramidal neurons were selectively shielded by GABA transporters with more efficient GABA uptake leaving very little GABA in the extracellular space. In neurons exposed to relatively low extracellular ambient GABA

concentrations, our results suggest that expression of α4β3δ receptors would contribute to tonic current by opening spontaneously, and that PKA phosphorylation would affect tonic α4β3δ current by elevating agonist-independent spontaneous tonic current. In contrast, in early development when there is less effective GABA uptake or in specific cell types in brain areas like the cerebellar granule cells that have unique morphology to trap GABA and impede diffusion of GABA away from release sites, we proposed that a stable micromolar ambient concentrations could work as a buffer system to minimize PKA modulation of total tonic α4β3δ currents.

PKA differentially modulated spontaneous and GABA-activated tonic α4β3δ and α4β3γ2L currents.

There is substantial evidence that αβδ and αβγ $GABA_A$ receptor currents have distinct properties and are differentially regulated by numerous drugs including neurosteroids, barbiturates and propofol. Here, we have shown a significant differential extent of effects of PKA-activation on α4β3δ and α4β3γ2L receptor tonic currents. The differential modulation of α4β3δ and α4β3γ2L currents by PKA could have physiological significance in normal or pathological conditions such as epilepsy by providing precise regulation of different receptor isoforms in their specific locations. For example, in the rat thalamus, α4 subunits preferentially co-assemble with δ subunits (Sur et al. 1999), suggesting a high capacity for PKA modulation in that brain area. In the pilocarpine rodent model of epilepsy, perisynaptic α4βδ receptors in the dentate granule cell dendrites were suggested to be replaced with

α4βγ2 receptors (Zhang et al. 2007), which would result in loss of sensitivity to PKA-modulation.

In addition, abnormalities in protein phosphorylation have been reported in several animal models of epilepsy, although a clear relationship between phosphorylation and seizures has not yet been established. For example, status epilepticus (SE) was demonstrated to cause decreased phosphorylation of β3 subunits associated with a deficit in receptor stability on the cell surface (Terunuma et al. 2008). PKA plays a particularly important role in phosphorylation of $GABA_A$ receptors relative to other protein kinases. There have also been reports of elevated cAMP levels as well as PKA activity in the hippocampus of experimental animal models with epilepsy (Tehrani and Barnes, Jr. 1995; Yechikhov et al. 2001), and repeated injections of an initially subconvulsant dose of cAMP into the rat amygdala, has been shown to produce seizures (Yokoyama et al. 1989). These data suggest that PKA activation may be involved in the pathogenesis of acquired epilepsies, and thus, further study of PKA-mediated phosphorylation of $GABA_A$ receptors before and after seizures, and determination of how PKA modulation of different $GABA_A$ receptor isoforms alters inhibitory signaling during seizures will help understanding the role of PKA in epileptogenesis.

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CHAPTER III

MODULATION OF THE KINETICS OF PHASIC CURRENTS MEDIATED BY α4β3γ2L AND α4β3δ $GABA_A$ RECEPTOR BY PROTEIN KINASE A

Introduction

$GABA_A$ receptors containing α4 subunits are highly expressed in the dentate gyrus and thalamus, with lower levels of expression in cortex, striatum and other brain areas (Chandra et al., 2006), and are up-regulated in many pathological conditions such as epilepsy and alcohol withdrawal syndrome and also in δ knock out mice (Brooks-Kayal et al., 1998;Peng et al., 2002;Cagetti et al., 2003;Peng et al., 2004;Zhang et al., 2007). α4 subunits were found coassembled with β subunits and with γ2 or δ subunits to form either ternary α4βγ2 and α4βδ in thalamus and dentate gyrus, while still a portion of α4 subunit-containing receptors in brain membrane extracts were believed to be binary α4β receptors (Bencsits et al., 1999). In rat thalamus immunoprecipitation experiments using antisera against multiple subunits showed coassembly of δ or γ2 subunits with α4 subunits and further pharmacological analyses confirmed the presence of both α4βγ2 and α4βδ receptor isoforms in this brain region (Sur et al., 1999). In addition to reports of extra-synaptic, tonic inhibition mediated by α4 subunit-containing receptors in thalamus and dentate gyrus (Chandra et al., 2006;Liang et al., 2008), α4βγ2 receptors also have synaptic localization in specific brain regions. Even more interesting, α4βγ2 receptors have

been reported to move between synaptic and extra-synaptic locations under certain pathological conditions (Hsu et al., 2003;Peng et al., 2004;Liang et al., 2006;Zhang et al., 2007). Therefore, it is important to understand the effects of protein phosphorylation on the properties occurring under different conditions including spontaneous openings, tonic currents activated by low GABA concentrations and phasic currents activated by high GABA concentrations. Any differences in the effects of PKA phosphorylation on α4β3γ2L and α4β3δ receptors and on other α subunit-containing receptors will help to clarify the role of PKA phosphorylation in regulating synaptic and extrasynaptic $GABA_A$ receptor inhibition in the central nervous system.

In the last Chapter, I reported that both α4β3δ and α4β3γ2L receptors displayed spontaneous currents, but that PKA-activation increased spontaneous α4β3δ currents substantially more than spontaneous α4β3γ2L currents. The increase in spontaneous α4β3δ currents was due to an increase in single channel open frequency. In contrast, PKA-activation did not alter steady state, tonic currents recorded in the presence of 1 μM GABA but altered the transient properties of α4β3δ currents more than those of α4β3γ2L currents. Specifically, fast phases of desensitization were accelerated, and current peak amplitudes were increased. Enhancement of α1β1,3γ2L $GABA_A$ receptor current desensitization by PKA has also been reported (Hinkle and Macdonald, 2003), suggesting that a common mechanism of PKA modulation to change the current kinetic properties of different $GABA_A$ receptor isoforms. As α4β3δ receptors have a higher affinity for GABA than α4β3γ2L receptors, the

differential alteration in time course of their currents at low concentrations of GABA might be due to different activation levels of the two receptor isoforms, and therefore, I activated $GABA_A$ receptors with a saturating concentration of GABA to study how PKA-activation modifies the kinetic properties of α4β3δ and α4β3γ2L receptor currents.

In vitro experiments using purified $GABA_A$ receptors or recombinant subunit polypeptides revealed that β and γ subunits are primary substrates for various protein kinases (McDonald and Moss, 1994;McDonald and Moss, 1997;Moss et al., 1992a). However, whether or not a specific site is phosphorylated by one protein kinase or another is likely dependent on the experimental situation. In HEK cells, β3 subunit residues S408 and S409 are phosphorylated by PKA and PKC. In cultured cortical neurons, those sites are basally phosphorylated in a PKC-dependent manner; however, in different cell types such as CA-1 pyramid cells, the receptor is phosphorylated by PKA (Brandon et al., 2000;Krishek et al., 1994;McDonald et al., 1998a;Moss et al., 1992c;Poisbeau et al., 1999). The γ2L subunit residues S327 and S343 can be phosphorylated by PKC, but not by PKA, *in vitro*, but the kinase responsible for basal phosphorylation of the two sites *in vivo* remains uncertain. There have been multiple studies of the functional effects of phosphorylation. For example, PKA was reported to increase the fast phases of macroscopic desensitization of α1β1γ2L and α1β3γ2L $GABA_A$ receptor currents by phosphorylation of the conserved β subunit phosphorylation residues described above. Here, I further studied and compared the effects of phosphorylation by PKA of α4β3γ2L and α4β3δ $GABA_A$ receptors. I

focused on the effects of PKA on the current kinetic properties of each receptor isoform, which reflects their specific gating properties as ion channels.

Methods and Materials

Transient transfection of $GABA_A$ receptor subunits into HEK 293T cells

The cDNAs encoding rat α4, β3, γ2L and δ $GABA_A$ receptor subunit subtypes were subcloned into the expression vector pCMVNeo. A 60 mm cell dish of HEK 293T cells was co-transfected with 1 or 2 μg of each subunit plasmid with a 1:1:1 cDNA ratio and 1 μg of the pHook-1 cDNA (Invitrogen, Carlsbad, CA) using a modified calcium phosphate precipitation method. pHook-1 was used as a marker of positively transfected cells, which were selected 24 hrs after transfection by magnetic hapten coated beads. In some experiment, mutated β subunits were transfected in place of wild-type β subunits. All point mutations in rat β3 subunit constructs were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing.

Electrophysiological recordings

All $GABA_A$ receptor currents were obtained using a lifted whole-cell voltage-clamp recording technique. Patch pipettes (resistance1-1.5 MΩ) were pulled from borosilicate capillary glass (World Precision Instruments, Sarasota, FL) on a P-2000 laser puller (Sutter Instrument Co., San Rafael, CA) and filled with an internal solution containing 153 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES pH 7.3, with 4 mM ATP added on the day of recording. The cells were subjected to

continuous background perfusion of the external solution consisting of 142 mM NaCl, 8 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4. All experiments were performed at room temperature. Signals were processed by using 200B amplifier (Molecular Devices, Union City, CA) in voltage-clamp mode. All cells were voltage clamped at -20 mV during recordings.

GABA application and kinase modulation

Activators and inhibitors of PKA were purchased from Calbiochem (San Diego, CA). PKA was activated by externally pre-applied 8Br-cAMP (500-1000 μM; 8-Bromoadenosine-3',5'-cyclic monophosphate, sodium salt) or alternatively by internally applied 8Br-cAMP in the recording pipette. PKA activity was blocked by adding the specific PKA inhibitory peptide (PKI; 100 μg/ml) into the internal recording solution.

GABA and kinase modulators were delivered through a four parallel glass square barrel to HEK 293T cells using a modified SF-77B Perfusion Fast-Step application system (Warner Instrument Corp., Hamden, CT) (Hinkle et al., 2003). The drug application was initiated by an analog pulse triggered by the pClamp 9.2 software (Axon Instruments, Union City, CA) that caused the motor of the Warner Fast-Step to reposition the multi-barrel array from one barrel to another (e.g., external solution to GABA). By using this modified application system, I consistently obtained rise times (10–90%) less than 800 μsec with open-tip electrodes stepped from standard bath solution to 90% bath solution.

A high (1 mM) or low (1 μM) concentration of GABA was applied to lifted whole cells for 2 sec or 28 sec, respectively. Between every two applications of GABA, external solution was applied for at least 3 min to ensure maximal recovery from the GABA exposure. I also added pre-application steps to the protocol for modulator experiments to enable us to pre-apply a membrane-permeable modulator such as 8Br-cAMP. In the pre-application experiments, GABA was first applied to lifted cells expressing α4β3γ2L or α4β3δ $GABA_A$ receptors as an initial pre-control. After washout of GABA, the PKA activator 8Br-cAMP (1 mM) was applied for 4 min and then the lifted cell was stepped back into external solution for a brief washout (3 sec) of any residual external 8Br-cAMP before it was subjected to a second GABA application. This second GABA-activated current recorded immediately after an 8Br-cAMP pre-application was defined as the experimental current. After the second GABA application, the cell was washed in external solution for more than 6 min before it was subjected to the final GABA application to evoke a post-control current. Due to the variability of currents among cells, the experimental currents were normalized to their matched pre-control currents so that differences made by pre-application were shown in percentage. To control for current run-down during the whole set of pre-application experiments that were recorded over 10 min, I superfused cells with external solution instead of 8Br-cAMP during the 4 min pre-application process. Significant differences between the 8Br-cAMP pre-application group and the external control group were used to identify specific and significant effects caused by PKA activators.

Mutagenesis

Subunit phosphorylation sites (β3 S408, 409) were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) either to block receptor phosphorylation entirely with alanine substitutions or to mimic phosphorylation with glutamate substitutions.

Data analysis

Macroscopic currents were low pass filtered at 2 kHz, digitized at 10 kHz, and analyzed using pClamp9.2 software. For comparison of desensitization among currents, currents were normalized first to their peak value (defined as a maximal response of 1). I plotted the relative residual current, which was the residual current at the end of a prolonged GABA application divided by the current peak value and used as indicator of the overall extent of desensitization. In addition, the desensitization time course of some phasic currents activated by 1 mM GABA was fitted to a sum of exponential functions (Lagrange et al., 2007). The entire desensitization time course was fitted with one to five exponential functions using the Levenberg–Marquardt least squares method with the form $\sum A_{n*}e^{-t/\tau_n} + C$, where *n* was the number of exponential components, *t* was time, *A* was relative amplitude of a given component at time 0, τ was the time constant for a given component, and *C* was a constant to account for residual current. The number of exponential functions required to obtain the best fit was determined when adding an additional component did not significantly improve the fitting result based on an *F* test automatically performed by the analysis software on the sum of squared residuals. All the two

second and eight second GABA-activated phasic currents were fitted best with two to four exponential functions. All numerical and bar graph data were expressed as mean ± standard error. Statistical significance was compared between the kinase modulator group and the control solution group or between wild-type and mutant condition being examined. Statistical analyses were performed using Graph Pad Prism 4. Student's paired or unpaired *t* tests were used to compare pairs of data, or appropriate ANOVA analyses were used for comparing three or more groups. Statistical significance was taken as: $*p<0.05$, $**p<0.01$, or $***p<0.001$.

Results

Spontaneous, GABA-evoked tonic and phasic α4β3δ and α4β3γ2L currents

To characterize α4β3δ and α4β3γ2L currents, I co-transfected HEK 293T cells with α4, β3 and δ or γ2L subunits and recorded from lifted whole cells. As reported in chapter II, I recorded large holding currents in cells transfected with α4β3δ or α4β3γ2L subunits in the absence of GABA, which were due to spontaneous opening of both receptor isoforms and that were blocked by the $GABA_A$ receptor antagonist picrotoxin (Fig. 3.1 A). To characterize GABA-evoked α4β3δ and α4β3γ2L currents, I applied a low concentration of GABA (1 μM) for a prolonged duration (28 sec), so that currents approached their steady state levels at the end of the application (Fig. 3.1 B) and applied a high GABA concentration (1 mM) for a briefer duration (2 sec) to characterize desensitization of these receptors that contributes to determining the time course of phasic, synaptic currents (Fig. 3.1 C). I used a longer application of

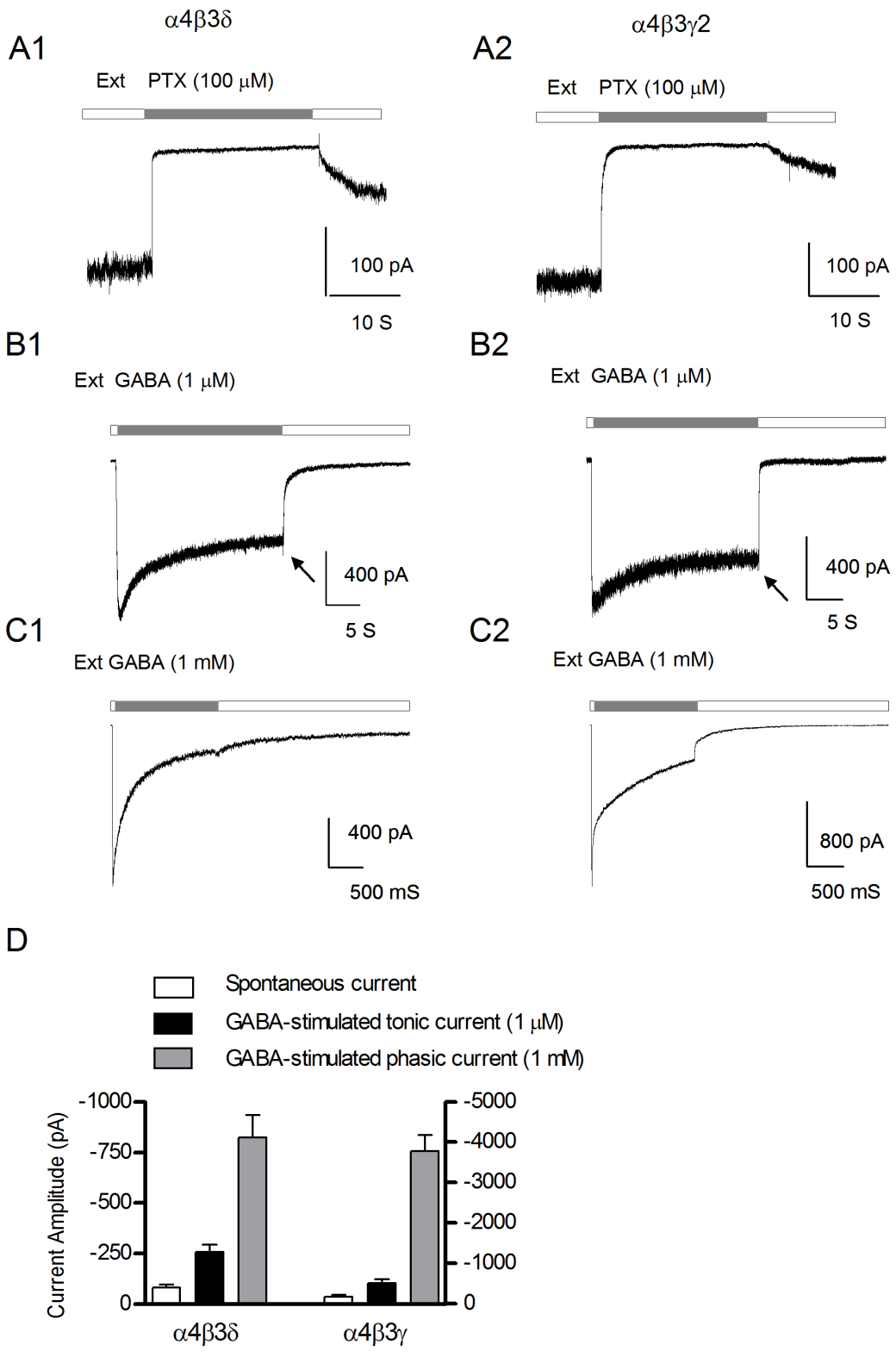

α4β3δ
A1
Ext PTX (100 μM)
100 pA
10 S
α4β3γ2
A2
Ext PTX (100 μM)
100 pA
10 S
B1
Ext GABA (1 μM)
400 pA
5 S
B2
Ext GABA (1 μM)
400 pA
5 S
C1
Ext GABA (1 mM)
400 pA
500 mS
C2
Ext GABA (1 mM)
800 pA
500 mS
D
Spontaneous current
GABA-stimulated tonic current (1 μM)
GABA-stimulated phasic current (1 mM)
Current Amplitude (pA)
-1000
-750
-500
-250
0
-5000
-4000
-3000
-2000
-1000
0
α4β3δ
α4β3γ

Figure 3.1. α4β3δ and α4β3γ2L receptors exhibit agonist-independent spontaneous currents and GABA-activated steady-state and phasic currents.

A, Spontaneous currents recorded from cells expressing α4β3δ (A1) and α4β3γ2L (A2) $GABA_A$ receptors were reduced reversibly by 100 μM picrotoxin. **B,** Steady-state currents recorded from cells expressing α4β3δ (B1) and α4β3γ2L (B2) $GABA_A$ receptors were activated by prolonged 28 sec applications of 1 μM GABA. The near steady state currents were indicated by the black arrows at the end of the GABA application. C, Phasic currents recorded from cells expressing α4β3δ (C1) and α4β3γ2L (C2) $GABA_A$ receptors were activated by brief, 2 sec applications of 1 mM GABA. **D,** The amplitudes of picrotoxin-sensitive spontaneous currents, GABA-activated steady-state tonic and phasic peak currents recorded from α4β3δ (left) and α4β3γ2L (right) receptors were plotted.

GABA than occurs during transmitter release (~1 msec) at synapses to permit characterization of PKA activation on desensitization as well as on the magnitude of the phasic currents.

Spontaneous α4β3δ and α4β3γ2L receptor currents were smaller than GABA-activated currents. Picrotoxin-sensitive α4β3δ and α4β3γ2L holding currents averaged 55.7 ± 11.7 pA (n = 12) and 126.6 ± 23.8 pA (n = 10), respectively (Fig. 3.1 D, open bars). Prolonged (28 sec) application of 1 μM GABA elicited α4β3δ (Fig. 3.1 B1) and α4β3γ2L (Fig. 3.1 B2) currents that desensitized moderately to near steady state currents (Fig. 3.1 B, arrows). The amplitudes of GABA-activated tonic α4β3γ2L currents (510.3 ± 101.9 pA, n = 7) at the end of application were twice as large as those of GABA-activated tonic α4β3δ currents (257.0 ± 35.5 pA, n = 12) (Fig. 3.1 D, solid bars). However, relative to their peak phasic currents, GABA-activated tonic currents were larger for α4β3δ (31.2%) than for α4β3γ2L (13.5%) receptors (Fig. 3.1 D), consistent with the observation that δ subunit-containing receptors conferred higher agonist sensitivity than γ2L subunit-containing receptors. Briefer (2 sec)

applications of 1 mM GABA elicited phasic α4β3δ (Fig. 3.1 C1) and α4β3γ2L (Fig.3.1 C2) currents that both desensitized rapidly and extensively. The extents of desensitization of phasic α4β3δ and α4β3γ2L currents were similar, except that desensitization of α4β3γ2L currents, but not α4β3δ currents, contained a fast phase. The peak amplitudes of phasic α4β3γ2L currents (3778 ± 390 pA, n = 23) were more than four times larger than those of phasic α4β3δ currents (824.2 ± 110.0 pA, n = 28), (Fig. 3.1D, gray bars). Picrotoxin-sensitive spontaneous α4β3δ and α4β3γ2L currents were less than 7% and 4% of their peak phasic currents, respectively, and thus, spontaneous currents have little effect on phasic synaptic currents. However, at low concentrations of GABA, spontaneous α4β3δ and α4β3γ2L currents were both more than 20% of their GABA-activated tonic currents. Thus, α4β3δ and α4β3γ2L receptor total tonic currents were composed of both GABA-activated tonic currents and spontaneous currents.

PKA activation regulates α4β3δ and α4β3γ2L $GABA_A$ receptor current kinetics.

We have demonstrated that PKA had a GABA concentration-dependent effect on α4β3δ and α4β3γ2L steady-state currents (Tang et al., 2010). Meanwhile, I also observed more alteration of transient α4β3δ currents than α4β3γ2L currents at 1 μM GABA (Fig. 3.2). Specifically, fast phases of desensitization were accelerated together with a potentiation of current peak amplitudes. I used pre-incubation of 8Br-cAMP (1 mM) (see methods) to determine PKA effects on α4β3δ or α4β3γ2 receptor currents. All three α4β3δ (Fig. 3.2 A1) and α4β3γ2L (Fig. 3.2 B1) currents, pre-control (green), 8Br-cAMP treated (black) and post-control (gray) currents, were

normalized to their peak amplitudes and superimposed to identify changes in their time course and extent of desensitization.

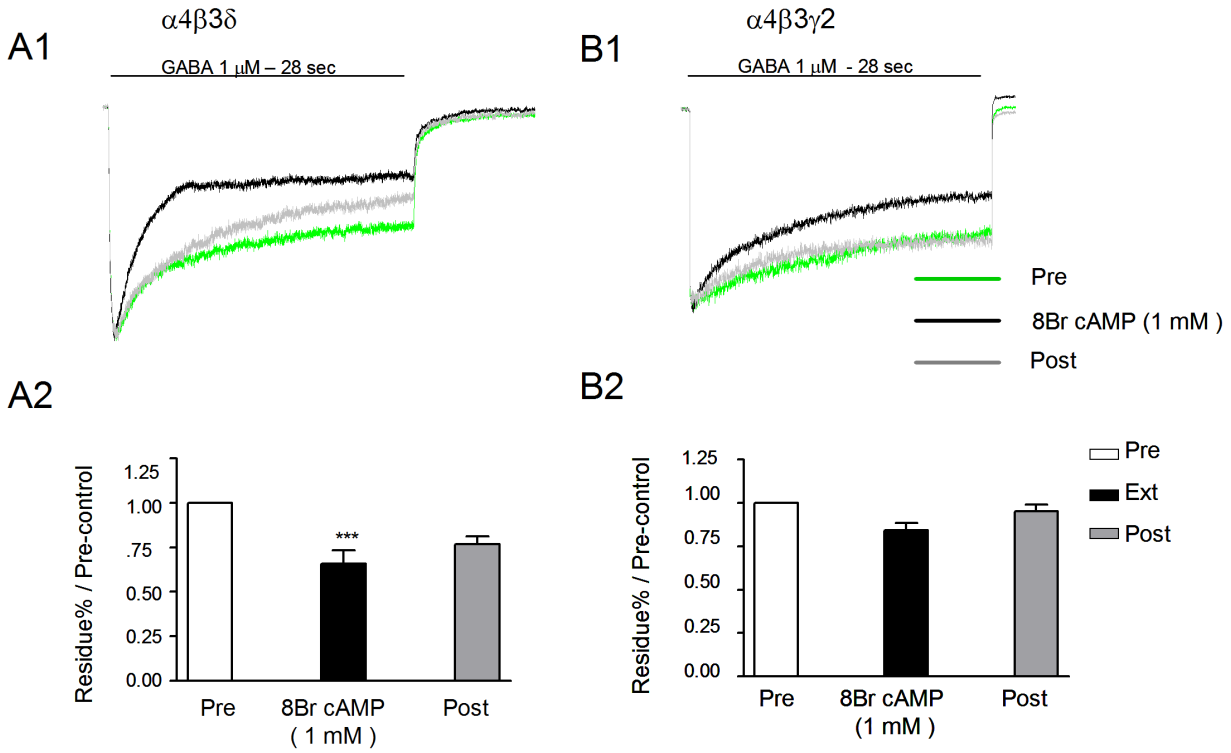


Figure 3.2. PKA-activation accelerated the fast phase of α4β3δ currents more extensively than α4β3γ2L currents at 1 μM GABA.

A and B, Pre-application of 8Br-cAMP (1 mM) increased the desensitization of α4β3δ (A1), to a lesser extent of α4β3γ2L, tonic currents (B1). Pre-control (green), 8Br-cAMP-treated (black) and post-control (gray) currents evoked by 1 μM GABA were normalized to peak currents and superposed. The relative residual of α4β3δ (A2) and α4β3γ2L (B2) 8Br-cAMP treatment (solid bars) and post-control (gray bars) currents were compared to the pre-control currents (open bar). Currents were significantly different from pre-control current at ***$p < 0.001$.

To quantitatively characterize changes in desensitization produced by PKA activation, I measured the relative residual current at the end of GABA application before and after a 1 mM 8Br-cAMP application and plotted them as a ratio of the relative residual current for matched, pre-control α4β3δ (Fig. 3.2 A1) or α4β3γ2L (Fig. 3.2 B1) currents. To control for current rundown during the experiments, I also

performed pre-application of external solution that did not change relative residual currents. After application of external solution, relative residual currents were within 5% of pre-control relative residual currents. In contrast, pre-application of 8Br-cAMP increased the extent of current desensitization, resulting in smaller relative residual currents. Compared to the pre-control currents (Fig. 3.2 A2, B2, open bars set as a ratio of 1), only 65.9 ± 7.6% (n = 13) (Fig 2A2, solid bar) residual currents of α4β3δ currents remained after pre-application of 8Br-cAMP, resulting in a significant 34.1% reduction of relative residual current. After PKA activation, α4β3γ2L receptors still had 84.9 ± 4.6% (n = 5) (Fig 2B2, solid bar) relative residual current, thus having only a 15.1% reduction. After wash with external control medium, the reduction of residual currents by PKA activation was reversed (Fig. 3.2 A2, B2, gray bars).

However, the larger effect of PKA on desensitization of α4β3δ currents than of α4β3γ2L currents might simply be because α4β3δ receptors were activated to a higher level than α4β3γ2L receptors at this low concentration of GABA. The acceleration of desensitization might be even strengthened when more receptors were activated to access desensitization states at a higher GABA concentration. Thus, I saturated α4β3δ and α4β3γ2L receptors with 1 mM GABA and tested PKA effects on the desensitization of their phasic currents (Fig. 3.3 A1, B1). For phasic currents activated by 1 mM GABA, pre-application of 8Br-cAMP produced larger reductions in both α4β3δ (49.2 ± 6.8%, n = 7) and α4β3γ2L (47.7 ± 5.8%, n = 8) relative residual currents (Fig. 3.3 A2, B2 solid bars). Thus, PKA had less effect on desensitization of currents with smaller extent of desensitization at a low activation level, and more

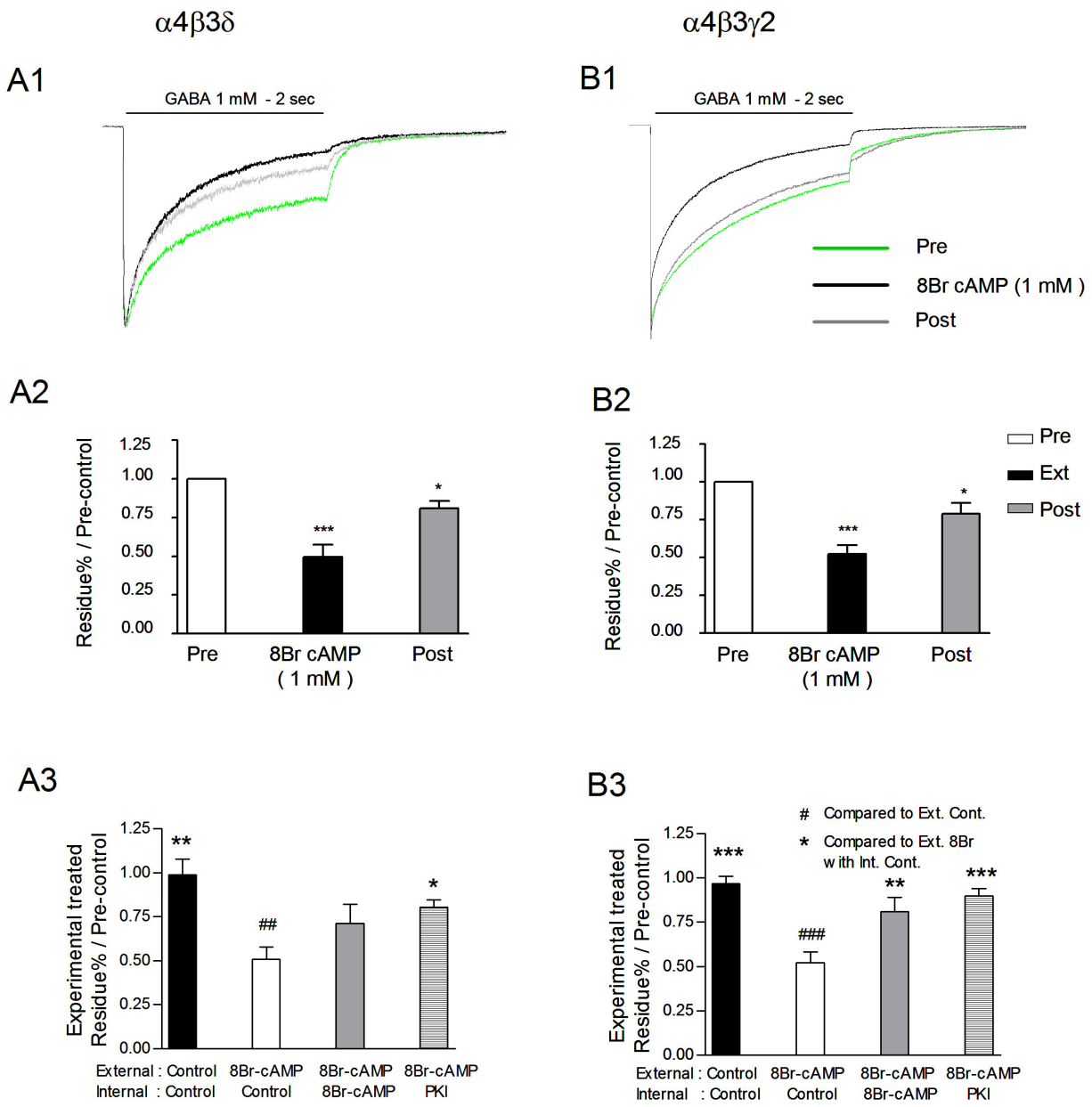


Figure 3.3. PKA-activation increase desensitization of both α4β3δ and α4β3γ2L phasic currents.

A1 and B1, Pre-control (green), 8Br-cAMP-treated (black) and post-control (gray) α4β3δ (A1) and α4β3γ2L (B1) currents evoked by 1 mM GABA were normalized to the peak currents and were superposed to facilitate comparison of the rate and extent of desensitization. **A2 and B2,** The relative residual of α4β3δ (A2) and α4β3γ2L (B2) 8Br-cAMP treated (solid bars) and post-control (gray bars) currents were compared to the pre-control currents (open bar). **A3 and B3,** Relative residual α4β3δ (A3) and α4β3γ2L (B3) currents following different experimental treatments were plotted as ratio of their pre-control relative residual currents. The current ratio for external control pre-application (solid bars) was close to 1 and was compared to the current ratio obtained for 8Br-cAMP pre-application in the absence (open bars) and presence of internal 8Br-cAMP (1 mM, gray bars) or PKI (100μg/ml, striped bars) in the recording pipette.

effect on currents with a larger extent of desensitization at a high activation level. This correlation between the enhancement of desensitization by PKA and receptor activation level suggests that PKA phosphorylation may affect states extensively accessed during application of high concentrations of GABA such as desensitized states.

8Br-cAMP did not have a direct, PKA independent, effect on α4β3δ or α4β3γ2L currents.

Externally applied cAMP analogs have been reported to decrease $GABA_A$ receptor currents independent of PKA phosphorylation via an extracellular site (Harrison and Lambert, 1989;Lambert and Harrison, 1990). However, in other studies, the analogs were reported to enhance $GABA_A$ receptor currents (Cheun and Yeh, 1992;Kano and Konnerth, 1992). Thus, it was important to distinguish PKA-dependent effects of 8Br-cAMP from any non-specific effects of the drug. In my experiments, 8Br-cAMP pre-application produced an increase, not a decrease, of peak currents (data not shown) and increased the rates and extents of current desensitization as described above. The reductions of relative residual phasic currents produced by 8Br-cAMP pre-application (Fig. 3.3 A3, B3; open bar) were significantly different from those produced by pre-control application of external solution (Fig. 3.3 A3, B3; solid bars) for α4β3δ (1.2 ± 9.1% $p<0.01$; $n = 4$) and α4β3γ2L (3.1± 4.0% $p<0.001$; $n = 13$) currents, respectively. I also performed several control experiments to determine if the effect of 8Br-cAMP on current desensitization was due to PKA activation. First, I added 8Br-cAMP (1 mM) to the internal recording solution to

activate PKA constitutively and to occlude the effect of extracellular application of 1 mM 8Br-cAMP. With inclusion of 8Br-cAMP in the recording pipette, the reduction of relative residual currents by extracellular 8Br-cAMP pre-application decreased from 49.2 ± 6.8% to 28.8 ± 10.8% (n = 4) for α4β3δ currents (Fig. 3.3 A1; open vs gray bar) and from 47.7 ± 5.8% to 19.0 ± 8.2% (n = 4) for α4β3γ2L currents (Fig. 3.3 B1; open vs gray bar). Second, I added the specific PKA inhibitor PKI (100 μg/ml) in the recording pipette solution to block PKA activation (Fig. 3.3 A3, B3; striped bars). With PKI in the pipette, pre-application of 8Br-cAMP only caused a 19.5 ± 4.2% (n = 3) reduction of relative residual α4β3δ current and a 10.0 ± 4.1% (n = 6) reduction of relative residual α4β3γ2L current, reductions that were significantly less than obtained with pre-application of 8Br-cAMP alone for both receptors. Thus, these data suggested that extracellular pre-application of 8Br-cAMP increased the rate and extent of desensitization of α4β3δ and α4β3γ2L currents, and that this effect was not due to a non-specific effect of 8Br-cAMP at extracellular sites but was specifically due to activation of PKA.

The effect of PKA on desensitization of α4β3γ2L, but not α4β3δ, currents was due to phosphorylation of two β3 subunit serines (408, 409).

Serines 408 and 409 have been reported to be the major $GABA_A$ receptor β3 subunit substrates for PKA *in vitro* or in heterologous expression systems (Browning et al., 1990;Moss et al., 1992b). Functional studies coupled with site-directed mutagenesis of potential PKA sites have also attributed PKA-induced changes in current amplitude to phosphorylation of these two adjacent β3 subunit serines

(McDonald et al., 1998b). To determine if the changes in current kinetics observed with α4β3δ and α4β3γ2L receptors was also due to phosphorylation of these β3 subunit residues, I mutated the phosphorylation substrate serines to alanines to block their phosphorylation, and pre-applied 8Br-cAMP (1 mM) to mutant $\alpha4\beta3_{AA}\delta$ and $\alpha4\beta3_{AA}\gamma2L$ receptors. 8Br-cAMP produced similar reductions of relative residual $\alpha4\beta3_{AA}\delta$ currents (40.6 ± 6.3%, n = 8) and α4β3δ currents (49.2 ± 6.8%) (Fig. 3.4 A). However, the reduction of relative residual $\alpha4\beta3_{AA}\gamma2L$ current by PKA (15.1 ± 3.7%, n = 5) was significantly less than the reduction of relative residual α4β3γ2L current (47.7 ± 5.8%) (Fig. 3.4 B). Thus, enhancement of phasic α4β3γ2L current

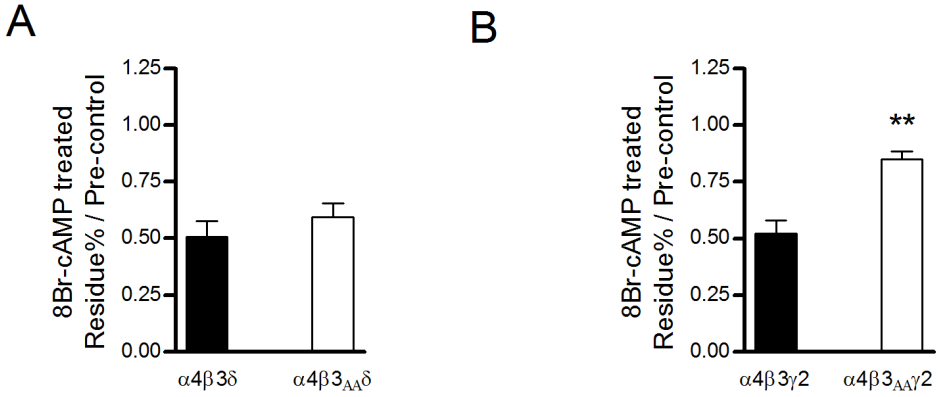


Figure 3.4. PKA enhancement of phasic current desensitization was due to phosphorylation of the β3 subunit for α4β3γ2L receptors but not for α4β3δ receptors.

A and B, Changes in phasic current desensitization produced by PKA activation between wild type and mutant α4β3δ (A) or α4β3γ2L (B) receptors were compared. Adjacent β3 subunit serines, S408 and 409, were mutated to alanines to block phosphorylation by PKA. Pre-application of 8Br-cAMP (4 min, 1 mM) caused similar reduction on the relative residual current of phasic wild-type α4β3δ and mutant $\alpha4\beta3_{AA}\delta$ currents. Mutant phasic $\alpha4\beta3_{AA}\gamma2L$ currents, however, had significantly larger relative residual currents after 8Br-cAMP treatment than phasic wild-type α4β3γ2L receptor currents. Currents were significantly different from control current at $^{**}p < 0.01$.

desensitization by 8Br-cAMP was due at least in part to direct phosphorylation of β3 subunit serines 408 and 409, while the effect of 8Br-cAMP on α4β3δ receptor current desensitization likely was not mediated by phosphorylation of the β3 subunits.

Knowing that increased desensitization of α4β3γ2L current caused by PKA-activation was mediated by phosphorylation of β3 subunit residues Ser 408 and 409, I compared PKA effects to direct receptor "phosphorylation" at those sites. To mimic direct phosphorylation, β3 subunit S408 and 409 were mutated to glutamate residues to mimic constitutive phosphorylation. Currents evoked by 8 sec applications of GABA (1 mM) to constitutively "dephosphorylated" $\alpha4\beta3_{AA}\gamma2L$ receptors and "phosphorylated" $\alpha4\beta3_{EE}\gamma2L$ receptors were normalized for comparison (Fig. 3.5 A), and $\alpha4\beta3_{EE}\gamma2L$ currents had an increased rate and extent of desensitization compared to $\alpha4\beta3_{AA}\gamma2L$ currents, similar to the enhancement of desensitization produced by PKA. I extended my study to determine the effects of the serine mutations on desensitization time courses by fitting the macroscopic desensitization decay with multiple exponential functions that were sorted into four discrete groups based on the fitted time constants: τ1 (< 20 ms), τ2 (20-150 ms), τ3 (150-800 ms) and τ4 (800-5000 ms) (Fig. 3.5 B) (Bianchi and Macdonald, 2002;Lagrange et al., 2007). Interestingly, there were no differences in the desensitization time constants (Fig. 3.5 B2). Current desensitization was accelerated mainly by increasing the contribution of the fastest desensitization component (A1) and small changes in relative proportion of the other components as well as a reduced residual current (C) (Fig. 3.5 B1).

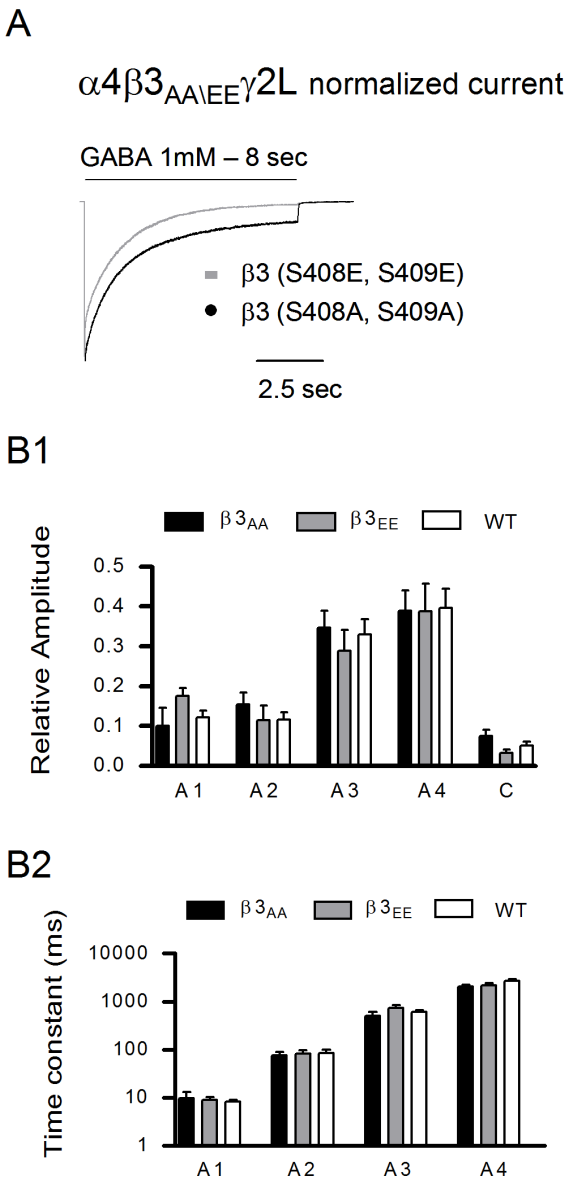


Figure 3.5. "Direct Phosphorylation" of β3 subunits increased desensitization of phasic α4β3γ2L currents.

A, $\alpha4\beta3_{AA}\gamma2L$ and $\alpha4\beta3_{EE}\gamma2L$ mutant receptors currents evoked by 8 sec application of GABA (1 mM) were normalized to the peak currents and overlaid. Constitutively "phosphorylated" mutant $\alpha4\beta3_{EE}\gamma2L$ receptor currents had faster and a larger extent of desensitization than constitutively "dephosphorylated" mutant $\alpha4\beta3_{AA}\gamma2L$ receptor currents. **B,** The wild type $\alpha4\beta3\gamma2$ and mutant $\alpha4\beta3_{AA}\gamma2L$ and $\alpha4\beta3_{EE}\gamma2L$ current desensitization phases were fit to the sum of four exponential functions, and the time constants (B1) and relative exponential component amplitudes (B2) were plotted. The faster desensitization of $\alpha4\beta3_{EE}\gamma2L$ receptor currents and slower desensitization of $\alpha4\beta3_{AA}\gamma2L$ receptor currents were due to different relative amplitudes of fitted exponential components and residual currents. The exponential component time constants were unchanged by the mutations.

This unique pattern of change in desensitization kinetics was also produced by 8Br-cAMP pre-application to α4β3γ2L and α4β3δ receptors (Fig. 3.6). There were changes (increases or decreases) in the relative amplitudes of the exponential components with a reduction of the residual component (Fig. 3.6 A1, B1), with no significant change in the time constants (Fig. 3.6 A2, B2). For α4β3γ2L receptors, the major change in desensitization was due to an increase in the relative amplitude of the fastest exponential component and small changes in the relative amplitudes of the other components, which was same as the results obtained for $\alpha4\beta3_{EE}\gamma2L$. Thus, the

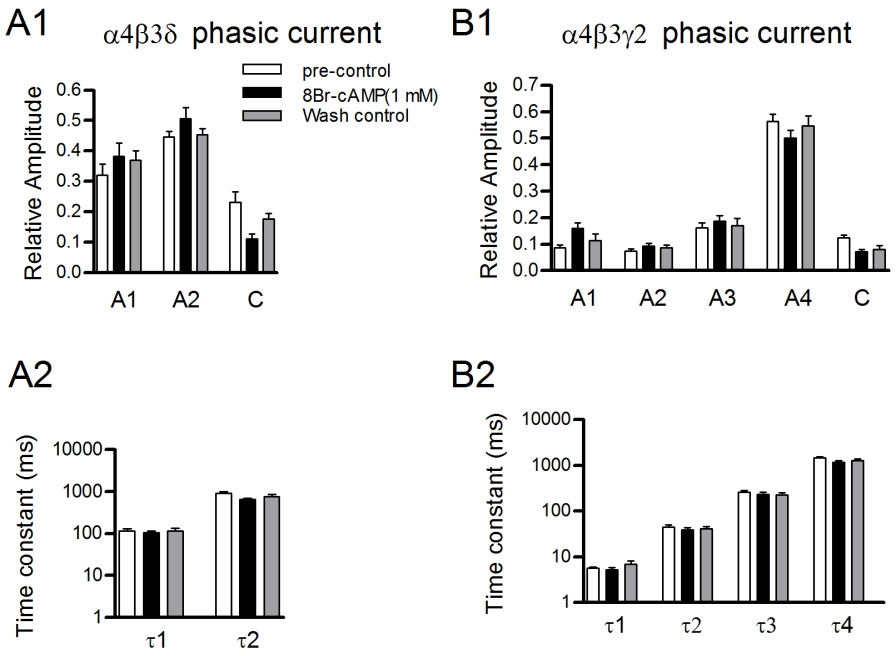


Figure 3.6. PKA-activation altered the kinetic properties of α4β3δ and α4β3γ2L receptor phasic currents.

A and B, Fitting results of 2 sec desensitization time course of pre-control (open bar), 8Br-cAMP treated (solid bar) and post-control (grey bar) α4β3δ (A1, B1) or α4β3γ2L (A2, B2) $GABA_A$ receptor currents. Desensitization enhancement of both of phasic α4β3δ and α4β3γ2L currents by 8Br-cAMP pre-application was caused by change of relative amplitude of fitting exponential components (B1, B2) with constant time constant (A1, A2).

results suggested that PKA might affect current kinetics of different receptor isoforms using a common mechanism to regulate the contribution of individual phases of desensitization without affecting their time constants. However, the similar modulation of current kinetics of both α4β3γ2L and α4β3δ receptor currents by PKA could not simply be attributed to phosphorylation on the same sites on their common β3 subunit, since the $\beta3_{AA}$ mutation blocked the PKA effects on the desensitization of α4β3γ2L, but not α4β3δ, currents.

Discussion

The aim of the present study was to investigate modulation by PKA of two α4 subunit-containing $GABA_A$ receptors and identify common and different PKA effects on different $GABA_A$ receptor isoforms. By executing parallel experiments on α4β3δ and α4β3γ2 $GABA_A$ receptor isoforms, I was able to identify different effects that were produced at multiple activation levels of the receptors. Combined with a previous study of α1β1/3γ2 receptors (Hinkle and Macdonald, 2003), I observed consistent modulation by PKA of current desensitization of different receptor isoforms including α4β3δ and α4β3γ2 receptors. The PKA-induced increase of desensitization of both α4β3δ and α4β3γ2 currents was due to alteration in the relative amplitudes of the exponential components (usually increase of relative amplitude of fast phases and decrease of relative amplitude of slow phases) with no change in the time constants. Thus, I propose a kinetic model to explain this unique pattern of change in current desensitization that could also explain the modulation of spontaneous and tonic currents by PKA as well.

Desensitization of phasic α4β3δ and α4β3γ2L currents was enhanced by PKA-mediated phosphorylation.

Although desensitization is usually considered inhibitory, current desensitization of $GABA_A$ receptor can prolong the deactivation time course of IPSCs with prolonged charge transfer due to slowing of dissociation of GABA from the receptor. Thus although total charge transfer is reduced, desensitization has an important role in determining the amplitude and time course of individual IPSC and of IPSCs activated by repetitive stimulation. PKA modulation of current desensitization could have a significant influence on synaptic α4β3γ2 currents and increase the plasticity of synaptic inhibition. PKA modulation of extrasynaptic α4β3δ receptors current desensitization also plays a critical role in regulating tonic inhibition. Although αβδ receptors that mediate tonic inhibition are relatively non-desensitizing receptors, extrasynaptic α4βδ receptors in thalamus and dentate gyrus and extrasynaptic α6βδ receptors in cerebellum both exhibited slow, but extensive, macroscopic desensitization (Bianchi et al., 2002). Desensitization of α4βδ and α6βδ currents actually preserved a population of receptors in non-conducting states that still could access open states in response to endogenous modulators such as phosphorylation. Thus, tonic inhibition mediated by desensitized αβδ receptor isoforms has more potential for modulation.

We have demonstrated differential modulation of steady-state (spontaneous and GABA-activated tonic currents) α4β3δ and α4β3γ2L currents by PKA (Tang et al., 2010). In Table 2, I summarized the effects by PKA on different components of the

different α4β3δ and α4β3γ2L receptor isoform currents. This is not surprising since αβδ and αβγ $GABA_A$ receptor currents have distinct properties and are differentially regulated by a variety of modulators such as neurosteroids, barbiturates and propofol. In this study of two α4 subunit-containing receptors and a previous study of α1β1,3γ2L receptors

Table 2. Comparison of PKA modulation of α4β3δ and α4β3γ2L *$GABA_A$ receptors.*

Activation level	No GABA	nM GABA	1 μM GABA	1 mM GABA
Current type	Spontaneous	GABA-activated tonic	Total tonic	Phasic
α4β3δ	Increase	Decrease	No change	Increased Desensitizatio
α4β3γ2L	Small increase	Small decrease	No change	Increased Desensitization

(Hinkle and Macdonald, 2003), I found similar effects of PKA to increase the rate and extent of desensitization of phasic, non-equilibrium macroscopic currents, suggesting that PKA always causes more current desensitization and implying a common effect on synaptic phasic currents not restricted to specific receptor isoforms. This consistency of PKA alteration of desensitization of different receptor isoforms could lead to uncovering a common regulatory mechanism for all $GABA_A$ receptors.

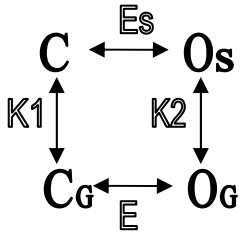
Modulation of α4β3δ and α4β3γ2L current desensitization by PKA was not due to phosphorylation of the same sites.

It was reasonable to ascribe the common effect on α4β3δ and α4β3γ2L current desensitization to the conserved PKA sites located in their common β3 subunit. However, I concluded the effects of PKA on α4β3δ and α4β3γ2L receptors were not

due to the same sites in the β3 subunit. Phosphorylation of the conserved β3 subunit sites (S408, 409) was responsible for the majority of the PKA effect on α4β3γ2L current desensitization but was not responsible for the PKA effect on α4β3δ current desensitization. The easiest explanation for this phenomenon was that β3 subunit serines 408 and 409 were the major targets of PKA in α4β3γ2L receptors, but for α4β3δ receptors, other phosphorylation sites were involved in the effect on desensitization. The alternate phosphorylation sites mediating the PKA effect on α4β3δ receptors could be located either on the receptor, such as on the δ subunit, or on other closely associated proteins. To test this hypothesis, biochemical experiments to distinguish different PKA phosphorylation patterns on α4β3δ and α4β3γ2L receptor complexes will be necessary.

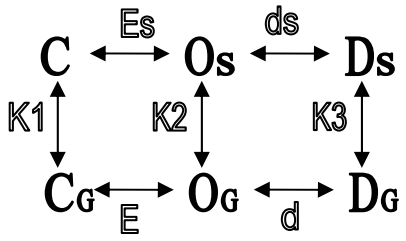
Mechanisms for PKA regulation of spontaneous, tonic and phasic α4β3δ currents

An allosteric activation scheme has been used previously to explain the presence of spontaneous openings (Chang and Weiss, 1999). In the scheme, a spontaneously gated open state (Os) was connected to an unbound closed state (C) and each of those states was connected to a GABA-bound open or closed state, respectively (kinetic scheme I). In this cyclic kinetic model, alteration of spontaneous opening efficacy (Es = βs/αs) was proposed to account for increased spontaneous openings, which for α4β3δ receptor channels obviously must also be modulated by PKA.



Kinetic Scheme I

To explain the results, I expanded this kinetic scheme to include desensitized states (kinetic scheme II). The three states in the upper layer of the cyclic model (C, Os and Ds) are unbound states, and the three in the lower layer (C_G, O_G and D_G) are GABA-bound states. It was also assumed that all connections between states in the two layers were driven by GABA binding. Thus, in the absence of GABA, channel opening rates would depend only on transitions among GABA-unbound states in the upper layer of the scheme. At saturating GABA concentrations, the transitions from GABA-unbound states (upper layer) to GABA-bound states (lower layer) is assumed to be very rapid (instantaneous) and complete, and thus occupancy of all three states in GABA-unbound layer would be immediately converted into the initial occupancy of their GABA-bound states in the lower layer at the onset of GABA application. This would be followed by redistribution among all GABA-bound states until the receptors reach equilibrium in the GABA-bound (lower) layer. I present this oversimplified model to qualitatively illustrate effects of PKA. Even relatively nondesensitizing α1β3δ channels have been shown to have at least two different open states and at least five closed states including several desensitized states (Haas and Macdonald, 1999). A more complete model would be needed to quantitatively determine current kinetics.



Kinetic Scheme II

Based on the results, PKA activation increased desensitization of both α4β3δ and α4β3γ2L currents at 1 mM GABA due to changes in relative amplitudes of the different exponential components of desensitization with no changes in time constants. Desensitization time constants reflect the rates at which open states relax into equilibrium with non-conducting (closed and desensitized) states and do not correspond to single microscopic transition rates; rather, they depend on both the forward and backward rates of the relevant transitions (Jones and Westbrook, 1996). Since receptors at this high GABA concentration should all be fully-liganded, the unaltered time constants of desensitization indicate that all of the rates in the GABA-gated scheme (lower layer) were unchanged. The coefficients of different desensitization exponential components, however, could be altered by changing the initial occupancy of different GABA-bound states (C_G, O_G and D_G) at the onset of GABA application, which was determined by the equilibrium occupancy of GABA-unbound states (C, Os and Ds) before GABA application. It is clear that the equilibrium occupancy of spontaneously gated open states (Os) of α4β3δ receptors was increased by PKA activation, as well as increased or decreased equilibrium occupancy of C and Ds states. As a result, the current would desensitize with the same exponential time constants, but because the distribution of starting states would

differ, the relative proportion of receptors relaxing into closed and desensitized states would differ with or without activation of PKA.

In contrast to non-equilibrium current properties such as desensitization, steady-state currents (tonic currents) reflect open probabilities at equilibrium and are only determined by the rate constants and not by initial occupancy of different states. Therefore, no matter how differently receptors enter the GABA-bound scheme from GABA-unbound states, the equilibrium current after all the receptors enter the GABA-bound states would not be affected. As 1 μM GABA has been shown to be a saturating concentration for steady-state α4β3δ currents (Lagrange and Macdonald, 2006), it was high enough to drive most unbound receptors to GABA-bound states at steady-state. With most receptors driven to GABA-bound states by 1 μM GABA and no alteration of the rate constants in GABA-bound layer, the equilibrium currents would be the same before and after PKA activation, consistent with that PKA activation produced no change in total tonic α4β3δ current activated by 1 μM GABA.

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CHAPTER IV

PROTEIN KINASE C HAS ADDITIVE EFFECTS ON DESENSITIZATION OF α4β3γ2L $GABA_A$ RECEPTOR CURRENTS

Introduction

Among all the phosphorylation sites found in $GABA_A$ receptors subunits, the most well characterized four sites (β3 S408,409 and γ2L S327,343) are subject to PKC phosphorylation. In cultured cortical neurons, the shared PKA and PKC phosphorylation sites in β3 subunits (S408,409) are basally phosphorylated in a PKC-dependent manner (McDonald et al., 1998;Brandon and Moss, 2000). For PKA, the *in vivo* and *in vitro* phosphorylation patterns of $GABA_A$ receptors could be different and subunit composition-dependent. For example, the β2 subunit, but not the β1 and β3 subunits, lacks the selective binding motif for A-kinase anchoring protein 150 (AKAP150) to recruit PKA (McDonald et al., 1998;Brandon et al., 2003), and therefore, can only be phosphorylated by PKA *in vitro*. Compared to the PKA phosphorylation sites, the four PKC phosphorylation sites characterized *in vitro* using GST-fusion proteins (S408,409 of the β3 and S327,343 of the γ2L) have been verified in native $GABA_A$ receptors and in recombinant $GABA_A$ receptors expressed in human embryonic kidney (HEK)-293 cells or cortical neurons (Brandon et al., 2000;Krishek et al., 1994;Moss et al., 1992;McDonald et al., 1998).

Although the PKC phosphorylation sites were relative clear, studies of physiological actions of PKC on $GABA_A$ receptor function have reported complex

and even contradictory effects. Activation of PKC by phorbol esters down-modulated $GABA_A$ receptors (Kellenberger et al., 1992;Krishek et al., 1994;Leidenheimer et al., 1992;Sigel et al., 1991). In contrast, the active catalytic domain of PKC (PKM) enhanced GABA-stimulated currents in L929 cells (Lin et al., 1994;Lin et al., 1996). These conflicting results may have been due to differences in methods used to activate PKC and diverse downstream signal pathways activated in different experimental cell systems, considering how many different isozymes of PKC might be activated by different approaches. Given that both β and γ2 subunits are PKC substrates, it could also contribute to the diversity of PKC modulation if PKC has different efficiency for phosphorylation of two subunits or if phosphorylation of one subunit affects the interaction of the other subunit with PKC.

Despite divergent results that have been reported, little effort has been made to compare the effects of different protein kinases on $GABA_A$ receptor function, and all of the studies of modulation by phosphorylation have been focused on α1βγ2 $GABA_A$ receptors. In studies of PKA modulation, a common effect on the current kinetics of several $GABA_A$ receptor isoforms including α4β3δ, α4β3γ2L and α1β1,3γ2L receptors was observed. Therefore, I was interested to determine if phosphorylation of the same β subunit serines by PKC caused increased current desensitization as PKA-activation did and to determine how the additional phosphorylation of γ2 subunits by PKC could affect the regulatory effects on β subunits. In the current study, I chose to study the effects of PKC-activation on α4βγ2L receptors. Although α4βγ receptors only comprise a small population of native $GABA_A$ receptors in the

brain, the α4 subtype is relatively abundant in brain regions involved in both partial and generalized epilepsies, including cortex, hippocampus, and thalamus (Pirker et al., 2000). Furthermore, the α4 subtype has been consistently shown to be up-regulated in multiple animal models of chronic epilepsy (Peng et al., 2004).

To understand the effects of PKC phosphorylation of β3 and γ2L subunits, I used several subunits with mutations of the phosphorylation sites. For example, the full range of PKC phosphorylation of α4β3γ2L receptors could vary from full dephosphorylation to full phosphorylation of all four sites; therefore, I used four quadruple mutant receptors ($\alpha4\beta3_{AA}\gamma2L_{AA}$, $\alpha4\beta3_{AA}\gamma2L_{EE}$, $\alpha4\beta3_{EE}\gamma2L_{AA}$ and $\alpha4\beta3_{EE}\gamma2L_{EE}$) to mimic the differential PKC phosphorylation levels. In the current study, I used a rapid-perfusion system to deliver GABA or kinases modulators to lifted whole cells with transient transfection of wildtype or mutant $GABA_A$ receptors subunits. By using rapid application with a short exchange time (<800 μs), I was able to obtain a rigorous study of current kinetics and to compare the modulatory effects of PKC on different subunits to PKA effects on different $GABA_A$ receptors.

Methods and Materials

Transient transfection of $GABA_A$ receptor subunits into HEK 293T cells

The cDNAs encoding rat α4, β3, γ2L and δ $GABA_A$ receptor subunit subtypes were subcloned into the expression vector pCMVNeo. Sixty mm cell dishes of HEK 293T cells were co-transfected with 1 or 2 μg of each subunit plasmid with a 1:1:1 cDNA ratio and 1 μg of the pHook-1 cDNA (Invitrogen, Carlsbad, CA) using a

modified calcium phosphate precipitation method. pHook-1 was used as a marker of positively transfected cells, which were selected 24 hrs after transfection by magnetic hapten coated beads. In some experiment, mutated β subunits were transfected in place of wildtype β subunits. All point mutations in rat β3 subunit constructs were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing.

Electrophysiological recordings

All $GABA_A$ receptor currents were obtained using a lifted whole-cell voltage-clamp recording technique. Patch pipettes (resistance1-1.5 MΩ) were pulled from borosilicate capillary glass (World Precision Instruments, Sarasota, FL) on a P-2000 laser puller (Sutter Instrument Co., San Rafael, CA) and filled with an internal solution containing 153 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES pH 7.3, with 4 mM ATP added on the day of recording. The cells were subjected to continuous background perfusion of the external solution consisting of 142 mM NaCl, 8 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4. All experiments were performed at room temperature. Signals were processed by using 200B amplifier (Molecular Devices, Union City, CA) in voltage-clamp mode. All cells were voltage clamped at -20 mV during recordings.

GABA and drug application system

GABA and kinase modulators were delivered through a four parallel glass square barrel to HEK 293T cells using a modified SF-77B Perfusion Fast-Step application system (Warner Instrument Corp., Hamden, CT) (Hinkle et al., 2003).

The drug application was initiated by an analog pulse triggered by the pClamp 9.2 software (Axon Instruments, Union City, CA) that caused the motor of the Warner Fast-Step to reposition the multi-barrel array from one barrel to another (e.g., external solution to GABA). By using this modified application system, I consistently obtained rise times (10–90%) less than 800 μsec with open-tip electrodes stepped from standard bath solution to 90% bath solution.

A high concentration of GABA (1 mM) was applied to lifted whole cells for 2 sec or 8 sec. Between every two applications of GABA, external solution was applied for at least 3 min to ensure maximal recovery from the GABA exposure. I also added pre-application steps to the protocol for modulator experiments to enable us to pre-apply a membrane-permeable modulator such as PMA. In the pre-application experiments, GABA was first applied to lifted cells expressing wildtype or mutant $GABA_A$ receptor subunits as an initial pre-control. After washout of GABA, the PKC activators or inhibitors were applied for different amounts of time to produce the effects and then the lifted cell was stepped back into external solution for a brief washout (3 sec) of any residual external PKC modulators before it was subjected to a second GABA application. This second GABA-activated current recorded immediately after a pre-application was defined as the experimental current. After the second GABA application, the cell was washed in external solution for more than 6 min before it was subjected to the final GABA application to evoke a post-control current. Due to the variability of currents among cells, the experimental currents were normalized to their matched pre-control currents so that differences made by pre-

application were shown in percentage. To control for current run-down during the whole set of pre-application experiment that were recorded over 10 min, I superfused cells with external solution during the pre-application process. Significant differences between the experimental pre-application group and the external control group were used to identify specific and significant effects caused by PKC modulation.

Kinase modulation

The effect of PKA and PKC on $GABA_A$ receptor currents was assessed by using their activators and inhibitors (Calbiochem, San Diego, CA). PKA and PKC were activated by extracellular 8-Br-cAMP (500-1000 μM; 8-Bromoadenosine-3',5'-cyclic monophosphate, Sodium Salt) and DOG (1-10 μM; sn-1,2-dioctanoylglycerol), respectively. PKA activity was blocked using extracellular application of H-89 (10 μM; N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl), and PKC activity was blocked by Calphostin C (1 μM). In some experiments, the specific PKA inhibitory peptide, PKI (100 μg/ml), and the specific PKC inhibitory peptide, $PKCI_{19-31}$ (50 μM) as well as some less specific but more potent protein kinase inhibitors like staurosporine were applied intracellularly by including them in the recording pipette to block PKA and PKC activity, respectively.

Mutagenesis

Subunit phosphorylation sites (β3 S408, 409 and γ2L S327, 343) were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) to either block receptor phosphorylation entirely with alanine substitutions or to mimic phosphorylation with glutamate substitutions. In some experiments, deletions of

portions of $GABA_A$ receptor subunits like the binding motif of PKC were introduced to β subunits to interrupt the phosphorylation produced by protein-protein interaction.

Data analysis

Macroscopic currents were low pass filtered at 2 kHz, digitized at 10 kHz, and analyzed using pClamp9.2 software. For comparison of desensitization among currents, currents were normalized first to their peak value (defined as a maximal response of 1). I plotted the relative residual current, which was the residual current at the end of a prolonged GABA application divided by the current peak value and used it as indicator of the overall extent of desensitization. In addition, the desensitization time course of some phasic currents activated by 1 mM GABA was fitted to a sum of exponential functions (Lagrange et al., 2007). The entire desensitization time course was fitted with one to five exponential functions using the Levenberg–Marquardt least squares method with the form $\sum A_n * e^{-t/\tau_n} + C$, where *n* was the number of exponential components, *t* was time, *A* was relative amplitude of a given component at time 0, τ was the time constant for a given component, and *C* was a constant to account for residual current. The number of exponential functions required to obtain the best fit was determined when adding an additional component did not significantly improve the fitting result based on an *F* test automatically performed by the analysis software on the sum of squared residuals. All the two second and eight second GABA-activated phasic currents were fitted best with two to four exponential functions. All numerical and bar graph data were expressed as mean ± standard error. Statistical significance was compared between kinase modulator

group and control solution group or between wild-type and mutant condition being examined. Statistical analyses were performed using Graph Pad Prism 4. Student's paired or unpaired *t* tests were used to compare pairs of data, or appropriate ANOVA analyses were used for comparing three or more groups. Statistical significance was taken as: *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

Results

"Phosphorylation" of both β3 and γ2L subunits had additive effects on increasing desensitization of α4β3γ2L currents.

The β3 subunit serines S408 and S409 and the γ2L subunit serines S327 and S343 subunits were mutated to alanine or glutamate residues either to block phosphorylation (S>A) or to mimic constitutive phosphorylation (S>E). Similar effects to increase α4β3γ2L current desensitization were observed with serine to glutamate mutation of the phosphorylation sites in both β3 (Fig. 4.1 A) and γ2L (Fig. 4.1 B) subunits. The mutant receptors with glutamate substitution (gray traces) in both β3 (Fig. 4.1 A1) and γ2L (Fig. 4.1 B1) subunits had faster and more extensive desensitization compared to currents from receptors with the corresponding alanine mutations (Fig. 4.1 A1, B1; black traces). I used the area under normalized currents as an indication of the relative rate and extent of current desensitization. If a treatment changes current amplitude but not desensitization, it will have no effect on

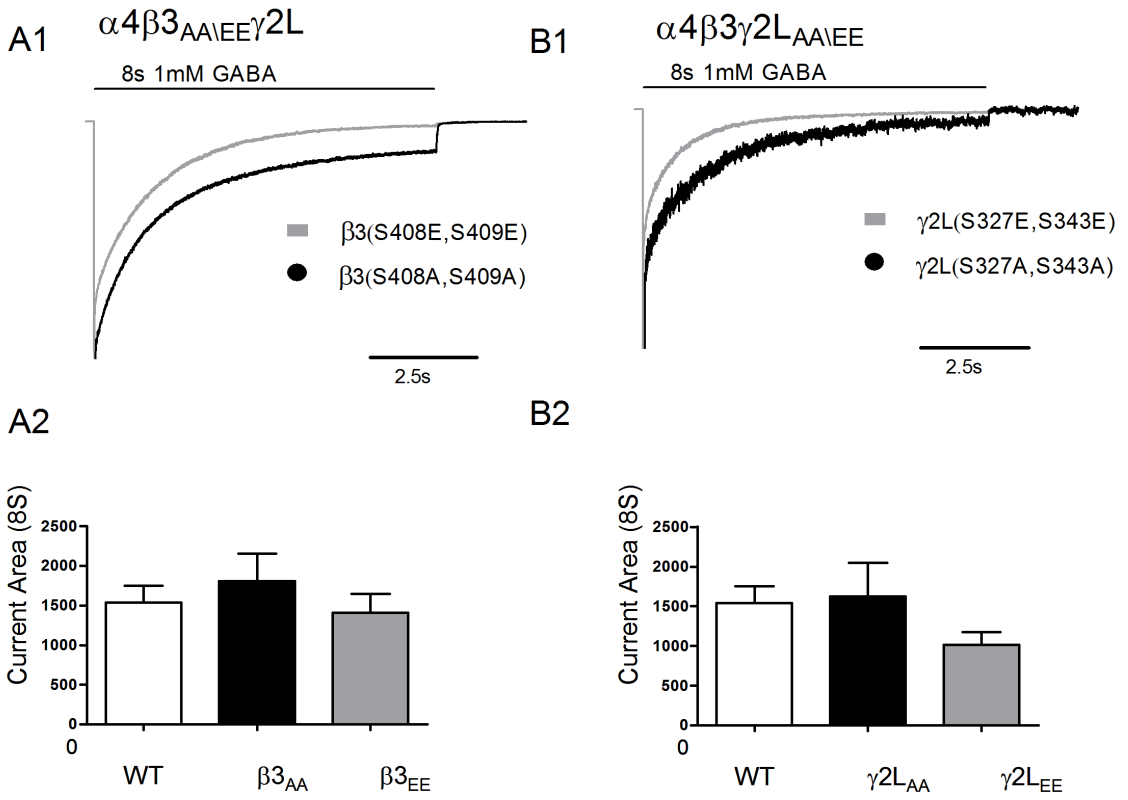

Figure 4.1. "Phosphorylation" of β3 and γ2L subunits respectively increased the desensitization of α4β3γ2L currents.

A1 and A2, Representative $\alpha4\beta3_{AA}\gamma2L$ and $\alpha4\beta3_{EE}\gamma2L$ currents evoked by 1 mM GABA were normalized and superposed for comparison (A1). Their current area under the 8 sec desensitization curve were measured and compared to wildtype α4β3γ2L receptor (A2). **B1 and B2**, Representative $\alpha4\beta3\gamma2L_{AA}$ and $\alpha4\beta3\gamma2L_{EE}$ currents evoked by 1 mM GABA were normalized and superposed for comparison (B1). Their current area under the 8 sec desensitization curve were measured and compared to wildtype α4β3γ2L receptor (B2).

the area under the normalized currents, but if it increases the rate or extent of desensitization, it will reduce the area. The areas of currents evoked by 8 sec GABA applications (1 mM) to $\alpha4\beta3_{EE}\gamma2L$ (Fig. 4.1 A2, gray bar) and $\alpha4\beta3\gamma2L_{EE}$ (Fig. 4.1 B2, gray bar) receptors were slightly less than the areas of currents evoked from $\alpha4\beta3_{AA}\gamma2L$ (Fig. 4.1 A2, solid bar) and $\alpha4\beta3\gamma2L_{AA}$ (Fig. 4.1 B2, solid bar) receptors. Wildtype α4β3γ2L receptor currents (Fig. 4.1 A2, B2, open bars) had areas that were

intermediate between those currents evoked from receptors containing subunits with either glutamate or alanine mutations.

Next, I studied current desensitization of α4β3γ2L receptors containing "dephosphorylated" and "phosphorylated" subunits combined in four ways: $\beta3_{AA}\gamma2L_{AA}$, $\beta3_{AA}\gamma2L_{EE}$, $\beta3_{EE}\gamma2L_{AA}$, $\beta3_{EE}\gamma2L_{EE}$. The $\alpha4\beta3_{AA}\gamma2L_{AA}$ receptor was considered to mimic full dephosphorylation of both β3 and γ2L subunits, the $\alpha4\beta3_{AA}\gamma2L_{EE}$ and $\alpha4\beta3_{EE}\gamma2L_{AA}$ receptors to mimic only phosphorylated γ2L or β3 subunits, respectively, and the $\alpha4\beta3_{EE}\gamma2L_{EE}$ receptor to mimic full phosphorylation of both subunits. Currents evoked by 8 sec applications of GABA (1 mM) to wildtype and the four mutant α4β3γ2L receptors were compared (Fig. 4.2 A). $\alpha4\beta3_{EE}\gamma2L_{EE}$ currents desensitized more rapidly and to a greater extent than wildtype currents and $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents desensitized less rapidly and to a lesser extent than wildtype currents. $\alpha4\beta3_{AA}\gamma2L_{EE}$ and $\alpha4\beta3_{EE}\gamma2L_{AA}$ currents desensitized at an intermediate rate and to an intermediate extent. The normalized current areas of the four mutant receptors decreased as the number of S>E mutation increased (Fig. 4.2 B), suggesting an additive effect of each "phosphorylated" subunit to increase desensitization. Wildtype α4β3γ2L receptor currents desensitized at a rate and extent that was similar to the partially "phosphorylated" $\alpha4\beta3_{AA}\gamma2L_{EE}$ and $\alpha4\beta3_{EE}\gamma2L_{AA}$ receptor currents, suggesting that wildtype α4β3γ2L receptors were likely partially phosphorylated under basal conditions in HEK293T cells.

"Phosphorylation" of both β3 and γ2L subunits additively increased the relative portion of fast components of desensitization without affecting desensitization time constants.

To understand the mechanism of increased desensitization produced by PKC-activation, I studied the desensitization kinetic properties of wildtype α4β3γ2L and four quadruple-mutation receptors ($\alpha4\beta3_{AA}\gamma2L_{AA}$, $\alpha4\beta3_{AA}\gamma2L_{EE}$, $\alpha4\beta3_{EE}\gamma2L_{AA}$, $\alpha4\beta3_{EE}\gamma2L_{EE}$) currents. The desensitization time course of all mutant and wildtype α4β3γ2L receptor currents were fitted best by four exponential components. Exponential relative proportions (Fig. 4.3 A), but not time constants (Fig. 4.3 B), were altered by the mutations. "Phosphorylation" of the β3 and γ2L subunit serines by glutamate substitutions caused an increase in the relative proportion of the fastest component (A1) and a decrease in the relative proportion of the slowest component (A4), resulting a smaller residual current (C) compared to wildtype currents. "Dephosphorylation" of the β3 and γ2L subunit serines by alanine substitutions resulted in the opposite result, a decrease in the relative proportion of the fastest component (A1) and an increase in the relative proportion of the slowest component (A4) with a larger residual currents (C), compared to wildtype currents. Interestingly, all the time constants of the four components of mutant receptors were the same as wildtype receptor. The slight increase of $\alpha4\beta3_{AA}\gamma2L_{AA}$ receptor in τ1, the fastest time constant, might not indicate a real difference but might have been due to a technical error occurring when the one component has only a very small proportion in the whole fitting or due to an error caused by multiple comparisons. This pattern of

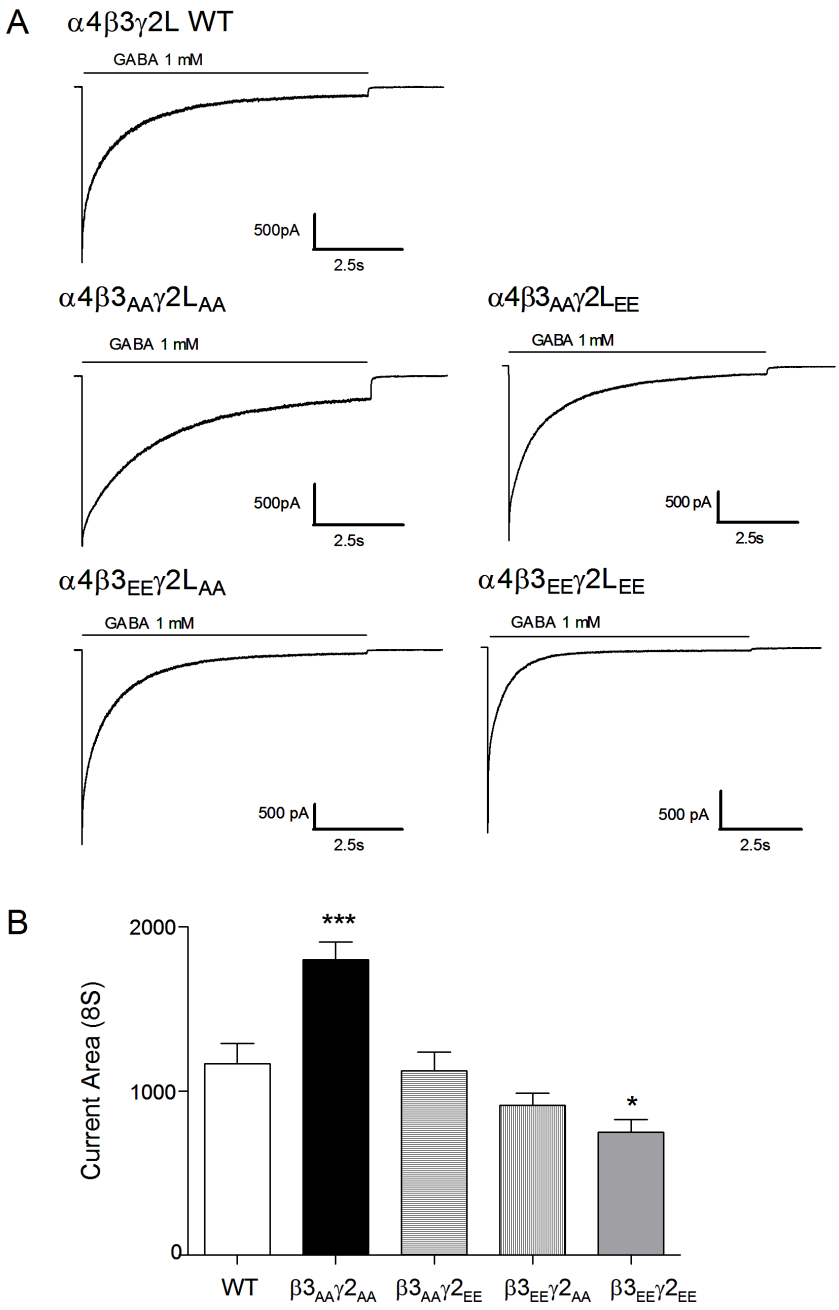

Figure 4.2. "Phosphorylation" of both β3 and γ2L subunits had additive effect on increasing the desensitization of α4β3γ2L current.

A, Representative wildtype α4β3γ2L and $\alpha4\beta3_{AA}\gamma2L_{AA}$, $\alpha4\beta3_{AA}\gamma2L_{EE}$, $\alpha4\beta3_{EE}\gamma2L_{AA}$ $\alpha4\beta3_{EE}\gamma2L_{EE}$ current were evoked by 1 mM GABA applied for 8 sec. **B**, The current areas under the 8 sec desensitization curve were plotted. Current area decreased with increased numbers of glutamates to mimic phosphorylation. Asterisks indicated significant differences compared to wildtype current areas.

kinetic changes was reported with PKA modulation of α4β3γ2L current desensitization mediated by phosphorylation of β subunits in chapter III. The quadruple mutation further suggested that phosphorylation of β3 and γ2 subunits mediate similar and additive effects to increase current desensitization.

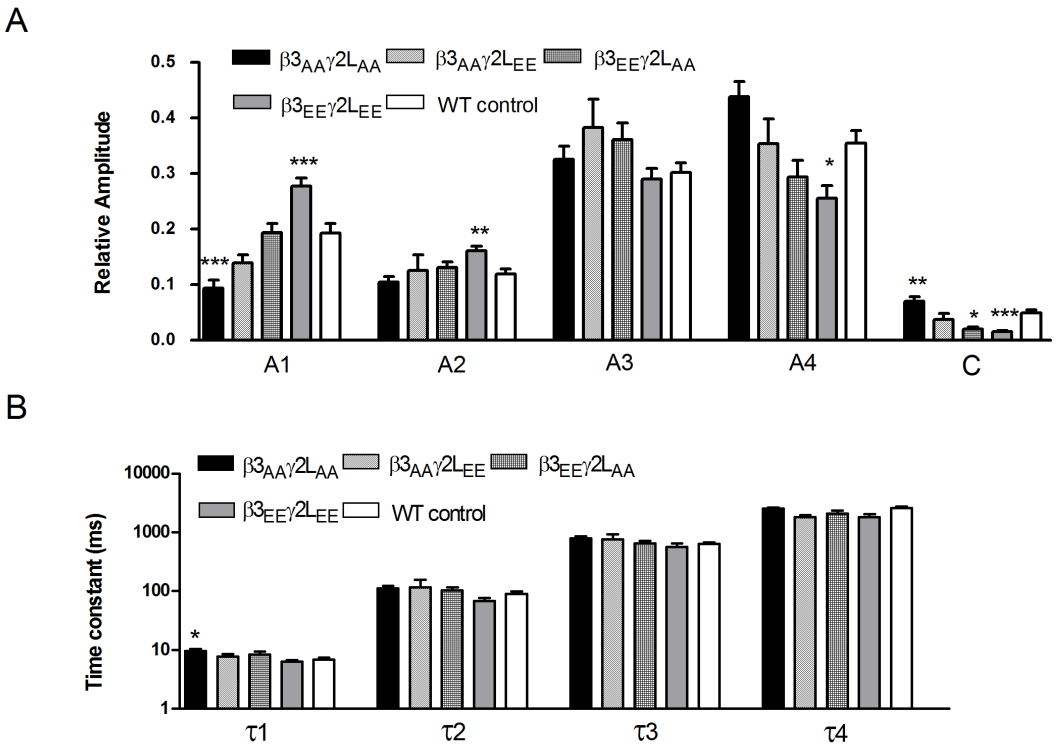

Figure 4.3. "Phosphorylation" of β3 and γ2L subunits additively increased the relative proportion of fast desensitization components without affecting the time constants.

A, Relative amplitudes plus residual currents and **B,** their corresponding time constants of each component were obtained from fitting the desensitization time courses were plotted. Asterisks indicated significant differences compared to wildtype.

PKC activation increased α4β3γ2L current desensitization.

To determine PKC effects on α4β3γ2L current kinetics, I applied both PKC activators and inhibitors using the pre-application protocol and compared the currents to those obtained with pre-application of external control solution (Fig. 4.4 A1). The normalized current areas evoked by 2 sec applications of GABA (1 mM) after pre-application (Fig. 4.4 B, solid bars) were plotted as a percentage of their pre-control areas (Fig. 4.4 B, open bars). Pre-application of external solution (Fig. 4.4 A1) did not cause any obvious change of current area (97.9 ± 2.2%, n=12; Fig. 4.4 B1, solid bar) and only a slight decrease of current area associated with rundown after a long time wash (86.1 ± 3.5%, n=12; Fig. 4.4 B1, gray bar). I used both phorbol ester (PMA) and diacylglycerol analogue (sn-1,2-dioctanoylglycerol, DOG) to activate PKC. The PKC inactive phorbol analogue (4-α phorbol) also did not alter current desensitization (Fig. 4.4 A2) with 95.1 ± 6.4% (n=11; Fig. 4.4 B2, solid bar) normalized area as a percentage of the pre-control area. Pre-application of both PMA (1 μM, Fig. 4.4 C1) and DOG (1-10 μM, Fig. 4.4 D1) for 90 sec led to faster desensitization accompanied with a reduced peak current (data not shown). Pre-application of PMA (1 μM) reduced normalized current area to 59.6 ± 7.3% (n=7; $p < 0.001$; Fig. 4.4 C2, solid bar) compared to the pre-control currents. However, this effect of PMA was not reversible (Fig. 4.4 C1, silver trace), resulting in 52.2 ± 6.8% (n=7; $p < 0.001$; Fig. 4.4 C2, gray bar) current area left after extensive wash. The other PKC activator, DOG (1 μM), reversibly increased current desensitization (Fig. 4.4 D1, sliver trace), and decreased the normalized current area to 61.3 ± 2.3% (n=40;

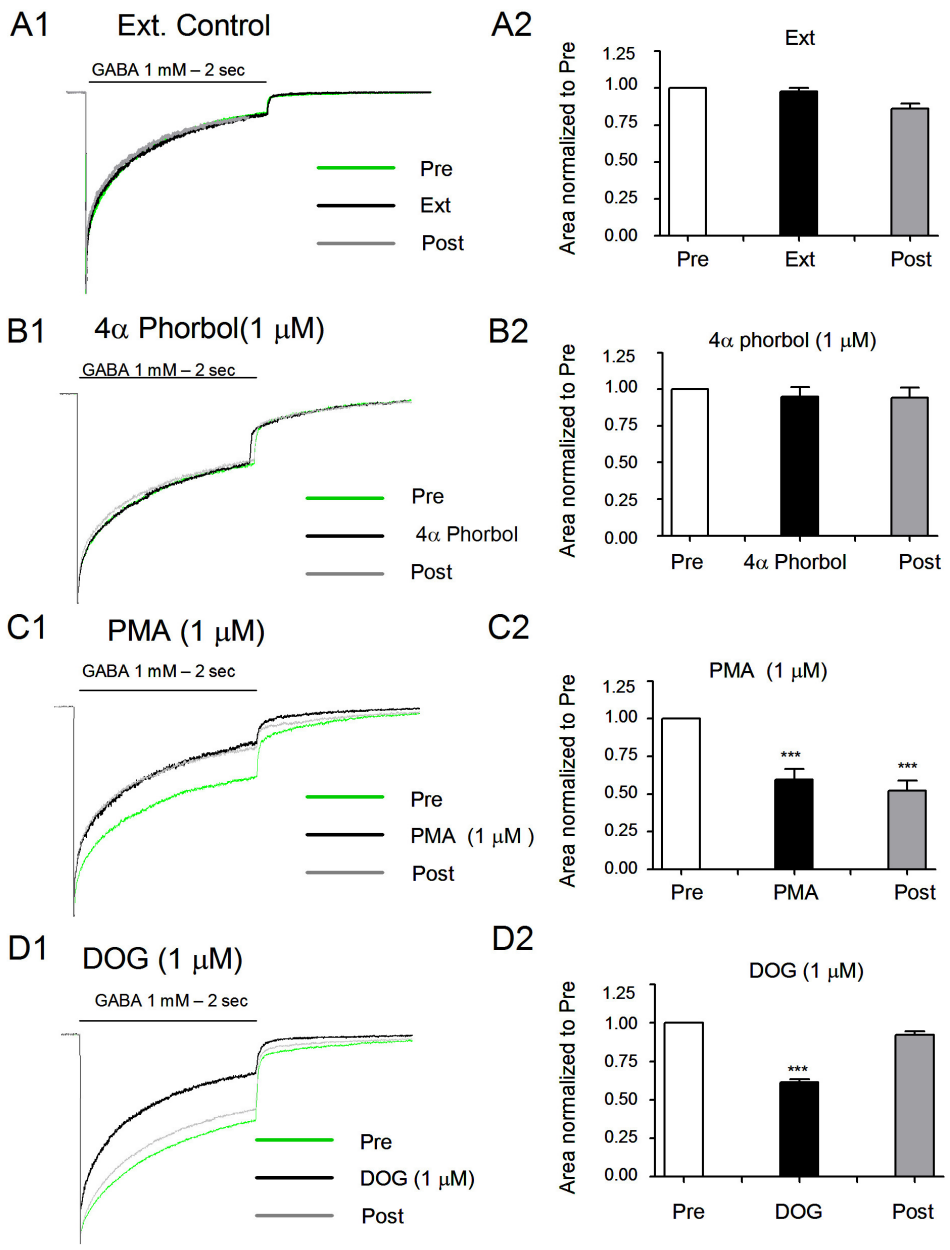

Figure 4.4. PKC activation increased the desensitization of α4β3γ2L current.

A, B, C and D, Pre-application of external control (A), PKC inactive analogue 4-α phorbol (B), and two PKC activators, PMA (C) and DOG (D) to lifted HEK293T cells expressing α4β3γ2L $GABA_A$ receptors. Pre-control (green), pre-application treated (black) and post-control (gray) α4β3γ2L currents (2 sec) evoked by 1 mM GABA were normalized to the peak currents and were superposed to facilitate comparison of the rate and extent of desensitization (A1, B1, C1 and D1). Current area under the 2 sec desensitization curve of pre-application treated (solid bar) and post-control (gray bar) were normalized to pre-control (open bar) and plotted in histogram (A2, B2, C2 and D2). Asterisks indicated significant differences compared to corresponding pre-control group.

$p < 0.001$; Fig. 4.4 D2, solid bar). The lack of effect of the inactive phorbol 4-α phorbol as well as similar area reduction by two different PKC activators (PMA and DOG) suggested that the increased α4β3γ2L current desensitization was due to PKC activation.

Next, I applied different concentrations of the PKC activator DOG (1, 5, 10 μM) to achieve low to high activation of PKC and also pre-applied the PKC inhibitor Calphostin C (1 μM) to down-regulate basal PKC activity (Fig. 4.5). Compared to the pre-application of external solution (96.4 ± 2.1%, n=16; Fig. 4.5, open bar), pre-application of Calphostin C (1 μM, 3 min) increased current area to 148.8 ± 2.0% (n=8, $p < 0.001$; Fig. 4.5, solid bar), while pre-application of DOG (Fig. 4.5, gray bars)

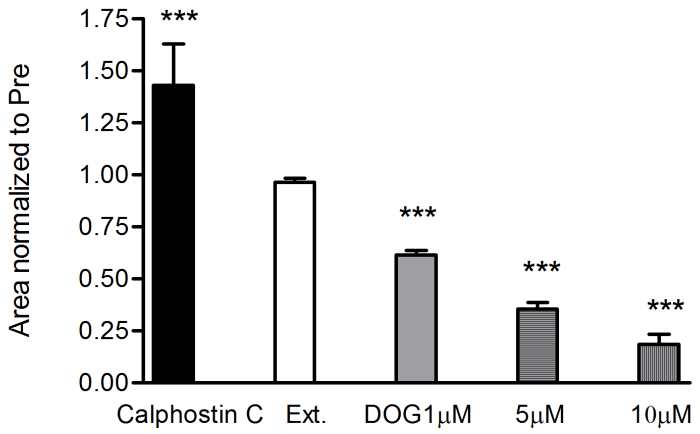

Figure 4.5. Pre-application of PKC activator DOG increased the relative proportion of fast desensitization component without affecting all the time constants.

Calphostin C, external control and increasing concentrations of DOG were pre-applied to wildtype α4β3γ2L $GABA_A$ receptors. The current area after each pre-application was normalized to its pre-control current and plotted in the histogram. Asterisks indicated significant differences compared to external control.

caused a DOG concentration-dependent reduction of current areas (at 1 μM, 61.3 ± 2.3%. n=40, $p < 0.001$; at 5 μM, 35.6 ± 2.9%. n=44, $p < 0.001$; at 10 μM, 18.5 ± 4.7%. n=40, $p < 0.001$). By comparing current areas after treatment with a PKC inhibitor and with increasing concentrations of PKC activators, I observed, a negative relationship of current area with PKC activation, which indicated a positive correlation of current desensitization with PKC activation level. The fact that currents desensitized more extensively at higher PKC activation levels was also consistent with the additive effect of the quadruple mutation of PKC phosphorylation sites to increase current desensitization.

To further compare the effects of PKC-activation to those of the quadruple mutation, I also fitted the desensitization of the pre-control (open bars), DOG-treated (solid bars) and post control (gray bars) currents with multiple exponential functions (Fig. 4.6). Consistently, I observed a change in current kinetic propertiesproduced by PKC-activation that was similar to that produced by the phosphorylation site mutations. The relative amplitudes of the four components were reversibly altered by DOG (1 μM) with a significant increase of the proportion of the fastest component (A1) and a significant decrease of the slowest component (A4) and residual currents (C) (Fig. 4.6 A). None of the time constants of the four components were altered significantly (Fig. 4.6 B). Based on comparable findings with PKC-activation and phosphorylation site mutation, I propose that PKC can phosphorylate β3 and γ2L subunits and cause an increase of α4β3γ2L current desensitization. When PKC is activated leading to phosphorylation of both β3 and γ2L current to a higher

stoichiometry, the current desensitization will be additively increased corresponding to the number of subunits being phosphorylated.

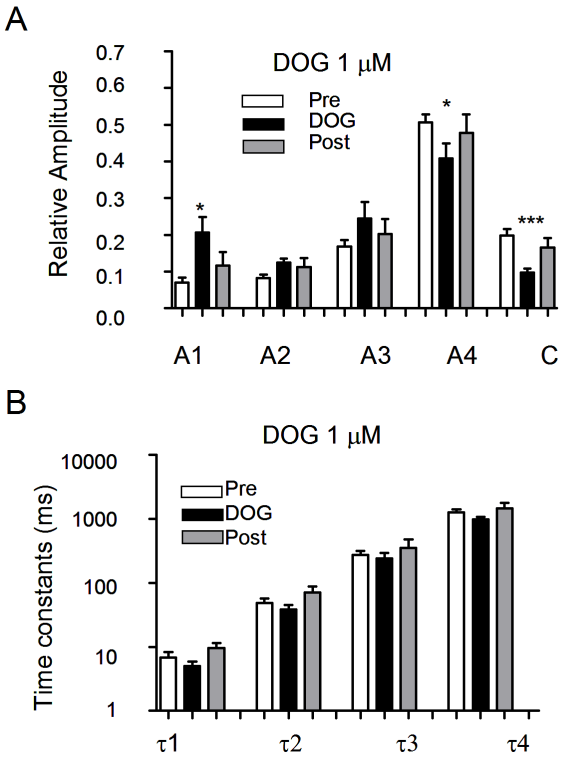

Figure 4.6. PKC enhancement of α4β3γ2L current desensitization was concentration-dependent.

A and B, Current areas of pre-control (open bar), pre-application treated (solid bar) and post-control (gray bar) showing the relative amplitude (A) and time constant (B) of four desensitization components were plotted. Asterisks indicated significant differences compared to wildtype.

PKC activation by DOG extensively increased the desensitization of α4β3γ2L receptor currents only partially due to phosphorylation of β3(S408, 409) and γ2L(S327, 343) subunits.

Although the results of the mutation at known phosphorylation sites can be well explained by the effects of DOG at low concentration like 1 μM, the increase of

desensitization by 10 μM DOG was too extensive even compared to the desensitization of fully "phosphorylated" $\alpha4\beta3_{EE}\gamma2L_{EE}$ receptors. This raised a concern that the effects of high concentrations of DOG may have arisen from

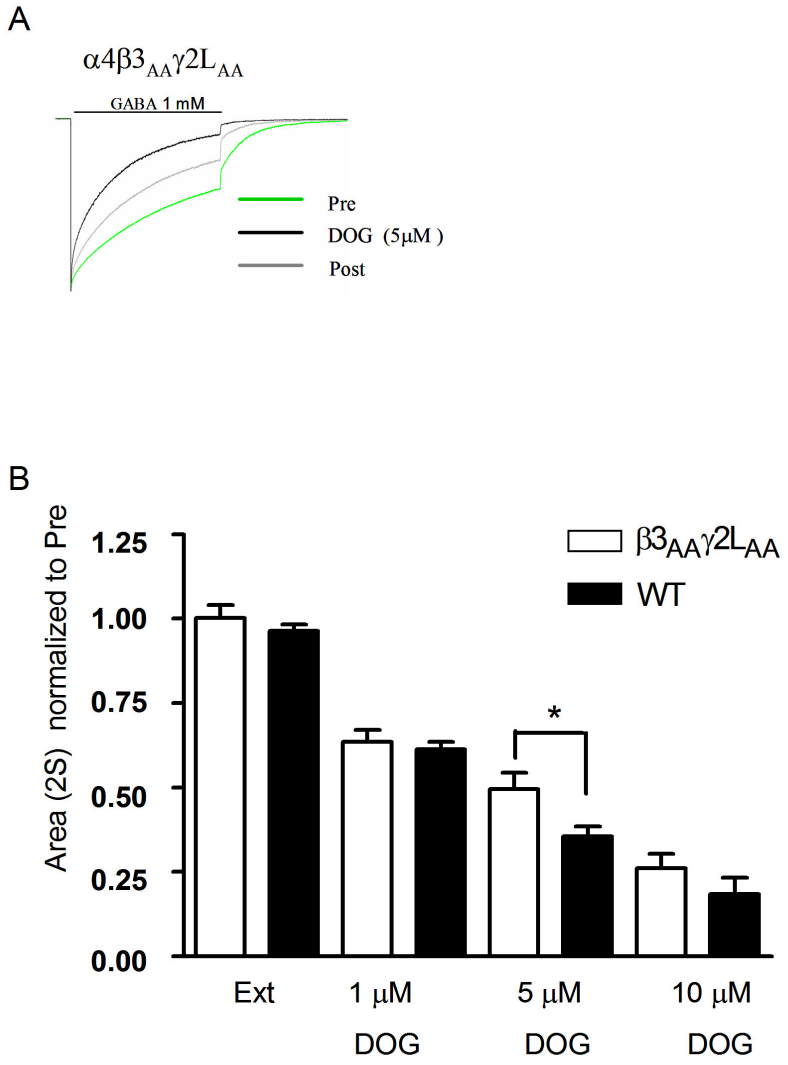

Figure 4.7. Mutation of the PKC sites on β3(S408,409) and γ2L(S327,343) subunits did not block entirely the effect of PKC activator DOG.

A, Pre-application of DOG still cause increase of desensitization of $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents. Pre-control (green), pre-application treated (black) and post-control (gray) $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents (2 sec) evoked by 1 mM GABA were normalized to the peak currents and were superposed. **B,** Comparison of normalized current area of $\alpha4\beta3\gamma2L$ (solid bars) and $\alpha4\beta3_{AA}\gamma2L_{AA}$ (open bars) current after pre-application of external control or three concentration of DOG.

mechanisms independent of phosphorylation of the four β3 and γ2L subunit PKC sites and if these effects are PKC-specific. First, to determine if the effects by DOG are mediated by the four β3 and γ2L subunit PKC sites, I applied three different concentrations (1, 5, 10 μM) of DOG to cells expressing $\alpha4\beta3_{AA}\gamma2L_{AA}$ receptors and compared them to the effects on wildtype α4β3γ2L receptors (Fig. 4.7). DOG still had clear effects on $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents. Concentration-dependent effect on both wildtype α4β3γ2L currents (Fig. 4.7 B, solid bars) and mutant $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents (Fig. 4.7B, open bars) were observed after pre-application of 1, 5 or 10 μM DOG (61.3 ± 2.3%, 35.6 ± 2.9%, 18.5 ± 4.7% vs. 63.4 ± 3.7%, 49.6 ± 4.8%, 26.1 ± 4.4%). Normalized current areas of α4β3γ2L and $\alpha4\beta3_{AA}\gamma2L_{AA}$ receptors only became slightly significantly different at 5 μM (35.6 ± 2.9%, n=44 vs. 49.6 ± 4.8%, n=21; $p < 0.5$) and 10 μM (18.5 ± 4.7%, n=9 vs. 26.1 ± 4.4%, n=10; $p > 0.5$), indicating that phosphorylation of the β3 subunit serines 408 and 409 and γ2L subunit serines 327 and 343 may only contribute marginally to the effects of DOG.

To determine PKC-specific effects of DOG, I applied PKC inhibitors in the pipette solution to suppress PKC activities inside the cell and subjected those cells to the DOG pre-application protocol (Fig. 4.8). I tested both the specific PKC inhibitory peptide ($PKCI_{19-31}$, 50 μM) and the non-specific PKC inhibitors (staurosporine, 5 μM) to block the effect of DOG at 1 (Fig. 4.8, solid bars) and 5 (Fig. 4.8, gray bars) μM. Both inhibitors significantly reduced the effect of DOG at 1 μM. Internal $PKCI_{19-31}$ reduced the effect of pre-application of 1 μM DOG (77.4 ± 2.9%, n=17 vs. 61.3 ± 2.3%, n=40; $p < 0.01$), although there was still a significantly smaller current area left

after DOG pre-application than with external solution pre-application (77.4 ± 2.9%, n=17 vs. 96.4 ± 2.1%, n=16; $p < 0.01$). Staurosporine is a potent inhibitor of PKC, but also a non-specific inhibitor that prevents ATP binding to the kinase and has a high affinity for other protein kinases including PKA, CaMKII, PKG, tyrosine kinase and myosin light chain kinase (MLCK). Internal staurosporine led to a greater reduction of the effect of 1 μM DOG.

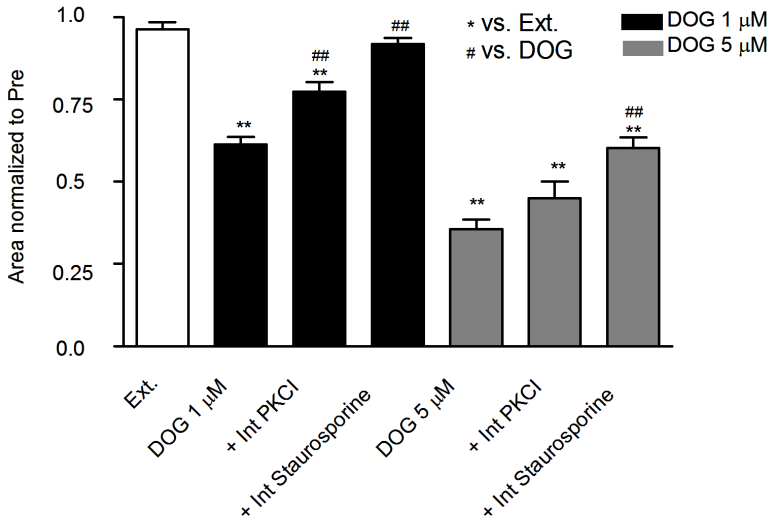

Figure 4.8. PKC inhibitors reduced effects of DOG pre-application on increasing the desensitization of α4β3γ2L current.

1 and 5 μM DOG were pre applied to cells expressing wildtype α4β3γ2L $GABA_A$ receptors in the absence or presence of $PKCI_{19-31}$ (50 μM) or staurosporine (5 μM). Normalized current areas to their pre-contorl current in each pre-application condition were plotted.

The current area with DOG and staurosporine application was not significantly different from that obtained with application of the external control (91.9 ± 1.8%, n=17 vs. 96.4 ± 2.1%, n=16; $p > 0.5$). At 5 μM DOG, internal PKCI and staurosporine showed similar trends, with less inhibition by PKCI than by

staurosporine. However, at this higher concentration of DOG, PKCI did not decrease significantly the area reduction produced by pre-application of 5 μM DOG (44.9 ± 5.2%, n=14 vs. 35.6 ± 2.9%, n=44; p >0.5), and staurosporine did not block the entire effect of DOG at 5 μM (60.2 ± 3.1%, n=14 vs. 5 μM DOG, 35.6 ± 2.9%, n=44; p < 0.01 and vs. external control, 96.4 ± 2.1%, n=16; p >0.5). It seemed that the inhibition of PKC by a chosen concentration of inhibitor had not been saturated, so that the more potent PKC non-specific inhibitor staurosporine always caused more inhibition of the DOG effect than that of the more specific but less potent PKC inhibitory peptide. It is possible that the extensive current area reduction produced by high concentrations of DOG might have been blocked completely by higher concentrations of PKCI or staurosporine, but these concentrations are very difficult to achieve since they disrupt patch formation and severely impair recording. Therefore, it remains uncertain if the effects of DOG at high concentrations are PKC specific or non-specific.

Enhancement of α4β3γ2L current desensitization by the PKC activator DOG was not due to additional PKC sites located specifically in α4 subunits.

In several studies, β3 subunit serines 408 and 409 and γ2L subunit serines 327 and 343 have been demonstrated to be the major sites of PKC phosphorylation of the two subunits, while α1 subunits have been shown to be poor PKC substrates. Although α4 subunits contain consensus sequences for PKC phosphorylation (Macdonald, 1995), and PKC modulation has been implied in many studies of the mechanisms of α4 subunit up-regulation, there is no direct evidence of PKC

phosphorylation of α4 subunits. To determine if PKC sites in α4 subunits could contribute to the extensive increase of desensitization by DOG on $\alpha4\beta3_{AA}\gamma2L_{AA}$ currents, I also pre-applied DOG to wildtype α1β3γ2L (Fig. 4.9 A1) and mutant $\alpha1\beta3_{AA}\gamma2L_{AA}$ (Fig. 4.9 B1) receptors. The time course of desensitization was similar for α1β3γ2L and α4β3γ2L currents, and the reduction in α1β3γ2L current area (to 21.1 ± 4.4%, n=3, Fig. 4.9 A2, solid bar) after pre-application of 10 μM DOG was

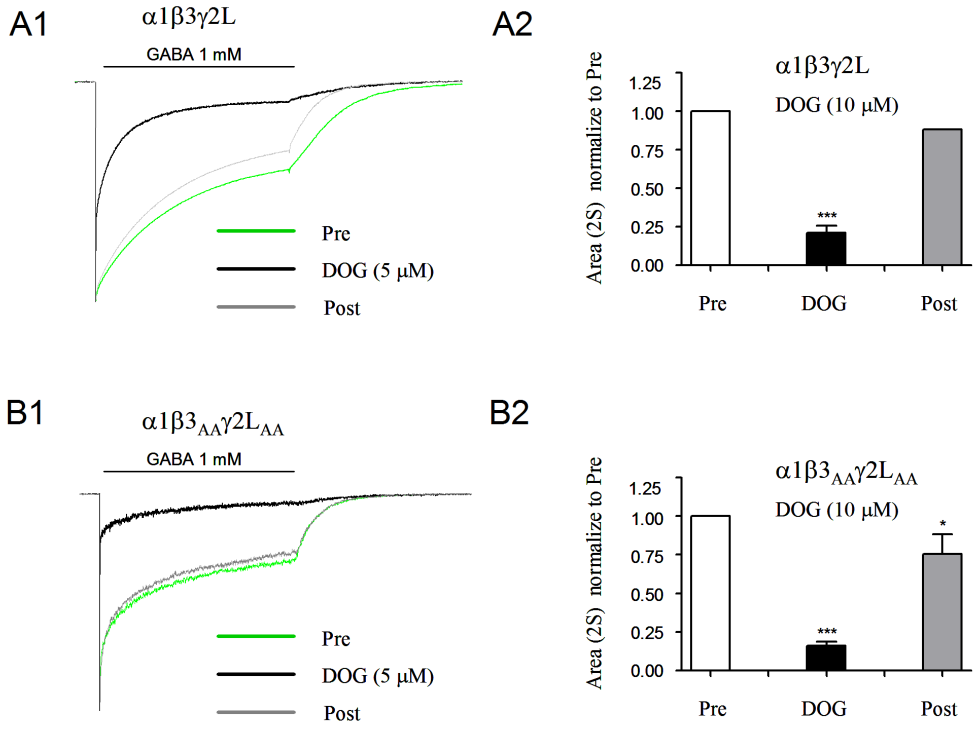

Figure 4.9. PKC activation increased the desensitization of α1β3γ2L and $\alpha1\beta3_{AA}\gamma2L_{AA}$ currents.

A and B, Pre-application of 10 μM DOG to α1β3γ2L (A) and $\alpha1\beta3_{AA}\gamma2L_{AA}$ (B) $GABA_A$ receptors. Pre-control (green), pre-application treated (black) and post-control (gray) α1β3γ2L (A1) or $\alpha1\beta3_{AA}\gamma2L_{AA}$ (B1) currents evoked by application of 1 mM GABA for 2 sec were normalized to their peak currents and were superposed. Current area under the 2 sec desensitization curve of pre-application treated (solid bar) and post-control (gray bar) were normalized to pre-control (open bar) and plotted in a histogram (A2 and B2). Asterisks indicated significant differences compared to the corresponding pre-control group.

also similar to that of α4β3γ2L currents (to 18.5 ± 4.7%). Pre-application of DOG (10 μM) also produced a similar reduction of $\alpha1\beta3_{AA}\gamma2L_{AA}$ current area (to 16.3 ± 2.6%, n = 4, Fig. 4.9 B2, solid bar), suggesting that the effects of DOG on current desensitization were not due to specific PKC phosphorylation of α4 subunits. If the residual DOG effects on $\alpha1/4\beta3_{AA}\gamma2L_{AA}$ currents were due to phosphorylation of extra sites beside the four investigated serines, they are not likely to be PKC sites located specifically in α4 subunits.

PKC βII binding sequences in β subunits were not required for mediating the effect of DOG on $GABA_A$ receptor current desensitization.

There are numerous examples of protein kinase modulation that were independent of receptor phosphorylation (Chapell et al., 1998;Connolly et al., 1999;Filippova et al., 2000), and so it is not surprising to discover that the β3 and γ2L subunit phosphorylation site mutations did not block the entire effect of DOG. Introduction of a charged phosphate residue to a receptor protein could have direct effects on receptor function by altering protein conformation. However, sometimes, receptor modulation is not directly due to the charge transfer. For example, PKC modulation of receptor internalization is mediated by the phospho-dependent binding of the clathrin AP2 adaptor complex to $GABA_A$ receptors (Kittler et al., 2005;Kittler et al., 2008). Indeed, phosphorylation of $GABA_A$ receptors or receptor-associated proteins that affect formation of protein complexes might affect multiple receptor properties through several different pathways. Because the receptor occurs in a dynamic protein complex, I should not focus on only receptor phosphorylation, but

should also determine how PKC could affect the whole receptor-protein complex. One receptor-protein interaction affected by PKC phosphorylation is the kinase itself. PKCβII was found to be associated with $GABA_A$ receptors by directly binding to all β subunits itself or indirectly through an anchoring protein RACK1, and this binding is impaired by phosphorylation of β subunits and decreased with PKC activation (Brandon et al., 2002).

To determine if disruption of PKCβII binding to $GABA_A$ receptors had any effect on GABA-activated currents, I deleted the PKCβII and/or RACK1 binding sequences located adjacent to each other in the M3-M4 loop of the β2 subunit to generate three mutant β2 subunits: β2(PKCΔ), β2(RACKΔ) and β2(PKC+RACKΔ). I co-transfected α4 and γ2L subunits with wildtype β2 or one of the three mutant β2 subunits. Only α4β2(PKC+RACKΔ)γ2L currents with the deletion of both β2 subunit motifs showed a slight increase of current desensitization. The normalized current areas of α4β2(PKC+RACKΔ)γ2L receptor currents (607.3 ± 79.2 ms, n=10; Fig. 4.10 B, open bar) were significantly smaller than those of α4β2γ2L receptor currents (882.7 ± 115.0 ms, n=9, $p < 0.5$; Fig. 4.10 B, solid bar), but neither α4β2(PKCΔ)γ2L or α4β2(RACKΔ)γ2L currents from receptors with deletion of only one motif were different from wildtype α4β2γ2L currents (Fig. 4.10 A). This is consistent with a report that phosphorylation of β subunits reduced PKC binding to the subunit (Brandon et al., 2002), since deletion of PKC and RACK binding motifs and phosphorylation of β subunits both disrupted binding of PKC to the receptor. Therefore, faster desensitization of α4β2(PKC+RACKΔ)γ2L currents similar to that

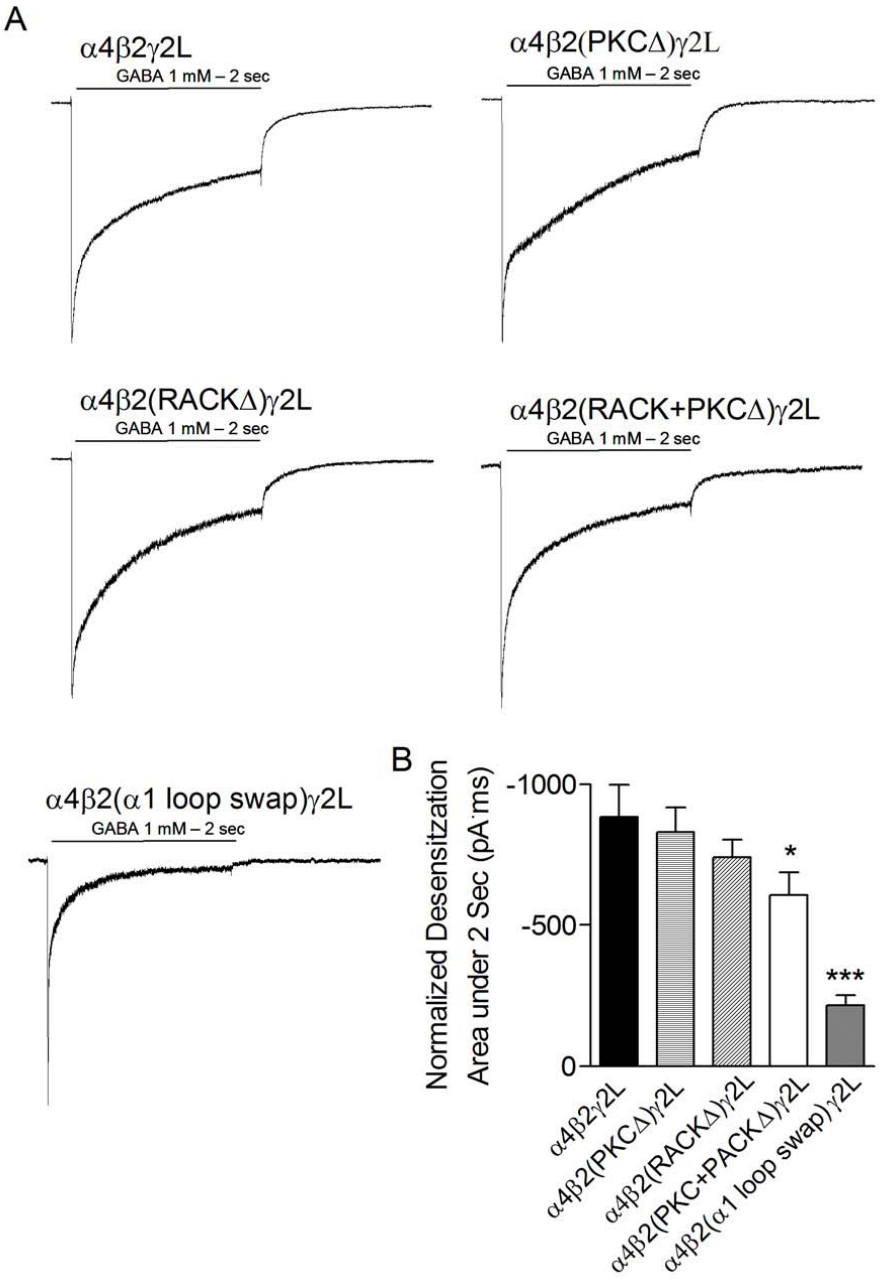

Figure 4.10. Deletion of PKC and RACK binding sequence in β2 subunit also caused an increase of the desensitization of α4β2γ2L current.

A, Representative current of wildtype α4β2γ2L and α4β2(PKCΔ)γ2L, α4β2(RACKΔ)γ2L, α4β2(PKC+RACKΔ)γ2L, α4β2(α1 loop swap)γ2L mutant receptors were stimulated by 1 mM GABA for prolong 2 sec. **B**, The current area under the 2 sec desensitization curve were plotted in histogram. Asterisks indicated significant differences compared to wildtype.

produced by the "phosphorylated" mutant receptor are consistent. However, the double deletion of PKC and RACK binding sequence only caused a small reduction of current area. I obtained a mutant subunit with replacement of the β2 subunit M3-M4 loop by the α1 subunit M3-M4 loop (β2(α1 loop swap)) (Dr. Wen-yi Lo).

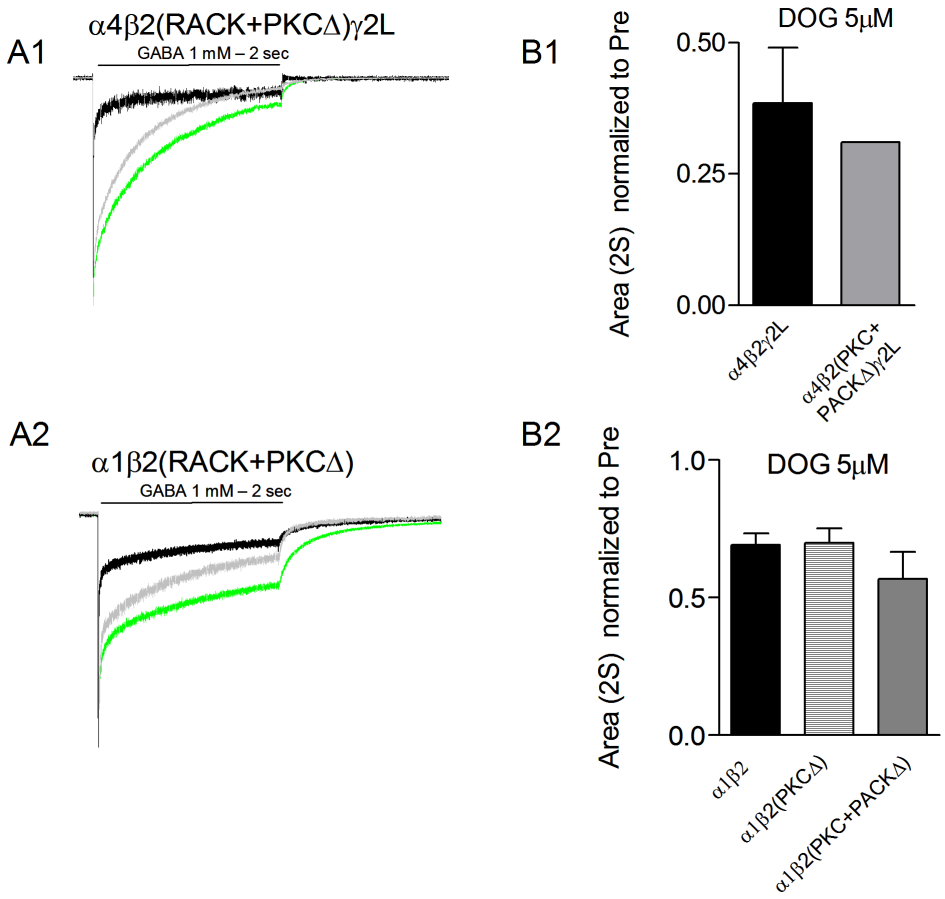

Figure 4.11. Deletion of PKC and RACK binding sequences in the β2 subunit did not eliminate DOG effects to increase desensitization of α4β2γ2L and α1β2 currents.

A1 and A2, Pre-application of 5 μM DOG still increased desensitization of α4β2(PKC+RACKΔ)γ2L (A1) and α1β2(PKC+RACKΔ) (A2) $GABA_A$ receptors. Pre-control (green), pre-application treated (black) and post-control (gray) α4β2(PKC+RACKΔ)γ2L or α1β2(PKC+RACKΔ) currents (2 sec) evoked by 1 mM GABA were normalized to the peak currents and were superposed. **B1 and B2,** Current areas of different receptor isoforms under the 2 sec desensitization curve after pre-application of DOG were normalized to pre-control (open bar) and plotted in a histogram.

α4β2(α1 loop swap)γ2L receptors had substantially decreased current area (215.1 ± 35.2 ms, n=6, $p < 0.001$; Fig. 4.10 B, gray bar), suggesting that the M3-M4 loop of β2 subunit was important in shaping macroscopic current desensitization, and that PKC binding had a minor effect on determining the desensitization rate. When I applied DOG (5 μM) to α4β2(PKC+RACKΔ)γ2L receptors, the current desensitization was still increased significantly (Fig. 4.11 A1). Current area after DOG pre-application to α4β2(PKC+RACKΔ)γ2L receptors (31.0%, n=1, Fig. 4.11 B1, gray bar) was similar to that of wildtype α4β2γ2L receptors (38.5 ± 10.5%, n=4, Fig. 4.11 B1, gray bar). Since γ2 subunits might also mediate protein-protein interactions with PKC, I applied DOG (5 μM) to α1β2, α1β2(PKCΔ) and α1β2(PKC+RACKΔ) receptors (Fig. 4.11 A2). However, neither of the two mutant α1β2 receptors (69.9 ± 5.2%, n=6, α1β2(PKCΔ), striped bar and 56.7 ± 7.4%, n=3, α1β2(PKC+RACKΔ), gray bar, Fig. 4.11 B2) was less insensitive to DOG than wildtype α1β2 receptors (69.1 ± 4.2%, n=7, Fig. 4.11 B2, solid bar), implying again that PKC binding to the receptor was not necessary for increased current desensitization caused by DOG.

Activation of other kinases may underlie a portion of the enhancement of current desensitization produced by DOG.

There is no clear evidence that the increased desensitization caused by DOG is PKC- specific or partially PKC-specific. It is possible that other protein kinases could be activated by DOG, dependent on or independent of PKC activation. There is substantial evidence that Ser/Thr and tyrosine kinases can interact. For example, PKC has been shown to influence the activity of the non-receptor tyrosine kinase Src in

neurons (Lu et al., 1999). Meanwhile, RasGPR1 was shown to be a high affinity diacylglycerol/phorbol-ester receptor, which acts as an activator of Ras and thus stimulates the Ras/Raf/MEK/ERK pathway. Thus, DOG could activate tyrosine kinases with or without PKC activation, to allow tyrosine phosphorylation of $GABA_A$ receptors, which might contribute to the effect on $\alpha4\beta3_{AA}\gamma2L_{AA}$ receptors.

To determine if other protein kinases were activated by DOG, the tyrosine kinase inhibitor, genistein (1 μM) or the CaMKII inhibitor, KN-93 (1 μM) was co-applied with DOG (5 μM). Co-application of genistein (46.8 ± 7.6%, n=5) or KN-93 (25.0%, n=1) did not decrease significantly the reduction in current area caused by DOG (35.6 ± 2.9%, n=44) (Fig. 4.12). There might be a slight decrease of area

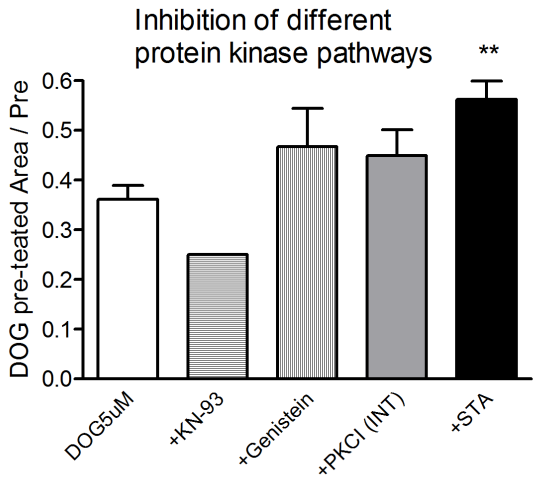

Figure 4.12. Different protein kinase inhibitors were used to reduce the effects of DOG pre-application on increasing the desensitization of α4β3γ2L current.

DOG (5 μM) was pre applied to cells expressing wildtype α4β3γ2L $GABA_A$ receptors with co-application of KN-93 (1 μM, horizontal striped bar) or genistein (1 μM, vertical striped bar) or with inclusion of internal $PKCI_{19-31}$ (50 μM, gray bar) or staurosporine (5 μM, solid bar). Normalized current areas to their pre-control currents in each pre-application condition were plotted.

reduction by co-application of genistein with DOG. Higher concentrations of genistein and longer pre-incubation time prior to DOG application might cause significant reduction of the DOG effect.

Using different receptor isoforms, mutant subunits and multiple protein kinase inhibitors, it is still not clear what mechanism is responsible for the major DOG effects on current desensitization. The extensive increase of desensitization produced by DOG was not mimicked by S to E mutations to produce full "phosphorylation" of all known PKC sites and was not blocked entirely by the investigated protein kinase inhibitors. A similar extent of increased desensitization was only observed with an increased calcium concentration in the internal solution. When the cells were broken into with an internal solution containing 5 mM Ca^{++} to form whole cell patches, increased current desensitization and decreased current amplitude were recorded that increased over several minutes (Fig. 4.13 A , normalized currents in the black circle). If I recorded with internal control solution that contained no calcium plus EGTA chelator, the initial current area (1 min after patch formation) averaged 1081 ± 148.3 (n=11, Fig. 4.13 B, red dot) and was relatively stable for up to 30 min. If I recorded with 2 mM Ca^{++} in the internal solution, the initial normalized current area (1 min after patch formation) was similar to internal control currents (1065 ± 196.8, n=2, Fig. 4.13 B, gray dot) but quickly declined (at 5 min) after patch formation (604.1 ± 183.5, n=2). With 5 mM Ca^{++} in the internal solution, the first current recorded was already desensitized extensively, and the normalized current area after patch formation was already reduced (295.6 ± 43.0, n=9, Fig. 4.13 B, black dot) and decreased to 159.5 ±

37.5 (n=5) at 3 min and to 103.3 ± 18.9 (n=3) at 5 min. At this time, all currents were highly desensitized and had very small amplitudes.

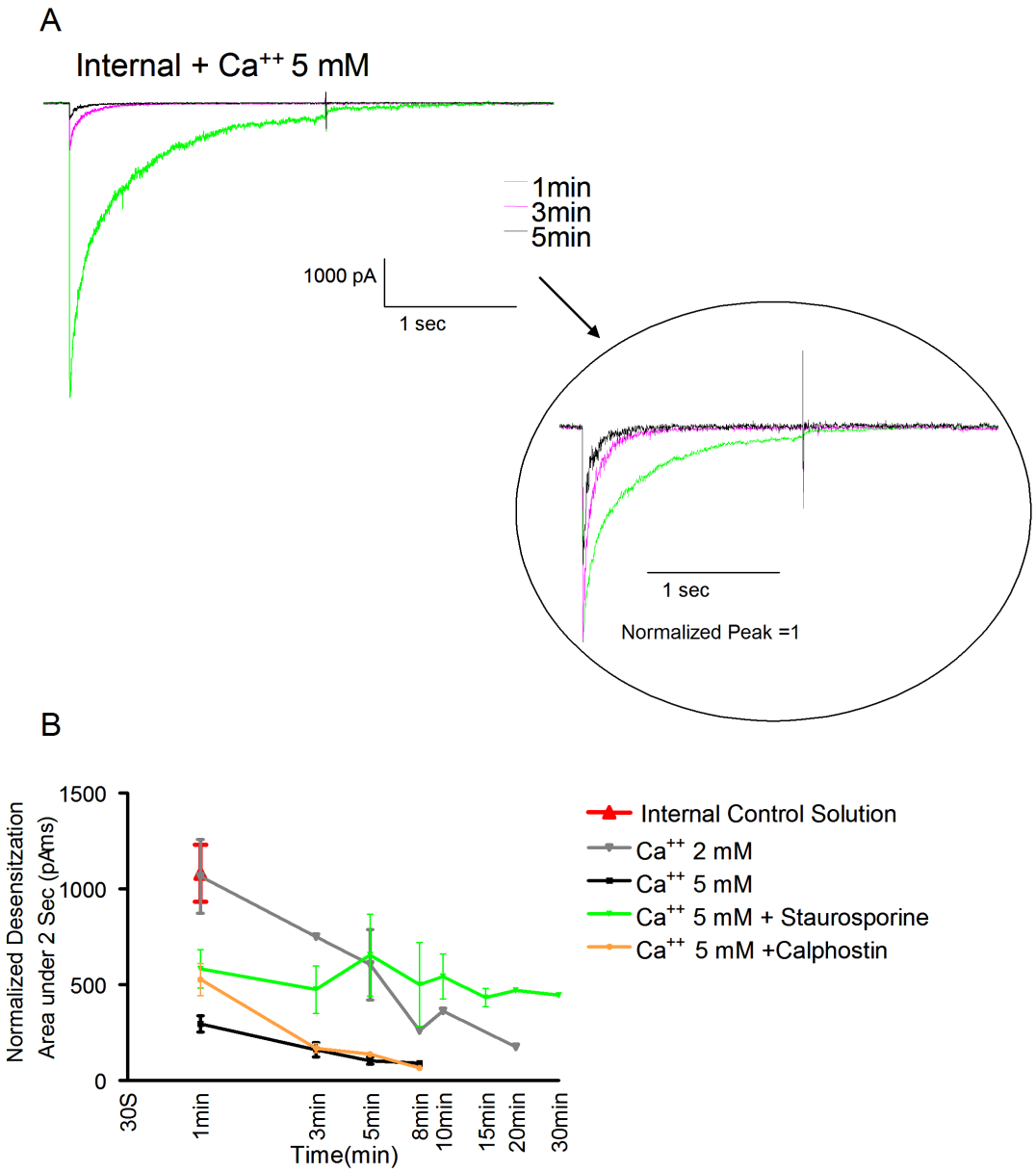

Figure 4.13. Increase of calcium ion inside cell activate similar pathway to increase the desensitization of α4β3γ2L current.

A, Smaller α4β3γ2L currents accompanied with increased current desensitization (see normalized currents in the circle) were recorded with increased time with whole-cell patch recording using an internal solution containing 5 mM Ca^{++}. **B,** Normalized current areas were plotted as a function of time after patch formation with internal control solution (red) or with 2 (gray) and 5 (black) or 5 mM Ca^{++} without or with staurosporine or Calphostin C.

To determine if the Ca^{++}-induced increase of desensitization was due to protein kinase phosphorylation, I included staurosporine (Fig. 4.13 B, green line) or the PKC inhibitor Calphostin C (Fig. 4.13 B, orange line) in the internal solution with 5 mM Ca^{++}. Although current areas recorded with 5 mM Ca^{++} and staurosporine (581.8 ± 100.1, n=11, Fig. 4.13 B, green dot) or Calphostin C (526.5 ± 82.4, n=3, Fig. 4.13 B, yellow dot) in the internal solution had larger initial current areas, only staurosporine maintained the current desensitization and slowed down the reduction of current area. Inclusion of Calphostin C in the internal solution did not alter the extent of desensitization (136.9 ± 13.3, n=2) compared to inclusion of only 5 mM Ca^{++} at 5 minutes. Therefore, this Ca^{++} induced-increase of current desensitization may be mediated also by a protein kinase sensitive to staurosporine. PKC might also have been involved in this Ca^{++} induced-effect since Calphostin C also had a smaller effect temporarily in the first few minutes after patch formation. The progressive extensive increased desensitization and decreased current amplitude produced by internal Ca^{++} was very similar to the reversible effect produced by pre-application of DOG. It is possible that a common mechanism is activated in the two different experiments and causes similar effects on the currents.

Discussion

Phosphorylation is an important mechanism of receptor regulation since it represents a covalent modification of receptors, which can have important implications for ion channel function (Moss and Smart, 1996;Levitan, 1994).

$GABA_A$ receptors, the major sites of synaptic inhibition in the brain, can be modulated by a variety of protein kinases that can have a significant functional impact. Previous studies have shown that PKA phosphorylation increased the fast phases of macroscopic desensitization of α1β1γ2L and α1β3γ2L $GABA_A$ receptor currents mediated by consensus phosphorylation sites β1 S409 and β3 S408, 409 in β subunits (Hinkle and Macdonald, 2003). In the current study, I found that phosphorylation by PKA or PKC affected the macroscopic kinetics of the α4β3γ2L $GABA_A$ receptor currents in the same manner, and that this effect may be related to the number of phosphate groups transferred to the PKA and PKC sites located in the major intracellular loop of β3 and γ2L subunits in the receptor.

Increased macroscopic current desensitization is a common effect of both PKA and PKC on several $GABA_A$ receptor isoforms.

Diversity is always the theme for modulation by various protein kinases. It is easy to image different downstream targets specifically activated by different protein kinases that produce receptor modulation. However, there should also be some common mechanisms shared by different protein kinases. The net charge brought by phosphate transfer and the resultant conformational change of the receptor protein are the most direct consequences shared by multiple protein kinases. There are clearly some regulatory mechanisms such as PKC modulation of receptor internalization that are independent of phosphorylation of the $GABA_A$ receptor itself (Chapell et al., 1998;Connolly et al., 1999), which might be mediated by regulation of associated protein involved in protein trafficking. The most direct consequence of charge

transfer and receptor conformational change by protein phosphorylation could be alteration of receptor gating properties, which usually can be reflected in current kinetics. In this study, I focused on modulation of current kinetics by protein phosphorylation to search for any shared mechanism of receptor modulation by both PKA and PKC. I compared the effects by PKA and PKC on current desensitization of α4β3γ2L currents. The change of current desensitization by PKA and PKC were similar and comparable. Both PKA and PKC increased current desensitization by altering the relative amplitude of different desensitization components and not altering the associated time constants. The different extents of increased α4β3γ2L current desensitization by PKA and PKC might be contributed by extra PKC sites located in γ2L subunits, or due to different permeability and potency of 8Br-cAMP and DOG that were used to activate PKA and PKC. Moreover, similar effects on desensitization kinetics were achieved by glutamate substitution to the phosphorylation sites of PKA and PKC to mimic phosphorylation. These results suggest that PKA and PKC share a regulatory mechanism to increase desensitization of α4β3γ2L $GABA_A$ receptor currents, which is very likely due to phosphorylation of $GABA_A$ receptor subunits.

This modulation of current kinetics was not observed only with one $GABA_A$ receptor isoform. Similar extents of current desensitization increase produced by PKC were found for different $GABA_A$ receptor isoforms including α1β2,3γ2L and α4β2,3γ2L receptors, and to a lesser extent for α1β2 receptors. Microscopic current kinetic studies revealed similar single channel properties of α4β2γ2L and α1β2/3γ2L receptors (Akk et al., 2004;Fisher and Macdonald, 1997;Steinbach and Akk, 2001),

and macroscopic kinetic studies found that the time constants for the desensitization of α4β3γ2L and α1β3γ2L currents were similar and could be divided into the same four discrete groups (Lagrange et al., 2006), indicating that the underlying single channel kinetic schemes and open, close and desensitization states of α4βγ2L and α1βγ2L receptors were likely similar. Thus, phosphorylation of β and γ2 subunits could favor entry into desensitization states of both α1β2,3γ2L and α4β2,3γ2L receptors. Since the α4 subunit is often up-regulated in epileptic animals, it is important to compare α4βγ2L and α1βγ2L currents to understand how their up-regulation may affect plasticity of receptor modulation and change seizure susceptibility in epileptic animals. Although pharmacological characterization has shown different sensitivity of α4 subunit- and α1 subunit-containing receptors to some allosteric modulators such as diazepam and zinc, the modulatory effects of protein phosphorylation were similar on α4βγ2L and α1βγ2L receptors.

Increased current desensitization mediated by phosphorylation of β and γ2L subunits were additive.

By comparison of the S>A and S>E mutations of β3 or γ2L subunits, I knew that "phosphorylation" of each subunit caused increased current desensitization, and the effects of "phosphorylation" of single subunits are additive leading to a maximal increase of current desensitization for $\alpha4\beta3_{EE}\gamma2L_{EE}$ receptors by glutamate substitution at all four sites to mimic full PKC phosphorylation. With pre-application of PKC activators, whether or not β or γ2L subunits were preferentially phosphorylated by PKC could not be controlled. However, the magnitude of the

desensitization increase was found to be concentration-dependent on pre-applied DOG, a PKC activator. The concentration-dependent effect of PKC activation suggested that α4β3γ2L currents desensitized more when the PKC activation level was higher and more receptor sites were phosphorylated by PKC. Although the four well known PKC sites (β3 S408,409 and γ2L S327,343) currents did not account for the major effect of DOG, it is possible that PKC can phosphorylate many additional sites that could not be revealed by conventional methods used to detect phosphorylation.

There is additional evidence suggesting that each β3 and γ2L subunit mediates partial effects of PKC. In receptors without γ2L subunits, like binary α4β3 and α1β2 receptors, current desensitization can still be increased by PKC-activation, but the area reduction was only about half of the reduction produced by PKC-activation of α4β3γ2L currents, consistent with the notion that partial effects of PKC are mediated by phosphorylation of both β3 and γ2L subunits and therefore more effect can be achieved with phosphorylation of α4β3γ2L than α4β3 receptors.

An unknown mechanism instead of direct phosphorylation of four PKC sites or disruption of PKC binding to the intracellular loop of receptor contributes to the major effects of DOG.

More extensive increases of α4β3γ2L current desensitization were achieved by application of high concentrations of DOG than were obtained with the β3 and γ2L S>E mutations. Therefore, there must be mechanisms other than "phosphorylation" of the four sites in β3 and γ2L subunits that causes the effect. I investigated the

possibility that extra phosphorylation sites in α4 subunits or phosphorylation-mediated PKC binding to β subunits contributes to the increased current desensitization. However, neither of the two mechanisms were found to be responsible for the major effects of DOG. Next, I studied the possibility that other protein kinases such as CaMKII or tyrosine kinase were stimulated by PKC-activation, therefore causing phosphorylation of more sites. I used reasonable concentrations of genistein and KN-93 to block tyrosine kinase and CaMKII respectively at the same time that PKC was activated. I failed to see any significant difference in the DOG effect in the presence of the two inhibitors, and only a slight inhibition of the DOG effect by genistein was observed.

Comparison of the DOG effect with results of another experiment, which also causes the similar increase of current desensitization and decrease of current amplitude in the presence of internal increase of Ca^{++} concentration, might provide some clue for the mechanism involved in both experiments. Interestingly, both effects of DOG and internal Ca^{++} were inhibited partially by the non-specific protein kinase inhibitor staurosporine and even less inhibition by PKC specific inhibitors. I think that PKC is activated in both two experiments, but the overall effects might due to protein phosphorylation of substrates in addition to the four known PKC sites. It is very likely other protein kinases like tyrosine kinase are activated in the cascade of PKC activation, which amplifies the signal triggered by PKC activation.

So far, I can not attribute the effects of DOG to any specific phosphorylation site or protein binding motif, but it should still be mediated by the major intracellular

loop of β3 and γ2L subunits given the fact that binary αβ currents were affected less by DOG than αβγ currents, and that the current desensitization was almost abolished by a β subunit mutation with the M3-M4 loop replaced by that from an α1 subunit. The large intracellular M3-M4 loops of $GABA_A$ subunits can contribute to receptor gating by affecting the diffusion rate of ions after crossing the transmembrane channel. Those loops also contain many protein phosphorylation sites and protein binding motifs including the sites and binding sequence for PKC, which are important in mediating modulatory effects on receptor proteins. I hypothesize that the complex of the large intracellular loop and its associated proteins provides a high energy threshold for receptor gating by conformational change of the whole complex, which might allow the receptor to be slowly turned off by slow desensitization. However, when the protein phosphorylation cascade is activated, it might cause a phosphorylation of the intracellular loop and the associated proteins leading to their dissociation. In this case, the intracellular loops are not burdened by the associated proteins and fully charged for intracellular gating purposes, which might allow the receptor to be turned off quickly by desensitization.

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CHAPTER V

CONCLUSION

Phosphorylation provides heterogeneous regulation of CNS $GABA_A$ receptors.

Protein phosphorylation is an important endogenous mechanism for modulating neuronal electrical activity by regulating ion channels on the cell surface. Regulation by protein phosphorylation is not restricted to one class of ion channels or one generic mechanism of modulation. There are reports of many different effects on ion channels ranging from regulation of subunit expression, current kinetics, pharmacological properties and postsynaptic clustering to regulation of receptor recycling. Many of the modulatory effects on receptor ion channels have been shown to be protein kinase-specific or receptor isoform-specific, which contributes to their heterogeneous mechanisms of modulation. In this dissertation, I demonstrated that the effects of protein kinase activation also depend on receptor localization as well as the receptor activation level that is determined by the GABA concentration to which neurons were exposed.

The heterogeneity of receptor modulation by phosphorylation provides various mechanisms for cell type-specific modulation by endogenous protein kinases and phosphatases, which has implications for the precise and dynamic regulation of neuronal network excitability. For example, receptor isoform-specific modulation of

α4β3δ and α4β3γ2L $GABA_A$ receptor currents may help clarify the pathogenesis of neurological diseases, since α4 subunit plasticity was found in several animal models of temporal lobe epilepsy (TLE) and the alcohol withdrawal syndrome (Brooks-Kayal et al., 1998;Cagetti et al., 2003;Peng et al., 2004;Zhang et al., 2007), and γ2 and δ subunits also showed plasticity in expression and might be co-regulated with α4 subunits in these animal models. The higher susceptibility of spontaneous α4β3δ currents than spontaneous α4β3γ2 currents to PKA modulation confers higher plasticity for modulation of tonic inhibition mediated by α4β3δ receptors. The difference in modulation between α4β3δ and α4β3γ2L receptors is most significant at low concentrations of extracellular GABA and becomes less significant with increasing concentrations of extracellular GABA. Tonic α4β3δ and α4β3γ2L currents should become more GABA-dependent and less PKA-sensitive at higher ambient GABA concentrations. At 1 μM GABA, tonic currents mediated by both α4β3δ and α4β3γ2L receptors lost sensitivity to PKA modulation but desensitization of α4β3δ and α4β3γ2L phasic currents were both accelerated by PKA. At a saturating concentration of GABA, PKA increased desensitization of phasic α4β3δ and α4β3γ2L currents to similar extents. In a study of the subcellular locations of δ, α4 and γ2 subunits on the dendrites of granule cells in the mouse pilocarpine model of TLE (Zhang et al., 2007), δ subunit labeling at perisynaptic locations in the molecular layer of the dentate gyrus was found to be decreased. In dentate granule cells from pilocarpine mice, increased α4 and γ2 subunit labeling was found at perisynaptic locations accompanied by a decrease of γ2 subunit labeling in the center of synapses.

Thus there was a shift of γ2 subunits from synaptic to perisynaptic locations and a potential replacement of perisynaptic α4βδ receptors by α4βγ2 receptors to mediate tonic inhibition in the epileptic animals. Interestingly, the magnitude of the tonic inhibition was not significantly affected, but neurosteroids modulation was reduced in dentate granule cells of pilocarpine-treated mice. This result indicated that compensation for the loss of preisynaptic α4βδ receptors by α4βγ2 receptors provided a mechanism to maintain tonic inhibition in pilocarpine-treated animals. However, the modulation of tonic inhibition in epileptic animals likely contributes to epilepsy pathogenesis. The differences in modulation of δ or γ2 subunit-containing receptors include different sensitivity to those allosteric modulators and different modulation by protein phosphorylation. According to my results, tonic currents mediated by peri-synaptic α4β3δ and α4β3γ2 receptors have different sensitivities to PKA modulation only at very low concentrations of ambient GABA. PKA activation can increase spontaneous tonic α4β3δ current in control animal to a greater extent than it can increase the compensatory α4β3γ2 receptor tonic current in epileptic mice. This loss of sensitivity to PKA modulation by α4β3γ2 receptor replacement might contribute to increased seizure susceptibility in the epileptic mice.

The difference in modulation of the kinetic properties of αβδ and αβγ2 currents would also provide a mechanism to regulate differentially phasic and tonic currents in the same neuron. In neurons with extrasynaptic α4β3δ and synaptic α4β3γ2 receptors, PKA could cause an increase of both phasic synaptic α4β3γ2 receptor current and tonic extrasynaptic α4β3δ receptor current when minimal GABA

is present in the extracellular space and only spontaneous α4β3δ currents contribute to the tonic current. However, when ambient GABA levels are elevated (as might occur during episodes of very high neuronal activity), PKA could also specifically enhance IPSCs by regulating phasic α4β3γ2 currents but would have no effect on tonic α4β3δ currents by buffering the extracellular GABA level to a relatively high micro-molar level. Under these conditions, α4β3δ tonic currents would be mainly contributed by GABA-activation and would be PKA-insensitive. This diversity of $GABA_A$ receptor modulation by protein phosphorylation would provide a mechanism to fine tune GABAergic inhibitory activities in specific brain areas with different receptor composition in response to various protein kinases.

Modulation of $GABA_A$ receptors by direct phosphorylation

There is substantial evidence indicating that the conserved $GABA_A$ receptor subunit phosphorylation sites (for example β3 subunit residues S408 and 409) are phosphorylated by multiple protein kinases including PKA and PKC *in vitro*. Until the development of phosphospecific antisera in the last decade, the two sites were shown to be phosphorylated in mouse brain and the basal phosphorylation was dependent on PKC activity (Brandon et al., 2002b). In studies of $GABA_A$ receptor modulation by phosphorylation, many effects have been reported to be mediated by phosphorylation of those sites. While the conclusions have been made in recombinant systems based on blockade of modulatory effects by mutation of the phosphorylation sites, none of the physiological studies in native neurons have demonstrated direct

phosphorylation of $GABA_A$ receptor subunits at the conserved sites. It is possible that auxiliary proteins were the actual targets of phosphorylation. Although there is no direct proof of the same phosphorylation-dependent pathway activated in native brain regions, it is still believed that there is a common action of kinases to alter current kinetics by direct phosphorylation. This alteration of receptor function is thought to be the consequence of a receptor conformational change due to changes in net charge by phosphate transfer.

By comparing the actions of two different protein kinases with shared phosphorylation sites, I observed that both PKA and PKC activation increased current desensitization of receptors with different subunit compositions. Moreover, glutamate substitution at PKA and/or PKC phosphorylation sites to mimic constitutive phosphorylation also produced similar changes in current desensitization, supporting the assumption that this change in current kinetics is due to direct phosphorylation of conserved sites. The greater increase of α4β3γ2L current desensitization by PKC than by PKA can be explained by the presence of additional PKC sites located in γ2L subunits. Consistent with this, I found that increasing concentrations of PKC and PKA activators resulted in a greater extent and rate of desensitization.

There is one result, however, that challenges the hypothesis that regulation of current kinetics is due to direct phosphorylation. Mutation of the conserved phosphorylation sites did not always block the kinase effects on current desensitization, suggesting a role for phosphorylation of associated proteins in the receptor-protein complexes. The large intracellular M3-M4 loops of $GABA_A$ subunits,

which contain binding sequences for several adaptor proteins, were proven important in mediating the effects of protein kinases on current kinetics. The PKC-induced increase of binary αβ current desensitization was less than the PKC-induced increase of αβγ current desensitization, and this effect was abolished almost completely by replacement of the β subunit M3-M4 loop with that of the α1 subunit. This major intracellular receptor protein domain may affect the diffusion rate of ions crossing the transmembrane channel. The interaction of this loop sequence with other associated proteins could be the most susceptible targets of modulation by protein phosphorylation in a cell type-specific manner, since specific, different cellular pathways and specific anchoring proteins may be involved in different neurons.

The physiological significance of plasticity of GABAergic inhibition regulated by protein phosphorylation

It is apparent that $GABA_A$ receptors at inhibitory synapses are dynamic entities with great plasticity for endogenous modulation. In the CNS, a prime mechanism for mediation of ion channel plasticity is phosphorylation (Levitan, 1999;Luscher and Keller, 2004)). It has been demonstrated in many studies that changes in the level of phosphorylation underlie some forms of activity-dependent GABAergic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), often by cross-talk with other receptors expressed in the same synapses. Several signaling pathways have been found to regulate $GABA_A$ receptor functions in the same neuron by acting through various protein kinase or phosphatases. Activation

of NMDA and AMPA receptors, voltage-dependent Ca^{2+} channels (VDCCs), D1 dopamine receptors, M1 muscarinic acetylcholine receptors, serotonin type 4 receptors, and insulin receptors and activation of TrKB receptors by brain-derived neurotrophic factor (BDNF) have been shown to regulate $GABA_A$ receptors by causing influx of Ca^{2+} inside cells as secondary messenger or through downstream signaling of G-protein coupled receptors (GPCRs), which contributes to plasticity of GABAergic inhibition. Involvement of several protein kinases or phosphatases has been implicated in different studies, obviously including PKA, PKC and protein kinase B (Akt), CaMKII, Ca^{2+}-sensitive phosphatase calcineurin (Kittler and Moss, 2003;Brandon et al., 2002a).

For example dopamine receptor activation results in $GABA_A$ receptor phosphorylation and functional modulation in neostriatal neurons. Activation of D1 receptors in neostriatal neurons results in a decrease in GABA-evoked currents, which can be mimicked by cyclic AMP analogues and blocked with PKA inhibitors. It has also been demonstrated that activation of D1 receptors in neostriatal neurons enhances $GABA_A$ receptor β1/3-subunit phosphorylation via a PKA-dependent mechanism (Yan & Surmeier,1997; Flores-Hernandez et al., 2000). Therefore, activation of D1 receptors increases phosphorylation of the $GABA_A$ receptor β1 subunit and inhibits receptor function, as predicted from studies of recombinant receptors. However, there are also examples without clear correlation of receptor activation with a specific protein kinase being involved in the signal pathway. In a similar study of olfactory bulb granule cells with predominant expression of β3 subunits, intracellular

application of purified PKA increased the amplitude of mIPSCs similar to the effect of PKA on β3 subunit-containing recombinant receptors in HEK-293 cells (Nusser et al., 1999; McDonald et al., 1998). In contrast to the potentiation of β3 subunit-containing receptor currents, activation of PKA via D1 receptors in olfactory bulb neurons decreased GABA-activated currents (Brunig et al. 1999). This discrepancy may due to the insensitivity of immunohistochemistry resulting in failure of detecting low levels of other $GABA_A$ receptor β subunits. Alternatively, there may be difference in the signal pathways stimulated by D1 receptor activation in different neuronal population, such as D2 receptor pathway that could also get involved through activation of D1/D2 heterodimer. Therefore, it is important to be aware of different expression pattern of $GABA_A$ receptor subunits in specific cell types, as well as the distinct modulatory effects associated with different receptor isoforms.

Additional evidence for $GABA_A$ receptor modulation by phosphorylation was obtained by studying activation of TrkB receptors by BDNF, which produces even more complex and profound regulation of GABAergic inhibition. The phosphorylation state of β3 subunits has been suggested to underlie BDNF-induced down-regulation of mIPSCs in cultured hippocampal and cortical neurons. However, instead of simple reduction of currents as reported earlier by Bru¨nig et al. (2001), a later report revealed, before a lasting depression of mIPSCs, a rapid and transient BDNF-induced up-regulation occurs (Jovanovic et al., 2004). Coincidently, this biphasic change in inhibitory synaptic activity occurred in parallel with phosphorylation status of $GABA_A$ receptor. Upon stimulus of BDNF, PKC and

anchoring protein receptor for activated C kinase-1 (RACK-1) are transiently recruited to β3 subunits, associated with a delayed but lasting association of PP2A with $GABA_A$ receptors. Sequential association of PKC/RACK-1 and PP2A with $GABA_A$ receptors result in transiently increase of receptor phosphorylation by PKC and then dephosphorylation by PP2A to below basal levels. Collectively, these observations indicate the delayed BDNF-induced and PP2A-mediated dephosphorylation of β3 subunits result in a major reduction in mIPSC amplitude despite the surface expression of $GABA_A$ receptor might be significantly increased. This conclusion conflicts with other studies, which argued that the BDNF-induced down-regulation of mIPSCs without the transient up-regulation was due to PKC-induced endocytosis of $GABA_A$ receptors that was independent of direct phosphorylation of $GABA_A$ receptors (Connolly et al., 1999; Cinar & Barnes, 2001). Thus, BDNF might induce effects on $GABA_A$ receptors by altering receptor surface expression or function, and acute stimulation by growth factors or drugs might have profound effects on GABAergic input in synapses.

The relationship of modulation by phosphorylation of $GABA_A$ receptors with epilepsy

A major challenge in epilepsy research is to understand how the initial injury involved in the induction of epileptogenesis can produce long-term changes in neuronal excitability. Although different types of injuries may lead to epileptogenesis, some of the underlying mechanisms may still be the same. The search for such

common pathways of epileptogenesis may thus provide the foundation for the development of novel antiepileptogenic drugs and thus preventative therapies. One common consequence of epileptic seizure is hyperactivation of excitatory glutamate receptors leading to accumulation of calcium ions inside cells that activate downstream signal cascades, which is widely accepted to be important in shaping the strength of inhibitory synapses. One consequence of the elevation of internal Ca^{2+} is activation of Ca^{2+}-dependent protein kinases and pathways linked to them.

In the introduction (Chapter I), I reviewed the abnormalities of protein phosphorylation reported in some animal models of epilepsy, such as elevated PKC activation after seizures during status epilepticus, and elevated cAMP levels and PKA activity in epileptic animal's hippocampus. The relationship between phosphorylation and seizures is not unidirectional because elevation of cAMP in rat amygdala also promotes seizures. Since protein phosphorylation by both PKA and PKC tend to decrease the response of $GABA_A$ receptors to prolonged or repetitive applications of GABA, the elevated PKA and PKC activities in epileptic animals could cause further disturbance of GABAergic inhibition and exacerbate the imbalance of excitatory and inhibitory signaling input that might contribute to the progress of status epilepticus. On the other hand, PKA increases spontaneous α4β3δ receptor currents, which would have the opposite effect by decreasing network excitability.

In synapses of epileptic animals, during high frequency bursts the overall efficiency of $GABA_A$ receptors in conducting Cl^- would be reduced due to the existence of more receptors in non-conducting desensitization states after

phosphorylation by PKA or PKC. The reduced efficiency of conducting chloride ion would result in less membrane hyperpolarization. When the membrane is not held below the action potential threshold, seizures could be triggered in the preferred phosphorylated condition. This provides a potential basis for maintenance of status epilepticus by promoting receptor phosphorylation after the initiation of status epilepticus. In this way, the endogenous protein kinases might become a good target for antiepileptogenic therapies to enhance GABAergic current by blocking receptor phosphorylation and preventing further development of status epilepticus.

This mechanism may also be relevant to modulation of mutant $GABA_A$ receptor in epileptic animals. While the membrane potential might be maintained below the threshold under basal conditions, protein phosphorylation might serve as a conditional trigger and cause more disinhibition that initiates seizures only in mutant animals. Therefore, treating epileptic animals with protein kinase antagonists to reduce the likelihood of seizures might be a novel approach to treat this disease.

Future directions

In this dissertation, I have described differential modulation by PKA and PKC of different $GABA_A$ receptors. However, there is no clear correlation of the modulatory effects of all the known phosphorylation sites in β and γ2 subunits. The conclusion is that receptor modulation is not necessarily due to direct phosphorylation of receptor proteins. I have shown some preliminary results indicating that only part of the observed effects, such as increased α4β3γ2 current desensitization by PKA, are

mediated by direct phosphorylation of those sites,. A number of effects of PKA and PKC are actually independent of phosphorylation of all of the investigated sites on β3 or γ2 subunits, such as the increased α4β3δ current desensitization produced by PKA and increased α4β3γ2 current desensitization produced by PKC. There are many examples of protein kinase modulation of receptor currents that are independent of receptor phosphorylation, such as phosphorylation-independent internalization of $GABA_A$ receptors induced by PKC (Chapell et al., 1998;Connolly et al., 1999;Filippova et al., 2000). I propose the hypothesis of association of a macromolecular complex with the intracellular loop of $GABA_A$ subunits that can be regulated by protein phosphorylation and may be involved in the regulation of receptor function.

Studies to explore receptor phosphorylation-independent effects should be focused on the regulation of associated proteins in the receptor-protein complex by specific protein kinases. I had tested the most likely protein, which is the protein kinase itself, because the interaction of PKC with $GABA_A$ receptors directly to all β subunits or indirectly through anchoring protein receptor for RACK-1 will be affected by PKC activation (Brandon et al., 2002b). I made three mutations with single or double binding motif deletions to disrupt the interactions of PKC and/or RACK-1 with $GABA_A$ receptor β subunits at their binding sequences (α4β2(PKCΔ)γ2L, α4β2(RACKΔ)γ2L, α4β2(PKC+RACKΔ)γ2L). The mutant with the double deletion (α4β2(PKC+RACKΔ)γ2L) that should produce complete abolition of PKC association at the two sites, failed to reduce PKC effects on current desensitization,

implying that the PKC interaction with the receptor was not a determinant in mediating the effects on current desensitization. Thus, the studies need to be expanded to other associated proteins. There are scattered reports of protein interactions with $GABA_A$ receptors that are regulated by PKC phosphorylation. For example, PKC can also modulate the binding of the clathrin AP2 adaptor complex to the YECL motif in the $GABA_A$ receptor γ2 subunits (Kittler et al., 2005;Kittler et al., 2008). It may be a large associated protein complex instead of each small protein that is involved in mediating the PKC effects on current desensitization. To further test the hypothesis of an associated protein that participates in $GABA_A$ receptor modulation, it will be necessary to have a more comprehensive understanding of how PKC regulates the interaction of all the associated proteins in the receptor-protein complex. Then, the effects of disrupting the interaction of the protein complex and the receptor could contribute to the effects on current kinetics by PKC could be determined.

$GABA_A$ receptors also contain consensus sequences for other protein kinases, such as CaMKII and PTK, and the two protein kinases have been reported to modulate $GABA_A$ receptors (Moss et al., 1995;Houston et al., 2008). I am also interested in determining how the other protein kinases affect $GABA_A$ receptor currents in comparison with the modulation by PKA and PKC. I need to investigate the effects of other protein kinases such as CaMKII and PTK on $GABA_A$ receptor current kinetic properties because phosphorylation signaling can be easily transduced and amplified by protein kinase signaling cascades in the interacting network of

protein kinase and phosphatase. In the experiments, I failed to block the effects of PKC activators by using multiple PKC-specific inhibitors. So far, the non-specific protein kinase inhibitor staurosporine was most effective in inhibiting effects stimulated by PKC activator, DOG. It is possible that other protein kinases were activated by PKC and the amplified protein kinase signaling caused a massive phosphorylation of $GABA_A$ receptors and its associated protein complex which contributes to the extensive kinetic effects on $GABA_A$ receptor currents.

Based on numerous investigations and my own study of modulation of $GABA_A$ receptors by protein phosphorylation, I confirmed the diversity and complexity of the effects conferred by this endogenous modulatory mechanism. The final goal is to collect all the information to fully understand how the protein kinases regulate neuronal excitability and affect brain electrical signaling by altering $GABA_A$ receptor function in their native state, and how modification of endogenous phosphorylation levels might contribute to the pathogenesis of neurological and/or neuropsychiatric diseases. The study of modulatory mechanism might be useful in developing new therapeutic methods to specifically target the dysfunctional receptor isoforms and modify the designed properties of the receptor.

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