PKC GAMMA REGULATES CONNEXIN 57

by

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Abstract

Spinocerebellar ataxia type 14 (SCA14) is a rare, autosomal dominant neurodegenerative disease caused by mutations in the gene encoding for protein kinase Cγ (PKCγ). These mutations affect the translocation and activation of the protein and are particularly damaging to the Purkinje cells of the cerebellum. This translocation and activation leads to the down regulation of gap junction activity by direct phosphorylation on the C-terminal tail of connexin proteins. This process is necessary in terminating the propagation of apoptotic signaling and is disrupted by SCA14-type mutations. Gap junctions allow the passive diffusion of small molecules from one adjoining cell to another. Gap junctions function as electrical synapses in neuronal tissue and are formed from connexin proteins. The connexin family of proteins contains approximately 20 members, each of which is expressed in a tissue dependent manner. One of the dominant connexin proteins expressed in Purkinje cells is connexin 57 (Cx57). Here, I have tested if Cx57 is regulated by PKCγ. This thesis shows that activation of PKC and PKCγ caused internalization of Cx57 gap junction plaques in HT-22 cell culture. PKC and PKCγ activation led to the phosphorylation of Cx57 primarily on serine residues. Furthermore, the expression of SCA14-type PKCγ led to increased sensitivity to oxidative stress, resulting decreased cell viability.

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Dedication

This work is dedicated to those suffering from orphan diseases and to their families.

CHAPTER 1 - Introduction

1.1 Ataxia

Ataxia, from the Greek "a taxis" or without order (OED) means a loss or deficit of coordinated motor function (Dictionary of Psychiatry, 1979) and is a term that encompasses a wide variety of diseases. Roughly 150,000 Americans suffer from some form of sporadic or hereditary ataxic disease (National Ataxia Foundation). The broad variety of ataxic diseases is delineated upon multiple variables. Ataxic symptoms can be caused by environmental as well as genetic factors. Prolonged ethanol consumption, stroke, and head trauma as well as multiple sclerosis and ALS are well known causes of motor coordination loss. Ataxias are imbued with more variation when one considers the variety of disease presentations. Ataxic symptoms may be sporadic or chronic, may develop early or late in life and may be caused by hyper- or hypotonic muscle tone. Furthermore, when the cause of ataxia is genetic, the allele in question may be autosomal dominant, autosomal recessive, X-linked, or mitochondrial (Mescalchi, 2008). Ataxic diseases may target different cerebellar systems including spinocerebellar (movement coordination), olivopontocerebellar (movement initiation), and vestibulocerebellar systems (balanced movement), each of which creates distinct symptoms. However, most forms of ataxias affect multiple cerebellar systems as well as cerebral systems (King, 1987). This thesis discusses an autosomal dominant form of spinocerebellar ataxia (SCA). Spinocerebellar ataxias are defined as "Inherited or sporadic diseases characterized by progressive dysfunction and loss of neuronal systems in the spinal cord, brainstem and cerebellum" (Mascalchi, 2008). Spinocerebellar ataxia type 14 (SCA14) is a rare, chronic neurodegenerative disorder caused by a mutation in the PRKCG gene (Chen et al., 2003). SCA14 is so rare that it accounts for less than 1% of all diagnosed cases of ataxia (National Ataxia Foundation). As with the 28 other known types of spinocerebellar ataxias, this disease is characterized primarily by progressive neurological disturbances in muscle coordination affecting gait, speech, limb and eye movements. Secondary to these, patients may also exhibit: Tremors, myoclonus, seizures, cognitive deficiencies, decreased sensation, and dystonia in later stages of the disease progression (Yamashita et al., 2000; Brkanac et al., 2002; Chen et al., 2003; Hiramoto et al., 2006; Vlak et al., 2006). Distinguishing SCA14 from other ataxic diseases such as the remarkably similar and much more prevalent SCA2, SCA3 and SCA6 on the basis of symptoms

is very difficult because age of onset and clinical features overlap (Schols, 2003). As such, clinical diagnosis requires genetic testing.

1.2 The Cerebellum

1.2.1 Cerebellar Input and Output

While the cerebellum cannot initiate movement, it functions to coordinate all somatic motor activity including not only concurrent movements with each other but also concurrent movements with intended movements (King, 1989). The cerebellum accomplishes this through multiple afferent connections between the cerebellum and the body and afferent and efferent projections between the cerebellum and the motor command centers in the cerebrum (pyramidal and extrapyramidal systems), respectively (King, 1989). These multiple neural pathways can be subdivided by afferent or efferent direction and their origination or destination, respectively. The corticopontocerebellar pathway, for example, is an afferent pathway that originates in the cerebral cortex, projects through the pons and terminates in the cerebellum (Nolte, 2000). Efferent pathways project from the cerebellum to the cerebral cortex, to feedback motor centers in the midbrain and to feedback motor centers in the hindbrain (King, 1989). No efferent projections from the cerebellum project directly to the spinal cord, which explains why the cerebellum is incapable of initiating motor activity. In short, the cerebellum integrates kinesthetic information from the body and cerebrum to produce balanced, graceful, synchronous movements. Protein phosphorylation is a key regulatory mechanism for tissue homeostasis. In the cerebellum, abnormal protein phosphorylation has been linked to autism, mental retardation, epilepsy and several forms of ataxia including SCA14 (Chizhikov et al., 2003).

1.2.2 The Cerebellum: A Microscopic View

A microscopic view of the cerebellar cortex demonstrates a uniform cellular organization. This uniform organization creates three distinct layers that persist throughout the cortex. From the outermost layer to the innermost layer, they are the molecular layer, the Purkinje layer and the granular layer (rev. by Ruigrok, 2010).

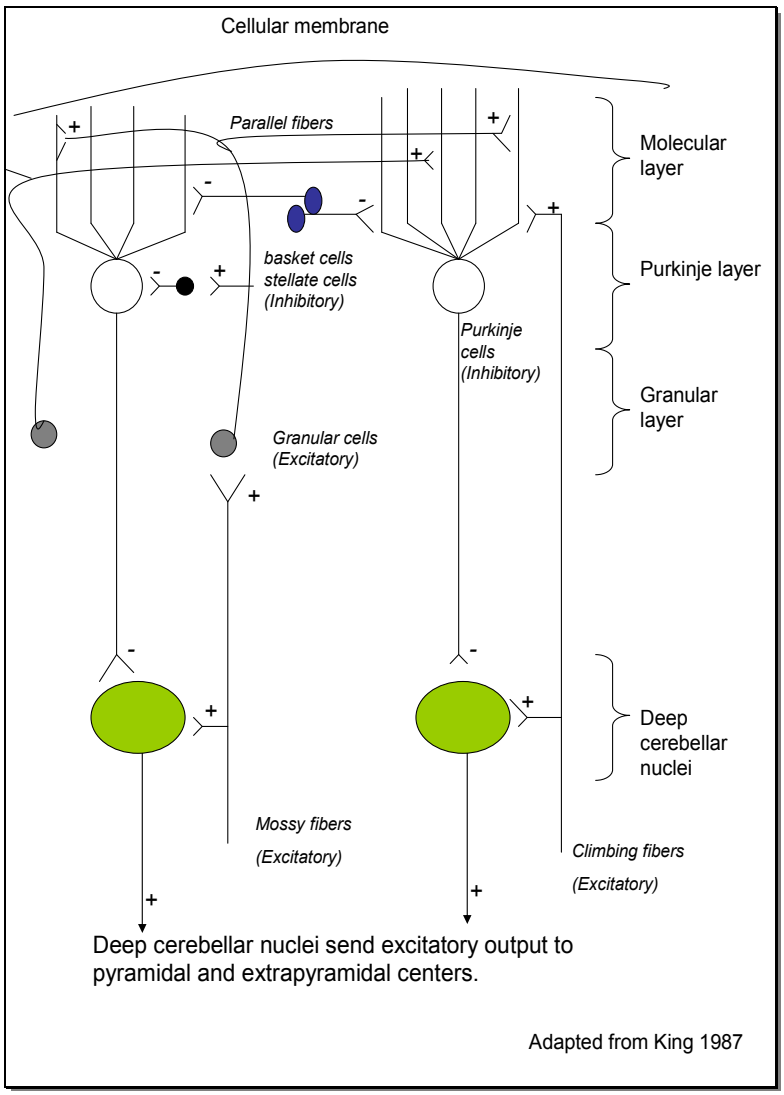


Figure 1 Cellular Organization of Cerebellar Cortex

The molecular layer contains stellate cells, basket cells, the dendritic arbor of Purkinje cells that originate in the Purkinje layer, and the parallel tracts of granular cells that originate in the granular layer. The parallel fibers are excitatory, whereas stellate and basket cells are inhibitory upon Purkinje cells.

The Purkinje layer contains Purkinje cells and interneurons. Interneurons receive excitatory information from granular cells and transmit inhibitory information to Purkinje cells. Purkinje cells are the sole provider of output from the cerebellar cortex to deep cerebellar nuclei. As such, Purkinje cells serve primarily to integrate cortical information. The Purkinje cells' extensive dendritic arbor extends into the molecular layer and is largely flat, forming a perpendicular angle to the parallel fibers. Purkinje cells can either be stimulated (by granular

cells) or inhibited (by stellate and basket cells) but are themselves exclusively inhibitory to deep cerebellar nuclei.

The granular layer contains granular cells, unipolar brush cells, Golgi cells and mossy fibers, which originate mainly in pontine nuclei. Mossy fibers form excitatory synapses directly to deep cerebellar nuclei and to granular cells (which, in turn, are excitatory to Purkinje cells and inhibitory to deep cerebellar nuclei). The functional relationship between the various nerve cell types is illustrated in the above figure (fig. 1). Abnormalities in Purkinje cell activity produce cerebellar dysfunction and consequently, ataxic symptoms (Walter et al., 2006)

1.2.3 Functional Divisions of the Cerebellum

The cerebellum is subdivided into three regions on the basis of input. Ataxic diseases affect at least one (if not all) of the three main cerebellar functional regions. Vestibulocerebellar lesions interfere with input information from the vestibular nuclei and produce disequilibrium and nystagmus. Lesions in the pontocerebellum interrupt feedback pathways between the cerebrum and cerebellum causing hypotonic tremors, dysmetria and asynergia. Spinocerebellar lesions, on the other hand produce hypertonic tremors and postural reflexes. In this way, spinocerebellar ataxias are further subdivided into three categories, namely SA (spinal atrophy), OPCA (olivopontocerebellar atrophy) and CCA (cortico-cerebellar atrophy) (Mascalchi, 2008). It should be noted that spinocerebellar ataxias often present with pontocerebellar and vestibulocerebellar symptoms, due to the diffuse nature of genetically derived lesions. This is the case for SCA14. While this disease is classified as a spinocerebellar type ataxia, pontocerebellar symptoms such as dysmetria and asynergia are common.

1.3 SCA14

1.3.1 ADCA's: A Brief History

In 1863 a German scientist named Nicholas Friedreich was the first to distinguish ataxias from multiple sclerosis. 30 years later a French scientist named Pierre Marie began classifying ataxias based on heredity. For the next 100 years (1893-1993), ataxias were genetically categorized as Friedreich's (recessive) or Marie's (dominant) ataxia. The search for a specific gene causing ataxia began in the 1950's by a neurologist named Dr. John Schut at the University of Minnesota Medical School. Dr. Schut was forced to abandon his research, however, because

of complications from a progressive and debilitative disease. In fact, the same disease killed his father at the age of 48, and would lead to Dr. Schut's death in 1972. Dr. John Schut, along with many members of his family suffered from "Marie's Ataxia". In the same year of Dr. Schut's death, researchers linked ataxia found in a large Mississippi family to a complex of genes on chromosome 6. In 1984, researchers at the University of Minnesota found that the chromosomal abnormality affecting this Mississippi family was one and the same as that affecting the Schut family. A combined effort by Dr. Harry Orr at the University of Minnesota and Dr. Huda Zoghbi at the Baylor led to the discovery of the first ataxic gene in 1993 (National Ataxia Foundation). Autosomal dominant cerebellar ataxias (ADCAs) are now classified on a genetic basis, rather than by inheritance. Until this point, ADCA's could only be classified by subset of cerebellar tissue most endangered by the specific disease and the inheritance pattern (Schols, 2003). The first gene to be identified as a causative agent of ataxia was discovered in 1993 and was named Ataxin-1. A CAG-trinucleotide repeat in Ataxin-1 causes SCA-1 (Orr et al., 1993). Since that time, 28 other genes have been identified to cause SCA and have been numbered accordingly.

Unlike many SCAs, SCA-14 is not caused by a CAG-trinucleotide repeat. Trinucleotide repeat mutations are a common suspect when considering any autosomal dominant, neurodegenerative disease because so many fit this category including at least 8 forms of SCA, along with muscular dystrophy and Huntington's disease. With trinucleotide repeat diseases, each successive generation develops a more severe phenotype earlier in life due to successive expansion of the trinucleotide repeat section. This phenomenon is referred to as anticipation. Dr. John Schut's father first noticed symptoms at age 32; Dr. Schut began suffering from SCA1 at the age of 29. SCA14, along with SCA-5, 11, 12, 13, and SCA-15 arise from point mutations, and so, anticipation does not occur (Brkanac et al., 2002; van de Warrenburg et al., 2004).

1.3.2 SCA-14: An Overview

SCA14 is caused by mutations in the gene encoding for protein kinase Cγ (PKCγ) and onset occurs between the ages of 2-70. (Chen et al., 2003; Vlak et al., 2006). PKCγ is an important kinase enzyme. Its expression is localized mainly in the brain, spinal cord, retina and lens. PKCγ is enriched in the Purkinje cells of the cerebellum and hippocampal pyramidal cells (Mellar et al.; 2005). Magnetic resonance imaging scans display mild to moderately severe

degeneration of the midline of the cerebellum, whereas the brain stem, cerebral cortex and spinal cord are unaffected (van de Warrenburg et al., 2003). Ataxic symptom severity correlates closely with the severity of atrophy (Mascalchi, 2008). Purkinje cell degeneration is a common feature among many ataxias and is demonstrated in SCA14 (Brkanac et al., 2002).

Children of SCA14 patients have a 50% probability of inheriting this disease. To date, only one patient has presented a homozygous SCA14 genotype resulting in a severe phenotype (Asai et al., 2009). The lack of common homozygosity is in part due to the rarity of this disease. It is also believed that these fetuses may not usually be viable. Homozygous transgenic mice develop hydrocephalus and die within a short period after birth. Generally, human patients remain ambulatory, but often require a cane or walker to prevent falling. As with many genetically derived ataxias, few treatments are available. Clonazepam or valproic acid can improve axial myoclonus. Physical therapy and speech therapy can improve quality of life. Weighted eating utensils, home grab bars, and dressing hooks help patients maintain independence. SCA14 does not decrease overall lifespan, and there is currently no cure.

The phenotypic presentation and severity of this disease may be dependent upon the specific mutated residue, as multiple mutations affecting the same residue present with similar symptoms. Examples include patients with the following mutations: H101Y/H101Q, S119P/S119F, and G123E/G123R. This is also supported by the observation that neurological abnormalities associated with this disorder vary to some extent, but are shared within patients of the same family (Morita et al., 2006). However, prognosis of the disease course may extend beyond the site of mutation, and have some dependence on the age of onset (Yamashita et al., 2000; Yabe et al., 2003). Currently, the overall features of SCA14 have yet to be completely analyzed, which prevents establishing a direct correlation between genotype and phenotype. Suspected but unconfirmed phenotypic characteristics of this disease include dysphonia, dysphasia, seizures and Parkinsonian features such as tremors and rigidity. Moreover, the full gamut of SCA14 causing mutations has yet to be determined. Currently, point mutations and point deletions are the only known sources of SCA14, with one exception. A recent report has shown that a mutation in the stop codon of the PRKCG gene leads to a 15 amino acid extension. However, it is possible that intronic duplications, deletions, and inversions that have yet to be identified could cause this disease (ncbi.nlm.nih.gov/bookshelf). If late onset cases are included, penetrance of this disease is high, but may be less than 100% (Klebe et al., 2005). Currently,

there are 22 known point mutations within PKCγ that result in SCA14. Notably, a majority of these mutations lie within a region of PKCγ known as the C1B domain.

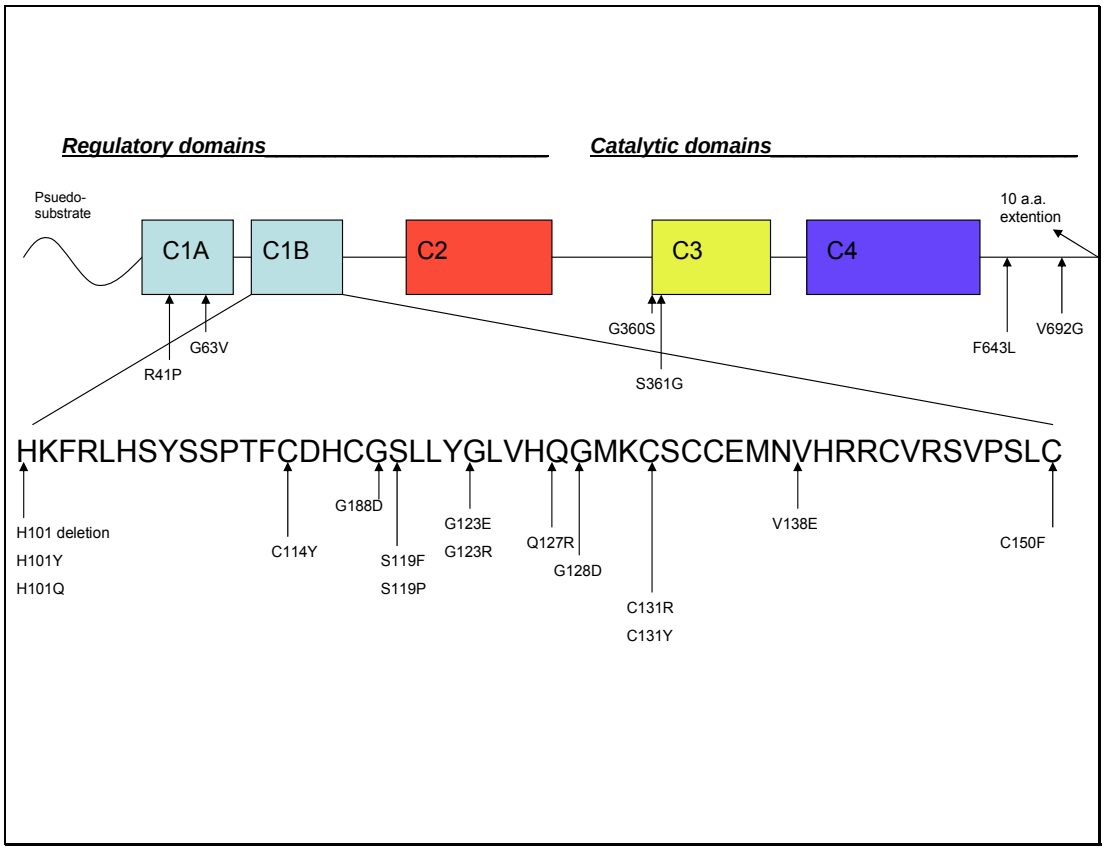


Figure 2: Domain Structure of cPKC and SCA14-Causing Mutations Found in PKCγ

1.4 PKC

1.4.1 An AGC Family Member and the Kinase Domain of PKC

Protein Kinase C (PKC) is a member of the AGC kinase super-family of kinase enzymes. The term AGC kinase defines a super-family of serine/threonine kinase enzymes based on sequence alignment within the catalytic domain and was originally composed of three members: protein kinase A, protein kinase G, and protein kinase C (Hanks and Hunter, 1995; rev. by Pearce et al., 2010). Currently, there are 60 known members of the AGC subfamily (Manning et al., 2002), 42 of which contain domains other than the catalytic core. The X-ray crystal structure for the kinase domain of cPKC was determined using PKCβII as a representative isoform (Grodsky et al., 2006).

The kinase domain of the AGC subfamily is composed of two lobes known as the N-lobe and C-lobe based on their orientation with regard to the amino and carboxy terminus, respectively (Knighton et al., 1991). In the case of PKC, these lobes are often referred to as the C3 and C4 domain. These two lobes sandwich together to create a substrate binding pocket and an ATP binding site. ATP serves as the phosphate donor compound for phosphorylation. The DFG (Asp-Phe-Gly) motif is a component of the C-lobe and functions to position ATP in the proper orientation for catalysis (Johnson et al., 1996).

Most members of the AGC kinase super-family, including PKC, contain two highly conserved regulatory segments that serve as phosphorylation sites of the enzyme and promote activation. The first regulatory segment is found within the C-lobe of the catalytic domain and is referred to as either the T-loop or the activation loop. The activation loop is a substrate for another AGC kinase, namely PDK1 (Newton, 2003). Phosphorylation of the activation loop by PDK1 contributes to the overall activation of the PKC. While the activation loop is part of the C-lobe, it is connected to the N-lobe through an αC helix. Phosphorylation of the activation loop by PDK1 causes a conformational change in the αC helix and by doing so allows for hydrogen bonding between Lys residues of the N-lobe, Glu residues of the αC helix and the phosphate of ATP (Yang et al., 2002; Komander et al., 2005). Moreover, phosphorylation of the activation loop is necessary for phosphorylation of other segments of PKC (Edwards et al., 1999). The second highly conserved regulatory segment is called the hydrophobic motif and is on the C-terminal tail. Once phosphorylated, this sequence wraps around the kinase domain and inserts two aromatic residues into a part of the N-lobe called the hydrophobic motif pocket. By doing so, the hydrophobic motif stabilizes the αC helix in the active conformation (Yang et al., 2002a; Yang et al., 2002b). PKCs and a number of other AGC kinases contain a lesser conserved, third phosphorylation site referred to as the turn motif. The turn motif is part of the N-lobe and phosphorylation of this segment promotes the overall integrity of the enzyme. Moreover, it may serve to prevent dephosphorylation of the hydrophobic region (rev. by Pearce et al., 2010). In the case of PKCs, phosphorylation of these three regions is a required component of overall enzyme activation, but is all together insufficient for kinetic activity. Like most AGC kinase super-family members, PKC contains domains outside the catalytic domain that are necessary for enzyme activity.

Protein Kinase C (PKC) is a multigene family of serine/threonine kinases that is further subdivided into three categories as conventional (PKCα, PKCβI/II, PKCγ), novel (PKCδ, PKCε, PKCη, PKCθ), and atypical (PKCξ, PKCr/λ) on the basis of these regulatory domains. Currently, there is some debate about whether or not to include PKN isoforms PKN1, PKN2 and PKN3 within the PKC family. Moreover, PKCβI and II are splice variants of the same gene and are sometimes regarded either as separate members of the PKC family or as a single member. The topic of this thesis is in regard to PKCγ and will only focus on the regulatory domains of conventional isoforms of PKC.

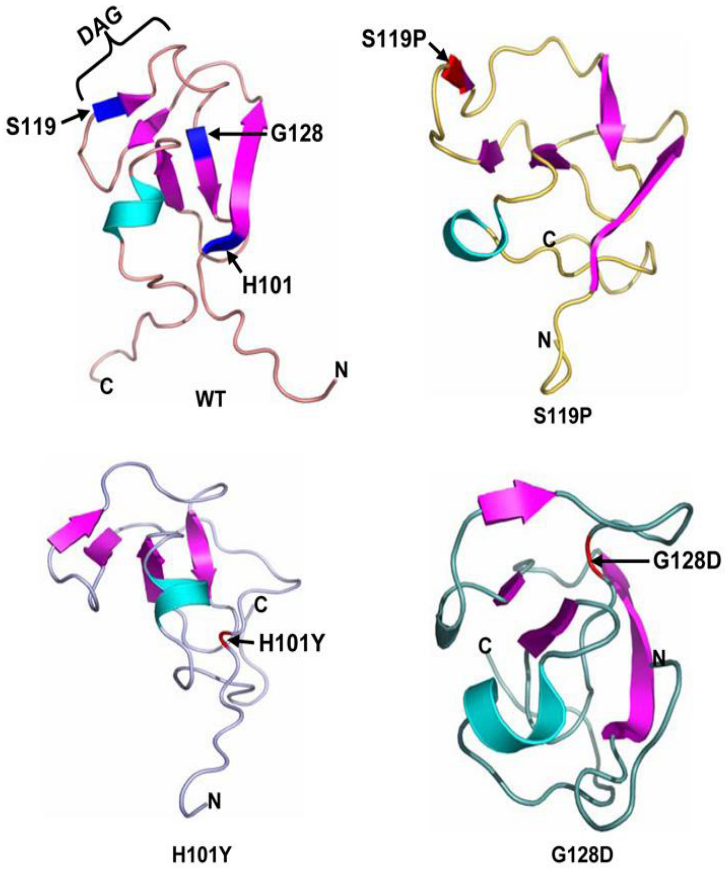
1.4.2 C1 Domains

All isoforms of PKC contain at least one C1 domain. In the case of conventional and novel isoforms, two C1 domains exist in tandem repeats and are dubbed the C1A and C1B domains. These domains are approximately 120 amino acids in total (rev. by Hurley et al., 1997). These tandem domains each contain a 50 amino acid sequence that functions as a zinc finger. Each zinc finger consists of 6 cysteines and 2 histidines at conserved distances ($HX_{12}CX_2CX_NCX_2CX_4HX_2CX_7C$, N=13-14) that facilitate the coordination of the zinc ion (Kikkawa et al., 1989; Bell and Burns, 1991; Dekker and Parker, 1994; Newton; 1995). The function of C1A and C1B is to bind diacylglycerol in order to translocate cPKC to the plasma membrane in the presence of calcium (rev. by Colon-Gonzalez and Kazanietz, 2006). By coordinating six hydrophobic amino acids between two fatty acid chains, PKC is tightly bound to the membrane (Cho, 2001). This type of lipid binding is a critical step in PKC activation. In general, these tandem repeats are not functionally redundant (Yongei, 2008). Moreover, not all lipid binding functions to activate PKCs. In the case of PKCγ, the C1A domain can interact with arachidonic acid to inhibit kinase activity (Ochoa, 2001), but the C1A and C1B domains equally bind PDBu (Phorbol 12,13-Dibutyrate) resulting in activation (Wang et al., 1996). Moreover, unlike PKC α, β and δ, PKCγ C1 domains do not show preferential binding to phosphatidylserine over other lipid species (Giorgione et al., 2006; Slater et al., 2002; Stahelin et al., 2005a; Stahelin et al., 2004; Stahelin et al., 2005b).

In PKCγ, the C1B domain interacts with a sequestrian-type inhibitory protein called 14-3-3ε. 14-3-3ε binds to binding partners through RSXpSXP or RXXXpSXP consensus sequences (Nguyen et al., 2004). Especially, though probably not exclusively, the first 12 amino acids of

the C1B domain facilitate this interaction. 14-3-3ε binds PKCγ in the cytoplasm and sequesters the inactive form away from target substrates to prevent unwanted kinase activity (Nguyen et al., 2004). In the presence of calcium, the multi-phosphorylated form of PKCγ is translocated to the membrane where it binds lipid substrates through the C1A and C1B domains. This holds the activated kinase enzyme to the membrane and near target substrates.

Unlike other classical isoforms of PKC, PKCγ does not require calcium signaling for activation, but instead can be activated by oxidative stress directly. Within the C1B domain, cysteine residues of PKCγ are oxidized by hydrogen peroxide and form a disulfide bond, causing a release from 14-3-3ε. This confers a method of activation independent of G-protein coupled receptor signaling (Lin and Takemoto, 2005). PKCγ is largely found in neuronal tissue, and not surprisingly, is a major responder to oxidative stress in the brain (Barnett et al., 2007). Of particular importance to this article, 15 of 22 mutations found in SCA14 are found within the C1B domain. These mutations affected the predicted structure as described by Lin et al. (2007). Usually, C1B domains form a binding pocket for diacylglycerol and six hydrophobic amino acids insert into the membrane (Hritz et al., 2004). The NMR structure for wild type PKCγ C1Bdomain was determined by Xu et al. (1997).



Reprinted from Experimental Eye Research, vol. 3, no. 362(4), Lin and Takemoto, Protection from ataxia-linked apoptosis by gap junction inhibitors, with permission from Elsevier.

Figure 3 Predicted Structure of the C1B Domain in SCA14-Type PKCγ

Wild type C1B domain shows a short α helix (V142-146) and five β strands (H101-S107, T112-C114, S119-L121, G128-C131, and M136-H139). The three SCA14 mutations studied in this work are at the first positions in strands 1, 3 and 4. The predicted three-dimensional structure of H101Y and G128D reveal conformational changes in the zinc-finger region. The predicted structure of S119P has a relaxed overall structure and may interrupt the Zn-finger oxidative stress switch function. Of interest, the S119P mutation is the least significant in terms of structural change but is the most severe in terms of phenotype. This may be due to collapsing of the DAG binding pocket (Lin et al., 2007).

A common feature in many kinase enzymes and present in all isoforms of PKC is the pseudo-substrate sequence. In the case of cPKCs like PKCγ, this sequence is found N-terminal to the C1 domain discussed above. This sequence conforms to substrate sequences with the substitution of Ala in the phospho-acceptor position. The pseudo-substrate is an intrinsic

component of PKCs that occupies the enzyme's active site when the kinase is in an inactive conformation (House and Kemp, 1987).

1.4.3 C2 Domains

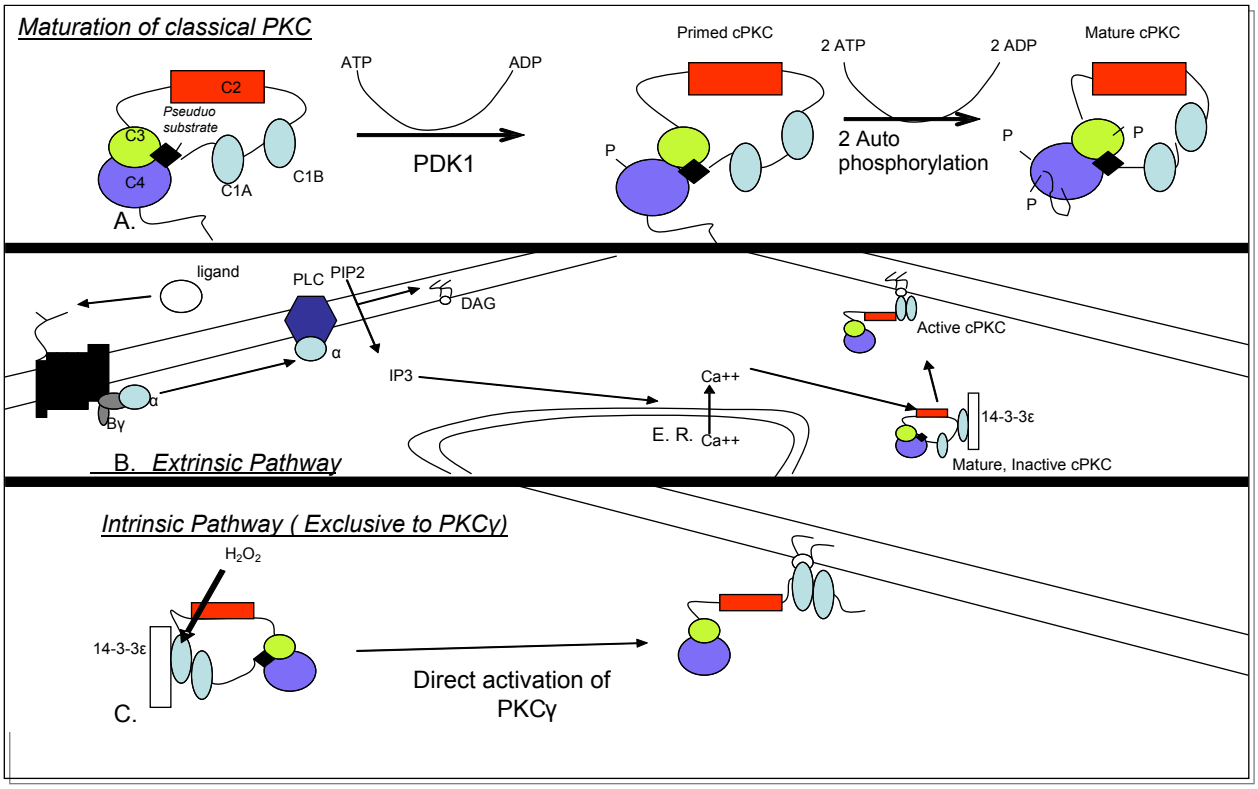


Figure 4 Maturation and Activation of PKC

The C2 domain is the second regulatory domain found within classical isoforms of protein kinase C. These are composed of approximately 130 amino acids that form eight anti-parallel β-strands connected by variable loops. The variability of these loops confers differential functional specificity. These loops function cooperatively to bind two or three calcium ions (Torrecillas et al., 2004). The binding of calcium allows the C2 domain to contribute a supplemental membrane interaction with the C1 domain. This promotes a conformational change that releases the pseudo-substrate. Thus, the C2 domain is responsible for calcium-dependent activation indicative of classical isoforms of PKC (rev. by Steinberg 2008; rev. by Cho, 2001; Medkova, 1998).

1.4.4 PKCγ Activation and Regulation

PKCγ can be activated in two distinct ways. In accordance with classical PKCs, a growth factor binds to a GPCR leads to the activation of phospholipase Cβ (PLCβ), which catalyzes the conversion of PIP_2 (phosphatidylinositol 4,5-bisphosphate) to PIP_3 (phosphatidylinositol 3,4,5 triphosphate) and DAG (diacylglycerol). PIP_3 interacts with the endoplasmic reticulum (E.R.) and releases calcium. Calcium releases PKCγ from 14-3-3ε by interaction in the C2 domain (Kohout et al., 2002; Nguyen et al., 2004). From here, PKCγ binds to DAG in the membrane. Binding of calcium and DAG by the C2 and C1 domains causes the release of the pseudo-substrate from the active site (Newton and Johnson 1998). Here, PKCγ is activated and translocated; thus, it can phosphorylate target substrates (fig. 4B) (Violin and Newton 2003). In experimental systems, phorbol esters such as TPA (12-O-tetradecanoylphorbol 13-acetate) or PMA (phorbol 12-myristate 13-acetate) are used as diacylglycerol mimetics in order to activate PKCs (Ono et al., 1989). These compounds are potent tumor promoters, implicating PKCs in tumorigenesis (Castagna et al., 1982). In fact, within the CNS, over expression or hyperactivity of PKCγ is a hallmark of malignancy (Bredel et al., 1997; Sharif et al., 1999).

The second method of activation describes the mechanism of activation by oxidative stress and is unique to PKCγ (fig. 4C) (Lin and Takemoto, 2005). In the presence of hydrogen peroxide, the two histidine residues and six cysteine residues of the C1B region are oxidized. This forms a disulfide bond between the two cysteine residues transforming PKCγ into an active form. In this conformation, PKCγ can bind DAG at the membrane and phosphorylate membrane substrates (fig. 4C).

While PKC maturation and activation is well understood, much less is understood about PKC down regulation. The maturation of PKC requires phosphorylation on the activation loop, the turn motif and the hydrophobic loop. The activation of cPKC requires cytosolic Ca^{++} and DAG. These necessary steps for maturation and activation are reversed in order to achieve down regulation. Translocation from the membrane (reverse translocation) is one of several ways PKCs are down regulated (Feng et al., 1998). When PKC is activated by a natural GPCR agonist, translocation to and from the membrane occurs rapidly, whereas when PKC is activated by phorbol ester stimulation, PKC persists at the membrane for a longer period (Feng et al., 1998). This reverse translocation can be caused by decreases in second messenger signaling. Increases in the level of Ca^{++} and DAG coincide with the activation of PKC. Similarly,

decreases in the level of these second messengers leads to decreases in PKC activity (rev. by Gould and Newton, 2008). Moreover, the mature, active form of PKC is more sensitive to dephosphorylation by phosphatases. Chronic PKC activation produces a decrease in phosphorylated PKC, which coincides with decreases in PKC activity. Over time, chronic PKC stimulation causes the degradation of PKC (Young et al., 1987). Activated PKCs have been shown to be degraded by both the proteosomal and lysosomal pathway, whereas PKC in the inactive conformation is relatively resistant to proteolysis (Herrmann et al., 2007).

The PKCγ gene is located on chromosome 17q13.4 in humans and 17q7 in mice. This gene is approximately 24.4 kb long and composed of 18 exons varying in length from 32 to 406 base pairs (Parker et al., 1986; Coussens et al., 1986). The 5' flanking region contains binding sites for transcription factors AP1, AP2 and SP1, but lacks any CAAT or TATA boxes (Chen et al., 1990; Takanaga et al., 1995; Kofler et al., 2002). As with all cPKCs, the AUG translation initiation site is located in exon-1. A detailed understanding of the genetic regulation of PKCγ remains unclear.

1.4.5 PKCγ Expression and Function in Various Tissues

PKCγ is found throughout the brain and spinal cord and is particularly enriched in cerebellar Purkinje cells and pyramidal cells (Huang et al., 1986; Shutoh et al., 2003). It is also found in the retina, peripheral nerves and the lens (rev. by Barnet, 2007). In each of these tissues, PKCγ performs an important role.

PKCγ has important functions within various eye tissues. This isoform has been shown to protect the lens from oxidative stress. Oxidative stress (100 μM, 30 min) activates PKCγ in the lens and results in the modulation of gap junction activity. This regulation was shown to be nullified when lens cells were transfected with SCA14-type mutants correlating with increases in caspase activity (Lin and Takemoto, 2007). PKCγ knockout mouse lenses are more susceptible to oxidative stress and subsequent cataractogenesis than wild type tissue (Lin et al., 2006). In the retina, PKCγ is important for development and hypoxia resistance. Knockout mice display increased thicknesses in the inner nuclear and ganglion cell layers, relative to wild type tissue. Hyperbaric oxygen was shown to damage the outer segments of the photoreceptor layer and ganglion cell layer in knockout mice but did not in wild type mice (Yevseyenkov et al., 2009).

These data indicate that PKCγ has a protective role in both ischemic- and reperfusion-induced stress in the eye.

PKCγ is involved in pain response (rev. by Saito and Shirai, 2002). This is demonstrated by the observation that μ-opioid receptor stimulation induces prolonged PKCγ translocation and activity in the dorsal horn of the spinal cord. Moreover, PKC inhibitors prevent acquired desensitivity to μ-opioid agonists, and knock out mice display a greater sensitivity to pain.

PKCγ is involved in the modulation of synaptic plasticity. It has been implicated in both long term potentiation (LTP) and long term depression (LTD) (Bliss et al., 1993; Ito, 1989). PKCγ's suspected involvement in LTP is based on the observations that PKCγ activation by phorbol esters stimulates synaptic transmission in hippocampal slices, PKCγ inhibitors inhibit LTP, and injection of PKCγ into post synaptic pyramidal cells of the hippocampus mimics LTP (Abeliovich et al., 1993a; Abeliovich et al., 1993b; Hu et al., 1987). Similarly, PKCγ is implicated in LTD by the observations that PKCγ activators induce LTD and PKC inhibitors inhibit LTD (Linden and Connor, 1991).

PKCγ has yet another role in the brain as a regulator of ischemia/reperfusion-induced damage. Knockout mice subjected to infarction followed by reperfusion showed a larger area of neuronal damage as compared to wild type mice. This indicates that PKCγ has a limiting effect on the volume of damage. When permanent ischemia was induced, knockout mice showed a significantly smaller infarct volume. These data indicate that PKCγ has a protective role in reperfusion but a deleterious role in ischemia in the brain (Aronowski and Labiche, 2003). However, Hambe et al. (2005) found that inhibitors of PKC increased neuronal damage in response to hypoxia as compared to tissues where PKCs were stimulated by insulin. In this study, only the γ isoform of PKC demonstrated increased translocation. These data contest the role of PKCγ in hypoxia as deleterious and suggest a protective role instead.

The role of PKCγ in the cerebellum is perhaps least well understood. A SCA14 transgenic mouse model has recently been developed, but has yet to be fully characterized. H101Y transgenic mice displayed an altered cerebellar phenotype and Purkinje cell degeneration. Caspase-12 induction was demonstrated when cerebellar tissue from the transgenic animal was treated with oxidative stress (100 μM, 30 min). The same levels of oxidative stress did not induce apoptotic signaling in wild type cerebellar tissue (Zhang et al., 2009). This indicates that SCA14 mutations increase cerebellar sensitivity to oxidative damage.

1.4.6 PKCγ Target Substrates and Sites of Phosphorylation

Generally, PKCs phosphorylate Ser or Thr residues that are surrounded by basic residues. However, PKCγ appears to be more target specific than other forms of PKC such as the co-expressed PKCε. PKCγ phosphorylation receptor sites require a proximal Arg on either side in order to demonstrate kinase activity (Marais et al., 1990). For example, one of the best characterized PKCγ substrates is connexin 43 (Cx43). Activated PKCγ is targeted to gap junction plaques though caveolin-1 containing lipid rafts in order to phosphorylate Cx43 (Lin et al., 2003). This gap junction is subsequently phosphorylated on S368 in the context of ARS-S_{368}-ARS. This is also seen within the pseudo-substrate. In the inactive conformation, the pseudosubstrate mimics a PKCγ target sequence with an alanine substitution in the place of Ser/Thr. This sequence is FCRKGALRQ.

PKCγ has been shown to target a number of substrates including receptors, structural proteins, ion channels and members of the connexin family, such as Cx43, Cx50, Cx56 and Cx46 (Saez et al., 1997; Berthoud et al., 2000; Lin and Takemoto, 2007). Overexpressed PKCγ was shown to decrease gap junction communication and increase disassembly of gap junction plaques (Saleh and Takemoto, 2000; Wagner et al., 2002).

1.5 Gap Junctions and Their Connexin Components

1.5.1 Gap Junction Overview

Gap junction communication is required in physiological processes such as cell synchronization, differentiation, growth and metabolic coordination (Mese et al., 2007; White and Paul, 1999; rev. by Mathias et al., 2010). Gap junctions play a role in a variety of diseases including peripheral neuropathy, deafness, skin disorders, cataracts and oculodentodigital dysplasia (rev. by Wei, 2004). Vertebrate gap junctions are formed primarily from connexin proteins. Invertebrates express the analogous innexin family of proteins, but these are unrelated to the connexin family by sequence similarity (Phalin et al., 2001). Vertebrate genomes do include an innexin-related family of gap junction forming proteins called pannexins, of which there are three family members (Panchin et al., 2000; Baranova et al., 2004). The expression of pannexin genes is somewhat limited and falls outside the focus of this thesis. To date, 20 human and 21 murine connexin proteins are known and distinguished by size (Sohl and Willecke, 2003). Connexin 43 (Cx43) is a 43 kilodalton connexin protein, Cx50 is 50 kDa etc.; 19 of these are direct orthologs. Mice express Cx33 whereas humans do not. Similarly, mice do not express any orthologs for human Cx22 and Cx59. Moreover, not all orthologs are of the same size. For example, mouse Cx57 is an ortholog of human Cx62, and human Cx46, mouse Cx46, bovine Cx44 and chicken Cx56 are all orthologous. Lastly, not all orthologs are expressed in similar tissues. For example, hCx30.2, hCx31.9, hCx40.1 and hCx62 are expressed in the human heart, but are not transcribed in the mouse heart (rev. by Saez et al., 2003). For the purposes of this thesis, all connexins will be referred to as they are found in mice. Nonetheless, most vertebrate cells express some form, if not multiple forms, of the connexin protein family. The only known exceptions are erythrocytes, spermatozoa and skeletal muscle cells in their fully differentiated state. However, the progenitors of these three cell types do express gap junctions (Constantin et al., 1997; Manthey et al., 1999; Proulx et al., 1997; Rosendaal et al., 1994). Gap junctions are not functionally redundant (White, 2003) and depending upon their connexin makeup gap junctions can respond to a variety of regulatory stimuli.

The connexin protein structure shows four membrane-spanning α helices, connected by one cytosolic loop and two extracellular loops (fig. 5A). These extracellular loops contain three cysteine residues and conserved intervals, [C-X_6-C-X_3-C] and [C-X_5-C-X_5-C] for the first and

second loops, respectively. These residues facilitate the docking of two hemichannels together to form a gap junction structure (Kistler et al., 1988; rev. by Sohl and Willecke, 2004). A short amino acid sequence at the N-terminus comprises the N-terminal tail, and an amino acid sequence of variable length comprises the C-terminal tail. The N-terminal tail, the cytoplasmic loop and the C-terminal tail have all been shown as sites of post translational modification and are highly variable between connexin family members (White et al.; 1995).

Six connexin proteins form a hemichannel, otherwise known as a connexon. The location of this oligimerization is connexin dependent. For example, Cx32 appears to oligomerize either in the E.R. or between the E.R. and Golgi. Cx43 based connexons, on the other hand, assemble in the trans-Golgi network (rev. by Saez, 2003). These connexons are trafficked through lipid rafts and conjoin with connexons from neighboring cells (Schubert et al., 2002; Makowski et al., 1977). These connexons form a tight seal with one another in order to exclude the exchange of small molecules with the extracellular milieu (Unger et al., 1999). In this way, each cell contributes half of what comprises a gap junction. A connexon may be made from identical connexin proteins (homomeric) or different connexin proteins (heteromeric), and a gap junction may be made from identical connexons (homotypic) or different connexons (heterotypic). In this way, a gap junction may be homomeric/homotypic, homomeric/heterotypic, heteromeric/homotypic, or heteromeric/heterotypic, in order to increase the variety of regulatory control (rev. by Wei et al., 2004). However, not all connexins can form heterotypic and heteromeric gap junctions. The variety of gap junction types in which each connexin protein is able to participate remains unknown and is likely connexin and tissue specific (rev. by Saez et al., 2003). Lastly, a gap junction does not function in isolation, but exists as a component of what is referred to as a gap junction plaque (McNutt and Weinstein, 1970; Friend and Gilula, 1972; Rash et al., 1974; Ginzberg and Gilula, 1979). These plaques can be as dense as 10,000 gap junctions per μm^2, and can range in size from several nanometers to several microns in diameter (McNutt and Weinstein, 1970). Each plaque is made from multiple gap junction types and is formed in a cooperative, self-assembling process. Researchers have yet to identify any obligatory proteins other than connexins in gap junction plaques.

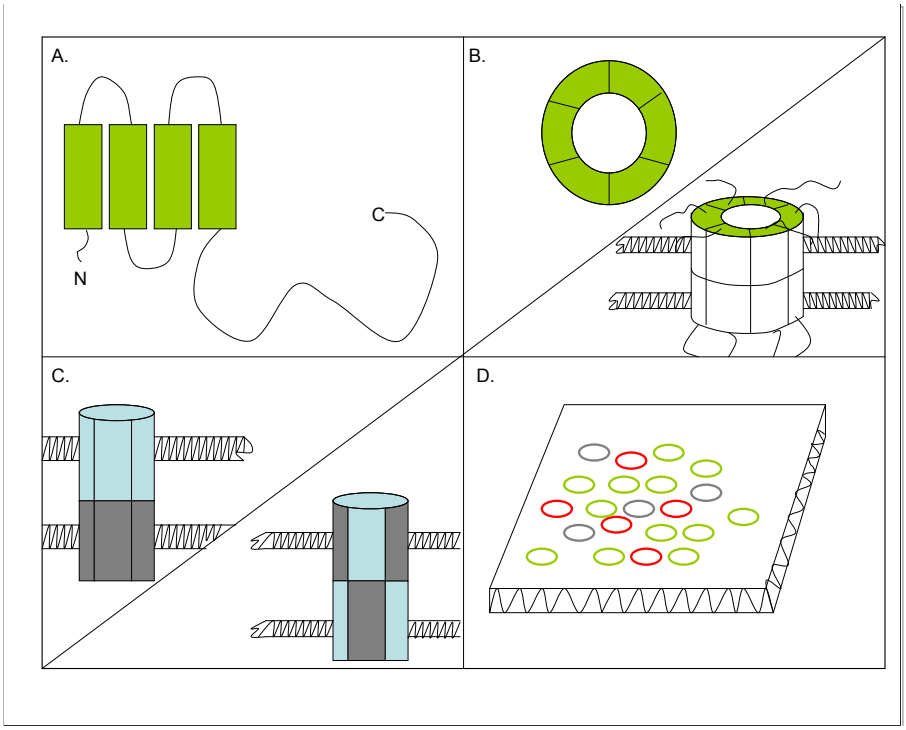


Figure 5 Structural Representations of a Connexin Protein, Gap Junction and Gap Junction Plaque

(A) The connexin protein is made from 4 α helices, two extracellular loops, one intracellular loop and an N-terminal and C-terminal tail. (B) 6 connexins form a connexon, and two connexons form a gap junction. (C) Shown here are heterotypic and heteromeric gap junctions. (D) Gap junctions are tightly packed together within gap junction plaques. A gap junction plaque is made from a multitude of connexin proteins.

Gap junctions function to allow the passive diffusion of small molecules, (approximately 1,000 Daltons or less) such as metabolites, water, ions and signaling molecules between cells by forming pores in the membranes of adjoining cells (Gilula et al., 1972; Kumar and Gilula, 1996; Alexander and Goldberg, 2003). Gap junctions coalesce in tightly packed regions of the plasma membrane, called gap junction plaques. In the brain, gap junction plaques function as electrical synapses and transmit an action potential from one neuron to another (rev. by Sohl et al., 2005). The majority of cells within the brain express gap junctions including glial cells, astrocytes and neurons (rev. by Nagy et al., 2004). These plaques hold adjacent cell membranes at a distance of approximately 3.5 nm and synchronize large neuronal modules at distinct frequencies (Hormuzdi et al., 2004; Gibson et al., 2005). The most abundant electrical synapse-forming connexins appear to be Cx36, Cx45 and Cx57 (Sohl et al., 2005).

1.5.2 Gap Junction Regulation

The regulation of gap junction cell-to-cell communication is critical for cell survival. Because these molecular pores indiscriminately allow small molecule diffusion, misregulated gap junctions can promote the propagation of cell death signaling. In fact, the passage of apoptotic signals such as calcium through gap junctions has been linked to neuronal cell death (Frantseva et al., 2002; Ripps, 2002), and perturbation of calcium homeostasis is implicated in SCA14 (Adachi et al.; 2008). When the internal cellular signaling for apoptosis is passed from an initial cell to a neighboring cell in a process referred to as the bystander effect, the normal physiological process of apoptosis is amplified beyond healthy limits. For this reason, gap junctions are regulated by a multitude of mechanisms.

From transcription to degradation, connexin proteins are intricately regulated. Initial investigations of the connexin gene structure demonstrated a somewhat simple organization. For many connexin genes, an untranslated 5'-exon is separated from a second exon that contains the entire connexin gene sequence (Willecke et al., 2002; Sohl and Willecke, 2004). Recent reports have shown that connexin genes are organized in at least four distinct ways. Some connexin genes contain multiple 5'-untranslated exons that are spliced either alternatively or in sequence with the encoding exon. In the case of Cx43, exon1a or exon1b is alternatively spliced to exon2 and respond to liver-specific and nervous system specific promoters, respectively. In the case of Cx45, three exons are spliced in sequence, but the final exon, exon3, still contains the entire coding sequence. In only a few cases are the coding regions found on multiple exons. The gene for Cx36 contains two exons that are spliced together to make the entire encoding sequence. The Cx57 gene contains 3 exons with the coding sequence on exon2 and 3. Salameh (2006) gives a complete review of connexin gene structure.

In most cases, the basal promoter is located within 300 bp from the transcriptional initiation site. This region contains binding sites for transcription factors such as Sp1/Sp3, AP-1, TATA box binding proteins, GATA4, Tbx5 as well as others (Chen et al., 1995; Sullivan et al., 1993; Yu et al., 1994). Transcription of connexin genes is further regulated by biochemical molecules such as cAMP, phorbol esters, and retinoids.

In addition to transcriptional regulation, many connexins are regulated on the translational level. For example, the internal ribosomal entry sites (IRES) for Gja1 and Gjb2 mRNAs are sufficiently potent to permit connexin translation under otherwise suppressive

conditions such as in density inhibited cells and in cap-dependent translation inhibition. Some studies have implicated microRNAs (miRNAs) as important negative regulators of translation. In the case of Cx43, two miRNAs, miR-206 and miR-1, bind to Cx43 mRNA during muscle differentiation and result in decreased levels of Cx43 expression (rev. by Saez et al., 2003).

Connexin proteins are synthesized as four transmembrane integral membrane proteins within the E.R. The endoplasmic reticulum (E.R.) functions as quality control for newly synthesized connexin proteins. When connexin proteins fail to fold properly, E.R.-associated degradation (ERAD) occurs by utilizing the ubiquitin/proteosomal system. Hemichannel oligomers are assembled within the E.R. Hemichannels are processed through the Golgi and travel on vesicles to the plasma membrane. These vesicles fuse with the membrane, making the hemichannel an intrinsic membrane component (rev. by Saez et al., 2003). Here, two hemichannels, each from a neighboring cell, dock together forming a gap junction. Once a dodecameric gap junction is formed, the two hemichannels do not separate under physiological conditions (Mazet et al., 1985; Dermietzel et al., 1991; Naus et al., 1993; Jordan et al., 2001). This gap junction is trafficked through lipid rafts where it is added to a gap junction plaque (Gaietta et al., 2002; Louf et al., 2002; Schubert et al., 2002). Small gap junction plaques can coalesce into larger plaques (Holm et al., 1999; Kistler et al., 1993).

The opening and closing of gap junction channels are highly regulated processes. This gating is responsive to transmembrane and transjunctional voltage, pH, Ca^{++} and phosphorylation state (Harris, 2001). A gap junction can be closed in a number of distinct ways. For example, gap junctions respond to voltage in one of two ways, fast gaiting or slow gaiting. In fast gaiting, the N-terminus functions as a voltage sensor and closes the gap junction channel to a sub conductance state, but the pore is only partially occluded (Weingart and Bukauskas, 1993; Moreno et al., 1994). On the other hand, slow gaiting appears to utilize the extracellular loop domains and completely closes the gap junction, leaving no residual activity (Trexler et al., 1996; Bukauskas and Verselis, 2004; Preacchia, 2004). This process is referred to as slow gating because it can take tens of milliseconds to complete. pH gaiting and phosphorylation gating utilize the "ball and chain" method for gap junction closure. In this model, the long C-terminal tail folds over the open pore in order to block diffusion through the pore (Lampe and Lau, 2004).

With the exception of Cx26, all connexin proteins are phosphorylated on the C-terminal tail by a broad array of kinase proteins, each of which responds to different stimuli. One of the

best studied is Cx43. Cx43 contains no less than 15 phosphorylation sites for at least six kinase enzymes. Phosphorylation by kinases like PKA and CK1 lead to increased gap junction communication, whereas phosphorylation by kinases like PKC, Src and MAPK lead to decreased gap junction communication. Each kinase has a specific phosphorylation site, and many kinases have multiple specific phosphorylation sites (Pahujaa et al., 2007). Phosphorylation patterns not only depend on the stimulus, but are also tissue dependent. For example, PKC phosphorylates Cx43 on S368 and S372, whereas MAPK phosphorylates Cx43 on S279 and S282. Moreover, in lens tissue PKCγ phosphorylates S368, whereas in the heart PKCε is responsible for the phosphorylation of this residue (rev. by Barnett et al., 2007). In retinal and lens tissues, PKCγ responds to oxidative stress by phosphorylating Cx43 and Cx50 and thus inhibiting gap junction activity (Lin et al., 2003; Lin et al., 2004; Lin et al., 2005).

This phosphorylation can not only lead to gap junction closure, but also to degradation. Gap junction proteins have relatively short half-lives of approximately 1-3 hours (Darrow et al., 1995). Gap junction degradation begins with internalization. Clathrin and actin filaments facilitate the endocytosis and trafficking of connexin containing vesicles destined for one of three fates. For Cx43, this internalization is stimulated by phosphorylation by a number of kinases including PKC, CK1 and ERK. Internalized gap junction vesicles can re-fuse with the plasma membrane resulting in a transient decrease in gap junction activity (rev. by Saez et al., 2003; rev. by Segratain et al., 2006). Vesicles that do not re-join the plasma membrane are degraded by either the lysosomal or the proteosomal system. In fact, both lysosomal and proteosomal degradation play a role in the Cx43 life cycle (Susil et al., 2000; Qin et al., 2003). In the case of Cx43, lysosomal degradation occurs on internalized gap junction vesicles, whereas proteosomal degradation occurs on phosphorylated, active Cx43 present in the plasma membrane. Treating cells with TPA has been shown to hyperphosphorylate Cx43, leading to ubiquitination, internalization and subsequent degradation by the proteosome (Leithe et al., 2004).

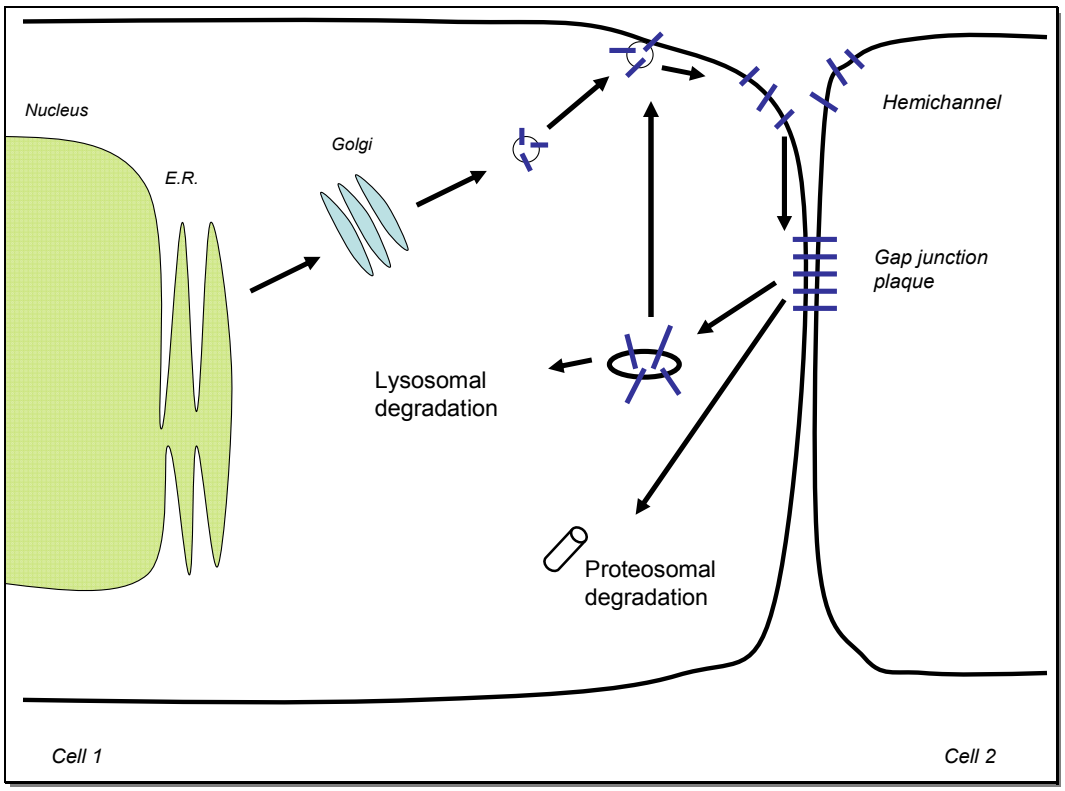


Figure 6 Gap Junction Life Cycle From Synthesis to Degradation
Connexin proteins are synthesized in the E.R. and assemble into hemichannels between the E.R. and trans-Golgi network. Connexons are trafficked to the plasma membrane where they bind connexons from a neighboring cell forming a gap junction. These gap junctions coalesce into gap junction plaques. Gap junction plaques are degraded by the lysosomal and proteosomal systems. Alternatively, internalized gap junctions can refuse with the membrane.

1.5.3 Connexin 57

While Cx43 has been studied at length, less is known about Cx57. Cx57 was originally thought to be a 57.115 kDa protein, but due to a previously unknown third exon, the last 25 amino acids described by Manthey et al. (1999) are not found as part of the protein. Instead, these 25 amino acids are replaced by 12 amino acids, making the total protein 55.64kDa (Hombach et al., 2004). This connexin still remains the largest of the connexin family. To avoid confusion, the name Cx57 is kept as the common nomenclature. Cx57 is expressed in the horizontal cells of the retina, thymic cells, embryonic kidney cells, ovarian cells and the Purkinje cells of the cerebellum. This connexin, like all connexins, responds to an array of regulatory

stimuli. Like most connexins, Cx57 responds to changes in voltage, pH and intracellular Ca^{++} levels. As previously stated, not all connexins are functionally redundant, and several differences between Cx43 and Cx57 have been shown. Palacios-Prado et al. (2009) demonstrated that Cx57 gap junctions allow the passive diffusion of positive and negative dyes up to 400 Da in size, indicating that Cx57 channels are size, but not charge selective. The permeability for Alexa fluor-350 through Cx57 gap junctions was shown to be approximately 90 fold smaller than the permeability through Cx43 channels. Cx57 single channel conductance is also smaller, but only approximately two fold (57 pS as compared to 110 pS). Cx57 is also remarkably sensitive to changes in pH. Increasing the pH from 7.2 to 7.8 increased the gap junctional conductions by about 100 times. An additional 12 histidine residues found in the C-terminus of Cx57 as compared to Cx43 may be involved in its increased sensitivity to changes in pH. Lastly, decreases in calcium below 0.3 mM was shown to open Cx57 gap junctions. Trypsinizing cell cultures under low calcium concentrations was shown to induce swelling and necrosis. When calcium concentrations were increased during the trypsinization process to 0.5mM swelling and necrosis were reduced. The C-terminal tail of Cx57 has the sequence 442-*QRHSDSGSSRSLNSS*-456. Based on serine density and arginine proximity, this sequence is a potential substrate for PKCγ.

1.6 Biochemical Mechanisms of SCA14, Mutant PKCγ and Cx57

Since the discovery of SCA14 in 2003, a number of scientific researchers have worked to explain the biomechanical etiology of this disease. The resulting data from this work is varied, and at times seemingly contradictory. Investigations of enzymatic activity have shown the most contradictory results. Loss of function, gain of function, haploinsufficiency, and a dominant negative effect have all been reported as effects of SCA14 mutations (Seki et al. 2005, Doran et al. 2008, Verbeek et al. 2005, Zhang et al. 2009). This is likely due in part to studies being done in differing cell lines as well as on differing mutations. In HT22 cells transfected with H101Y, S119P and G128D type PKCγ as well as in the H101Y transgenic mouse model, a dominant negative effect was shown (Lin and Takemoto, 2007; Zhang et al., 2009). Within this mouse model, Cx57 and PKCγ showed similar enrichment in the dendritic arbor and soma of Purkinje cells. Protein aggregation has also been reported to be involved in SCA14. Large protein

aggregation was incurred in various cells transfected with SCA14 type PKCγ (Seki et al., 2007; Lin and Takemoto, 2007). In HT22 cells, the dominant negative effect of SCA14-type PKCγ showed an inverse correlation with gap junction activity (Lin and Takemoto, 2007). These mutant kinases failed to release from 14-3-3ε and were not phosphorylated on the activation loop (Nguyen et al., 2004). CHO cells transfected with SCA14 mutants were shown to be more sensitive to oxidative stress-induced DNA damage and demonstrated perturbations of the proteosomal pathway (Hirohide et al., 2009; Seki et al., 2007). Within the cerebellum, PKCγ is a regulator for nuclear import of aprataxin (APTX). APTX is a DNA repair protein and is also implicated in autosomal recessive ataxia (Asai et al., 2009). This may lead to E.R. stress and caspase activity (Seki et al., 2007; Lin et al., 2007). However, protein aggregation is not observed in primary cultures of Purkinje cells. These primary cells from E18 embryos demonstrated maldevelopment that was shown to be independent of protein aggregation (Seki et al., 2009). Finally, SCA14-type PKCγ has been shown to negatively affect Ca^{++} homeostasis and gap junction signaling. The dominant negative effect of SCA14-type mutants (H101Y, S119P, and G128D) in HT22 cells prevented normal gap junction regulation by PKCγ and increased the levels of E.R. stress markers and apoptotic markers (Lin et al., 2007). Cx57 is an abundant connexin isoform in Purkinje cells. The purpose of this thesis was to investigate if Cx57 is regulated by PKCγ.

1.7 Goals of This Study

In this study, immortalized mouse hippocampal cells were used to demonstrate that PKCγ regulates Cx57 in response to oxidative stress and phorbol ester stimulation, and the presence of SCA14 PKCγ, transfected cells have decreased viability. Confocal microscopic analysis showed that activators of PKC and of PKCγ caused internalization of Cx57 gap junction plaques. Extended activation of PKC and PKCγ resulted in post-translational modification of Cx57. Immunoprecipitation followed by western blotting demonstrated that this internalization correlated with increases in serine but not threonine phosphorylation. Finally, these activators induced cell death in cells transfected with SCA14-type PKCγ, but did not induce decreased viability in untransfected cells or in cells transfected with wild type PKCγ.

CHAPTER 2 - Materials and Methods

2.1 Cell Culture

Murine hippocampal HT22 cells were cultured in high glucose (4.5 g/L) Dulbecco's modified eagle's medium (DMEM) pH 7.4, supplemented with 10% fetal bovine serum (FBS), 0.05 U/ml penicillin, 50 μg/ml gentamicin, and 50 μg/ml streptomycin. These cells were incubated at 37˚C in 95% air and 5% CO_2.

2.2 Reagents

HT22 cells as well as SCA14 plasmid were graciously provided by Dr. Dingo Lin. DMEM, trypsin-EDTA, gentamicin and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). BSA was purchased from Fisher Scientific (Hampton, NH). Protease and phosphatase inhibitor cocktails were purchased Sigma-Aldrich (St. Louis, MO). Super-Signal West Pico Substrate kit as well as anti-mouse and anti-chicken antisera conjugated to horseradish peroxidase (HRP) were purchased from Pierce (Rockford, IL). Goat anti-chicken antiserum conjugated to Alexa Fluor-568 was purchased from Molecular Probes (Eugene, OR). Electrophoresis equipment, reagents and protein marker were purchased from BioRad (Hercules, CA). Whatman Optitran BA-S-85 nitrocellulose 0.45 μm membrane was purchased from MidSci (Lt. Louis, MO). Chicken anti-Cx57 antiserum was purchased from Diatheva (Italy). Protein A/G agarose beads were purchased from Santa Cruz Biotech (Santa Cruz, CA). Mouse anti-phosphoserine and anti-phosphothreonine antisera were purchased from CalBiochem (EMD Biosciences, #539165). Cell Titer-Blue cell viability assay kit was purchased from Promega (Madison, WI).

2.3 Confocal Analyses

Glass coverslips were boiled in water for 10 minutes then sterilized by ethanol and flame and placed in 6-well plates. HT22 cells were passed into these plates at a dilution factor of 1:8 to 1:10. Cells were grown to a confluence between 70-85% and treated with 100 μM H_2O_2 for 30 minutes, 100 nM TPA for 30 minutes or 24 hour hypoxia (1% O_2, 5% CO_2). Cells were fixed with 4% paraformaldahyde in phosphate buffered saline (PBS) for 10 minutes. Cells were then

quenched with cold 50 mM glycine in PBS 5 times for 5 minutes. Cells were permeabilized with 0.05% Triton X100 in PBS for 30 minutes at room temperature and then rinsed in 3% bovine serum albumin (BSA) diluted in PBS 3 times for 5 minutes. Blocking was done in 3% BSA in PBS overnight at 4˚C. Cell preparations were not kept longer than 3 days. Cell preparations were treated with chicken anti-Cx57 antiserum (4 μg/ml in 3% BSA solution) and incubated at 4˚C overnight. Cells were then rinsed 3 times for 5 minutes in cold PBS and treated with 3 μg/ml goat anti-chicken antiserum conjugated to Alexa fluor-568 for 90 minutes in the dark at room temperature. Cell coverslips were again rinsed in cold PBS 3 times for 5 minutes, a small drop of anti-fade solution was placed on a microscope slide and the coverslip (cells facing down) was applied to the slide. Gain measurements were taken for each set of experiments and were consistently between 6.4 and 6.7 indicating a low degree of photo-bleaching and a high degree of binding from the secondary antibody. Confocal images were taken on a Nikon Eclipse TE2000-U confocal microscope. A slide for each treatment was made and 10 images per slide were taken. This experiment was repeated three times. Images were then analyzed using ImageJ software. For this analysis, the threshold level for all pictures was kept equal. Within each picture 10-15 cells were counted by eye. Using imageJ software, membrane plaques were delineated from internal Cx57. Both internalized and membrane associated Cx57 plaques were counted. The threshold was set at 77/155 and the range was set from 0.01 μm-infinity. To be considered a functional gap junction plaque, two or more punctate dots had to be within 5 μm and directly between two cellular membranes.

2.4 Time Course and Western Blotting

HT22 cells were grown to approximately 80% confluence in 75cm^3 flasks. Media was replaced with 100 μM H_2O_2 or 200 nM TPA in DMEM supplemented with 10% FBS. Cells were scrape harvested in cold PBS and centrifuged at 4˚C. Cells were then lysed using 300 μl of cold RIPA (RadioImmunoPrecipitation Assay) buffer with protease and phosphatase inhibitors (diluted 1:100). Lysis was done by sonication three times for 3 repetitions at 1 second intervals with 10 minute lapses between sonications. Cell lysates were centrifuged at 4˚C at 15,000 rmp for 15 minutes. From here, the Bradford assay was used to equalize total protein concentration between cell lysates. 300 μl of 5X gel loading buffer containing 500mM DTT (DL-dithiothreitol) was applied and tubes were boiled for 7 minutes. Electrophoresis was done on these lysates using an

8% acrylamide gel, run at 150V for 45-55 minutes. Gel protein was transferred to a nitrocellulose membrane, run at 100V for 1hr. Blots were rinsed in Towbin-T buffer (1X Towbin buffer contained 0.5% Tween 20, 0.05 M NaCl, 0.01M Tris, pH 7.5) then blocked in 5% powdered milk in Towbin buffer (without Tween-20) overnight. Blots were then washed 6 times over 45 minutes with Towbin-T buffer and primary antibody was applied. Chicken anti-Cx57 antiserum was diluted in 5% powdered milk in Towbin-T buffer, added to the blot, and allowed to incubate overnight at 4°C. Blots were washed as described above and incubated in secondary antibody for 90 minutes at room temperature. Mouse anti-chicken-HRP solution (horseradish peroxidase conjugated to mouse anti-chicken monoclonal antibody) was prepared by diluting concentrated antibody 1:15,000 in 5% powdered milk in Towbin-T. Blots were washed again and incubated in Super-Signal West Pico Substrate for 8 minutes. Blots were then developed on autoradiography film and analyzed using UN-SCAN-IT software (Silk Scientific. Orem, UT)

2.5 Phosphorylation Profile Analysis

HT22 cells were grown, harvested, pelleted, lysed and the protein concentration was equalized as described in section 2.4. Cell lysates were treated with 0.8 µg of chicken anti-Cx57 antibody and placed on a rocker at 4˚C for 6 hours. To this 50 µl of protein A/G agarose bead slurry was added and tubes were incubated at 4˚C for 10-12 hours. The beads were then washed 3 times in PBS buffer for 5 minutes and centrifuged at 2,000 rpm, 4˚C for 1 minute each time. 80 µl of 2X loading buffer was added and tubes were boiled for 7 minutes. Electrophoresis, nitrocellulose transfer and blotting for Cx57 was done as described in sec. 2.4. Blotting for phosphoserine-Cx57 and phosphothreonine-Cx57 required 0.3 µg/ml mouse anti-phosphoserine and anti-phosphothreonine antisera diluted in 5% BSA in Towbin-T. These blots were washed and treated with rabbit anti-mouse antiserum diluted in 5% BSA in Towbin-T. Substrate incubation, development and analysis were performed as described in sec 2.4 of this thesis.

2.6 Cell Viability Assay

Transfections were performed according to the manufacturer's protocol. Briefly, HT22 cells were grown to 60% confluency in 96-well plates in 100 µl of antibiotic free DMEM supplemented with 10% FBS. 70 µl of Lipofectamine was added to 300µl of Opti-MEM media without serum. 5 µg of plasmid was added to 300µl of Opti-MEM media and incubated for 15

minutes. Pre-complexed plasmid was combined with Lipofectamine dilution and allowed to incubate for 25 minutes at room temperature. 25 µl of this complex was added to each well. 96-well plates were incubated for 4 hours, at which time growth media was replaced and cells were allowed to recover for 36 hours. Cells were then treated with 100 µM H_2O_2 for 1 hour, 100 nM TPA for one hour or hypoxia (1% O_2, 5% CO_2) for 24 hours. Cell viability was measured using the Cell Titer-Blue Cell Viability Assay in accordance with the manufacturer's instructions. Briefly, 20 µl of resazurin reagent solution was added to 100 µl of media in each well and incubated for 3 hours. Fluorescence recordings were taken (560/590). 5 wells per transfection per treatment were recorded. This experiment was repeated three times.

2.7Statistical Analysis

All analyses represent at least triplicate experiments. The Student's t-test was used to investigate statistical significance, and a value of $\rho \leq 0.05$ was considered statistically significant.

CHAPTER 3 - Results

3.1 Confocal Analysis and Cx57 Plaque Count

Internalization is one of the hallmarks of connexin regulation. In this study, HT22 cells were treated with activators of PKC (TPA), PKCγ (H_2O_2), and PKCε (hypoxia) (fig. 7-11). Upon PKC and PKCγ stimulation for 30 minutes (200 nm TPA and 100 μM H_2O_2, respectively), Cx57 gap junction plaques at the membrane decreased by a significant amount (comparing fig. 7 to 8 and 9, quantified in fig. 11). This coincided with an increase of Cx57 found in the cytoplasm. Phorbol esters and oxidative stress not only activate PKC and PKCγ, respectively, they are also a source of cellular stress. Because connexin regulation has been demonstrated in response to a wide variety of cellular stress, hypoxic confocal images provide an appropriate control in order to demonstrate that this regulation is not a general mechanism of cellular stress. Moreover, hypoxia has been shown to induce another isoform of PKC, namely PKCε. Hypoxia was shown to induce Cx57 internalization significantly, but not to the extent of TPA or oxidative stress (fig. 10, quantified in fig. 11). These images and their corresponding quantification indicate that Cx57 plaque internalization in response to PKC activation is largely PKCγ specific.

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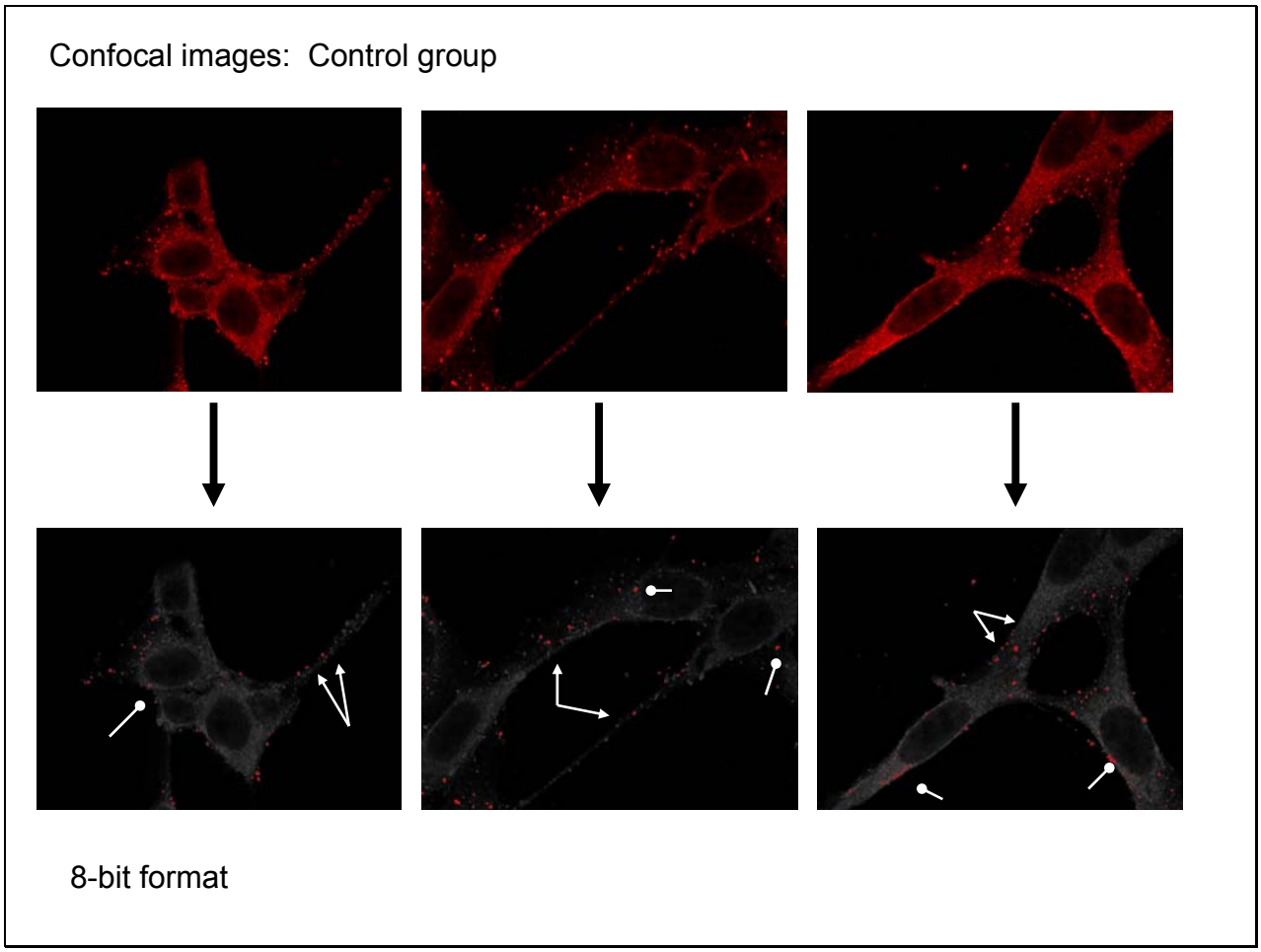


Figure 7 Confocal Images of Untreated HT22 Cells

Three representative images are shown here (above) accompanied by their 8 bit, threshold adjusted copies. Cx57 plaques are largely found on the membrane. Pointed arrows indicate plaques found at the membrane. Circular arrows indicate internalized Cx57.

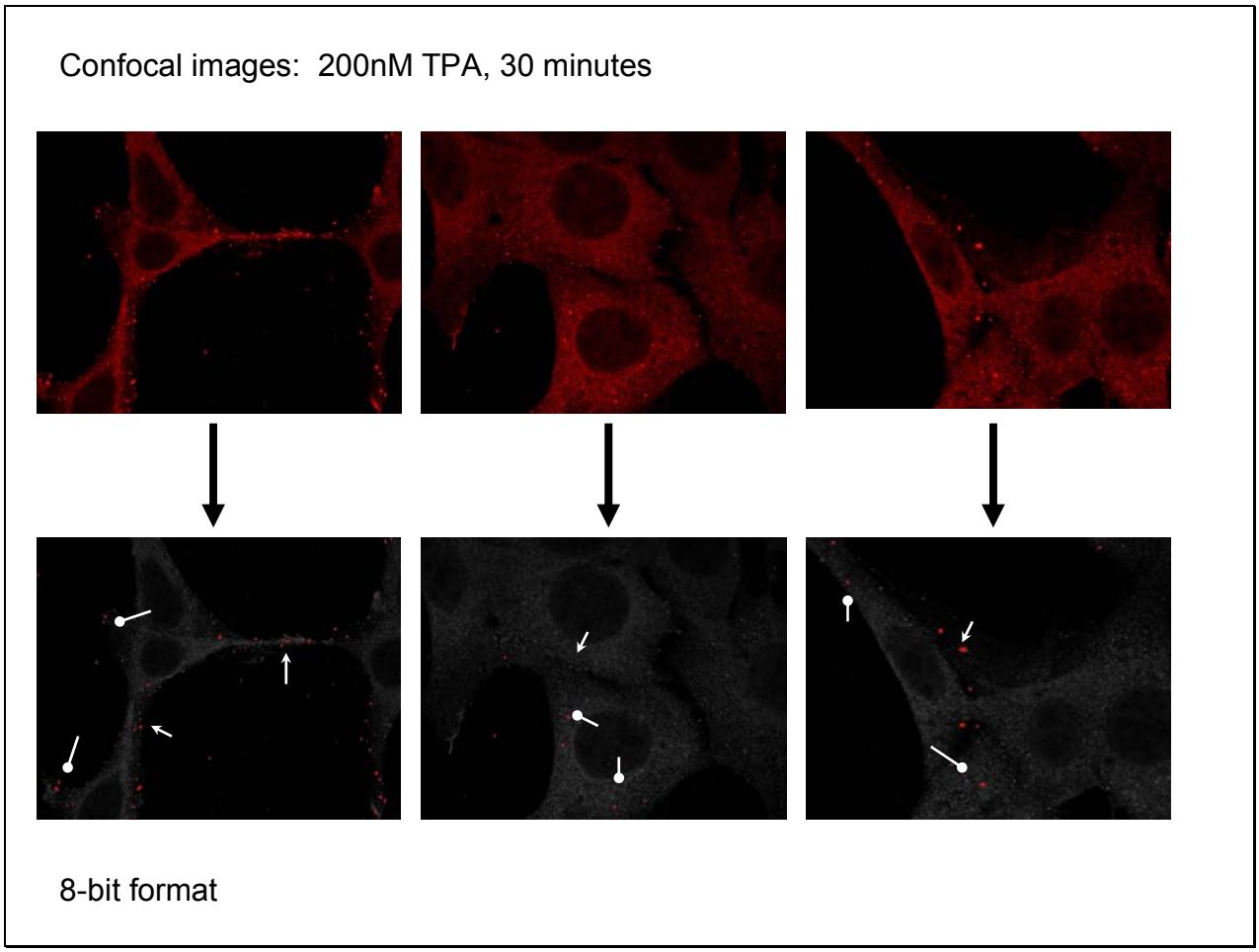


Figure 8 Confocal Images of TPA Treated HT22 Cells

HT22 cells treated with 200 nM TPA for 30 minutes displayed a decrease in Cx57 plaques and an increase in internalized Cx57. Shown here are three representative confocal images (above) and their threshold adjusted 8 bit copy (below). Pointed arrows indicate membrane-associated plaques. Circular arrows indicate cytosolic Cx57.

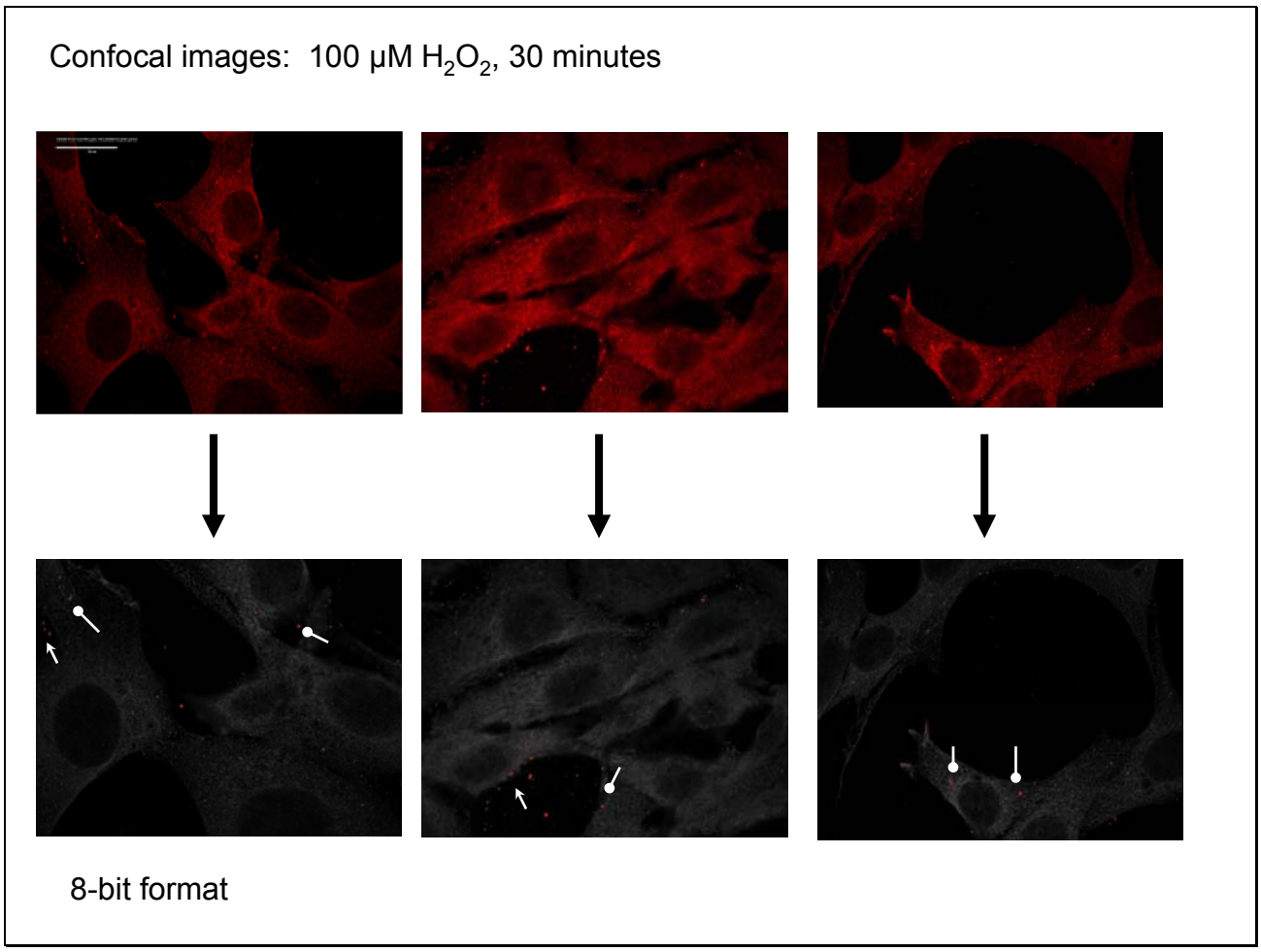


Figure 9 Confocal Images of H_2O_2 Treated HT22 Cells

HT22 cells treated with 100 μM H_2O_2 for 30 minutes display similar Cx57 plaque patterns to TPA treated HT22 cells. Most Cx57 plaques are internalized, whereas only a few are found on the membrane.

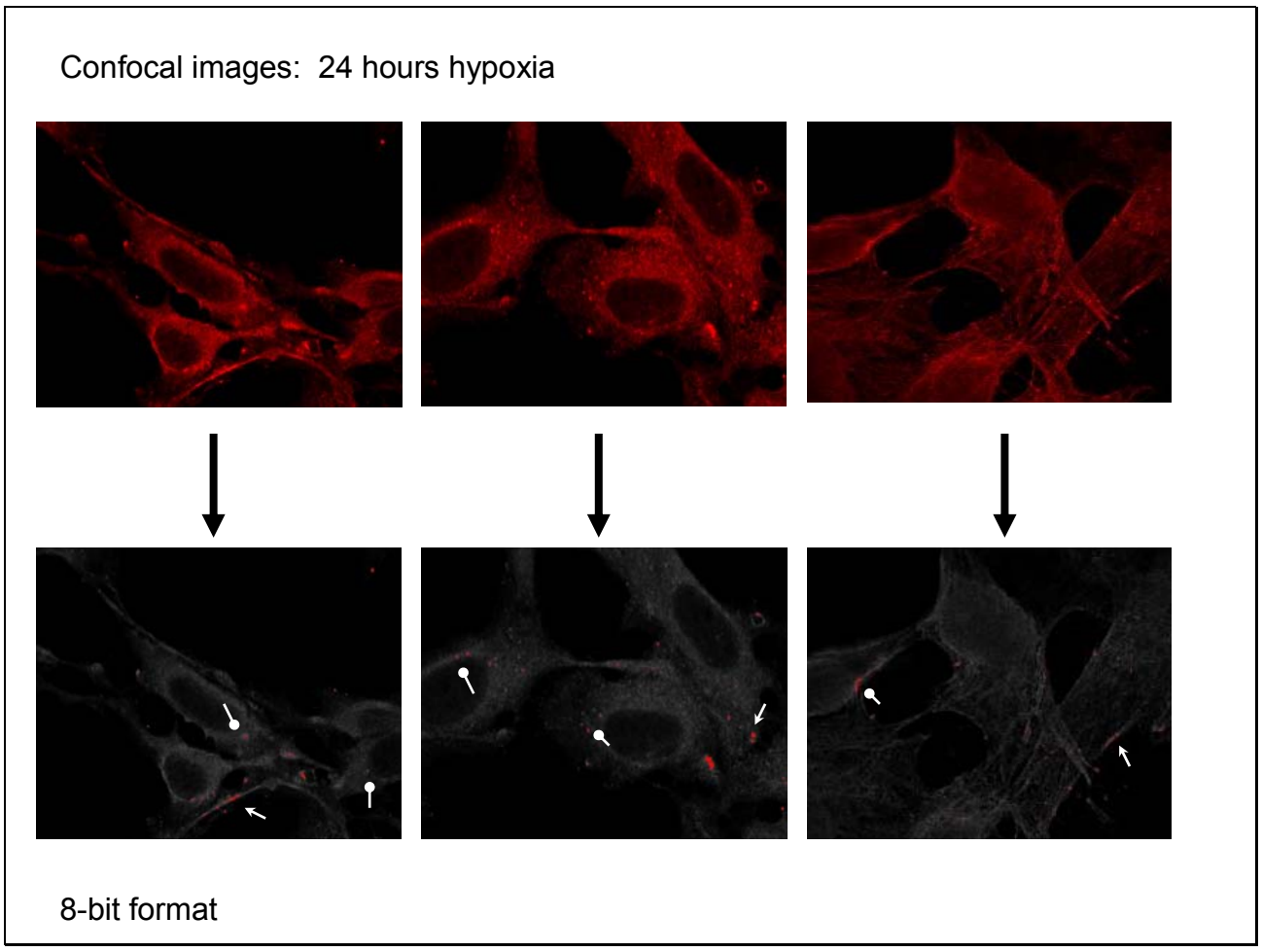


Figure 10 Confocal Images of Hypoxia treated HT22 Cells
HT22 Cells treated with 24 hours of hypoxia show a decreased number of Cx57-gap junction plaques at the membrane and a slightly increased number of internal Cx57-plaques. These plaques are larger in number than those treated with oxidative stress or TPA.

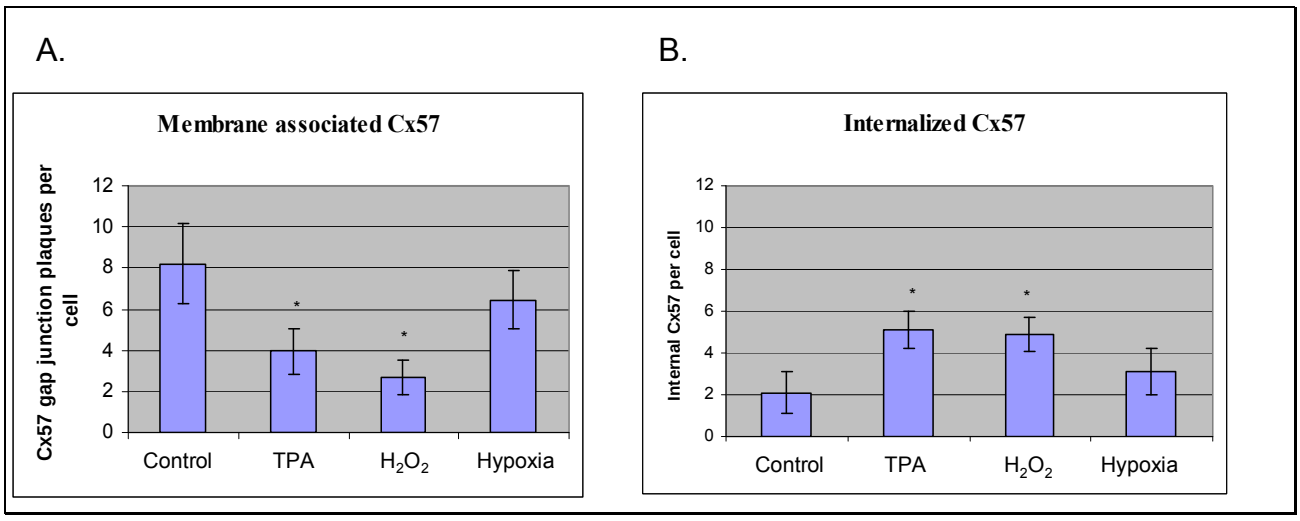


Figure 11 Quantification of Confocal Images

Treatment with 100 µM H_2O_2 or 200 nM TPA for 30 minutes significantly decreased the amount of membrane-associated Cx57 plaques and increased the amount of cytoplasmic Cx57. Hypoxia had a more limited effect. Hypoxia did not significantly decrease the amount of Cx57 plaques or significantly increase internalized Cx57. Shown here are the numbers of plaques or internal Cx57 per cell. Error bars represent +/- one standard deviation. (*) indicates statistical significance of $\rho \leq 0.05$.

3.2 PKC and PKCγ Induce Post Translational Modification of Cx57

As discussed in the introduction, a number of connexin proteins are regulated by PKCγ. The phosphorylation of these membrane proteins leads to internalization and may lead to degradation or alternatively, plaque reformation. The previous study (figures 7-11) indicated that PKC, PKCγ, and PKCε activation caused the internalization of Cx57 gap junction plaques. In order to determine if post-translational modifications such as phosphorylation and subsequent degradation of Cx57 are caused by PKCγ activity, HT22 cells were treated with 200 nM TPA or 100 μM H_2O_2 for 0-60 minutes. Whole cell lysates were western blotted for Cx57 and α-tubulin. Beginning at approximately 30 minutes after TPA or H_2O_2 treatments, a progressive increase in the molecular weight of Cx57 was demonstrated. TPA treatment significantly decreased the amount of Cx57 after 1 hour, but H_2O_2 treatment did not. This suggests that Cx57 degradation requires the activation of not only by PKCγ but other PKC isoforms as well.

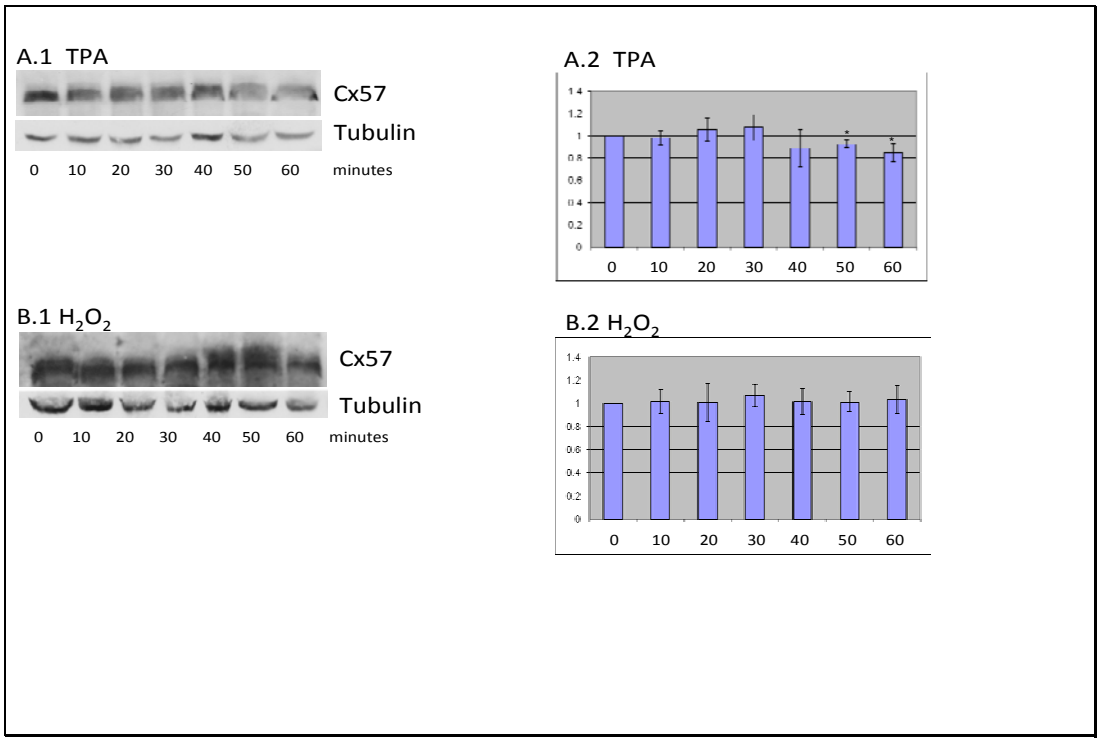


Figure 12 TPA and H_2O_2 Time Course

HT22 cells were treated with 200 nM TPA or 100 μM H_2O_2 for up to one hour. Cell lysates were harvested at 10 minute intervals and western blotted for Cx57 and α-tubulin. This experiment was repeated three times and densitometrically scanned. The ratio of Cx57 to α-tubulin at 0 minutes was normalized to one. At approximately 30 minutes, Cx57 bands

began to show an increase in molecular weight. Densitometric analysis showed that 200 nM TPA treatement caused the down regulation of Cx57, whereas 100 μM H_2O_2 treatment did not down regulate Cx57 within a one hour time frame. (*) indicates statistical significance of $\rho \leq 0.05$.

3.3 Phosphorylation Profile

Connexin phosphorylation is a common mechanism for gap junction regulation. The previous experiment (figure 12) suggested that the activation of PKC and PKCγ may induce post-translational modification of Cx57. Because a number of connexin isoforms are phosphorylated by PKC and specifically PKCγ, phosphorylation was the suspected type of post-translational modification. In order to determine if Cx57 is phosphorylated in response to oxidative stress, HT22 cells were treated with 200 nM TPA, 100 μM H_2O_2, (30minutes) or hypoxia (24 hours). Serine phosphorylation in response to both PKC and PKCγ activation was demonstrated. In the case of threonine phosphorylation, PKC and PKCγ activation did not cause a significant increase, although hypoxia treatment induced a significant decrease of phosphothreonine-Cx57. This suggests that PKCγ is the main isoform responsible for Cx57 phosphorylation.

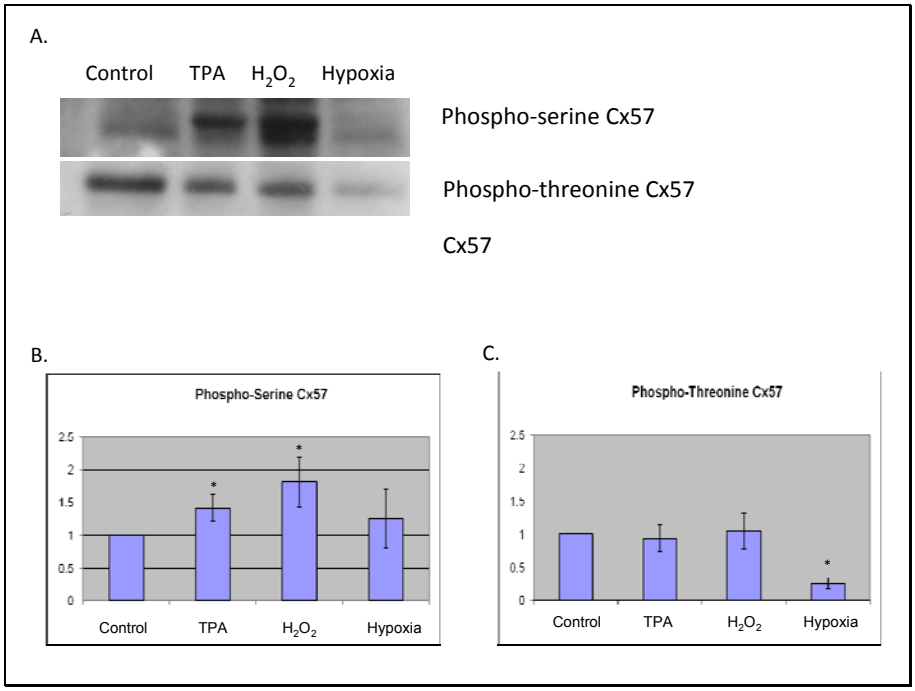


Figure 13 Phosphorylation Profile

HT22 cells were treated with 200 nM TPA, 100 µM H_2O_2, (30 minutes) or hypoxia (1% O_2, 5% CO_2, 24 hours). Cx57 was immunoprecipitated and western blotted with anti-phosphoserine, anti-phosphothreonine and anti-Cx57 antisera. This experiment was repeated three times and densitometrically scanned. The ratio of phospho-Cx57 (either serine or threonine) to total Cx57 was normalized to a value of one. The activation of PKC and PKCγ caused a significant increase in the level of serine phosphorylation on Cx57, but did not significantly alter phospho-threonine levels. Interestingly, PKCε activation led to a decrease in phosphorylated threonine residues on Cx57. (*) indicates statistical significance of $\rho \leq 0.05$.

3.4 Cell Viability

A multitude of scientific research has covered the nature of SCA14-associated cellular stress. These mutations have been shown to induce E.R. stress, calcium misregulation, proteosomal inhibition, oxidative stress sensitivity and increases in apoptotic markers. In order to gain a broader view on the effects of SCA14 transfection, a cell viability assay was preformed. In this experiment, HT22 cells were transfected with wild type, H101Y, S119P and G128D constructs of PKCγ. These cells were then treated with activators for PKC (TPA), PKCγ (H_2O_2) and PKCε (hypoxia). Using a resazurin based assay, cell viability was measured (fig. 14). Each treatment was normalized to the wild type. This experiment showed that HT22 cells, transiently transfected with SCA14-type PKCγ, are more sensitive to TPA and H_2O_2 (fig. 14B and 14C) than those left untreated or those treated with 24 hour hypoxia (fig. 14A and 14D).

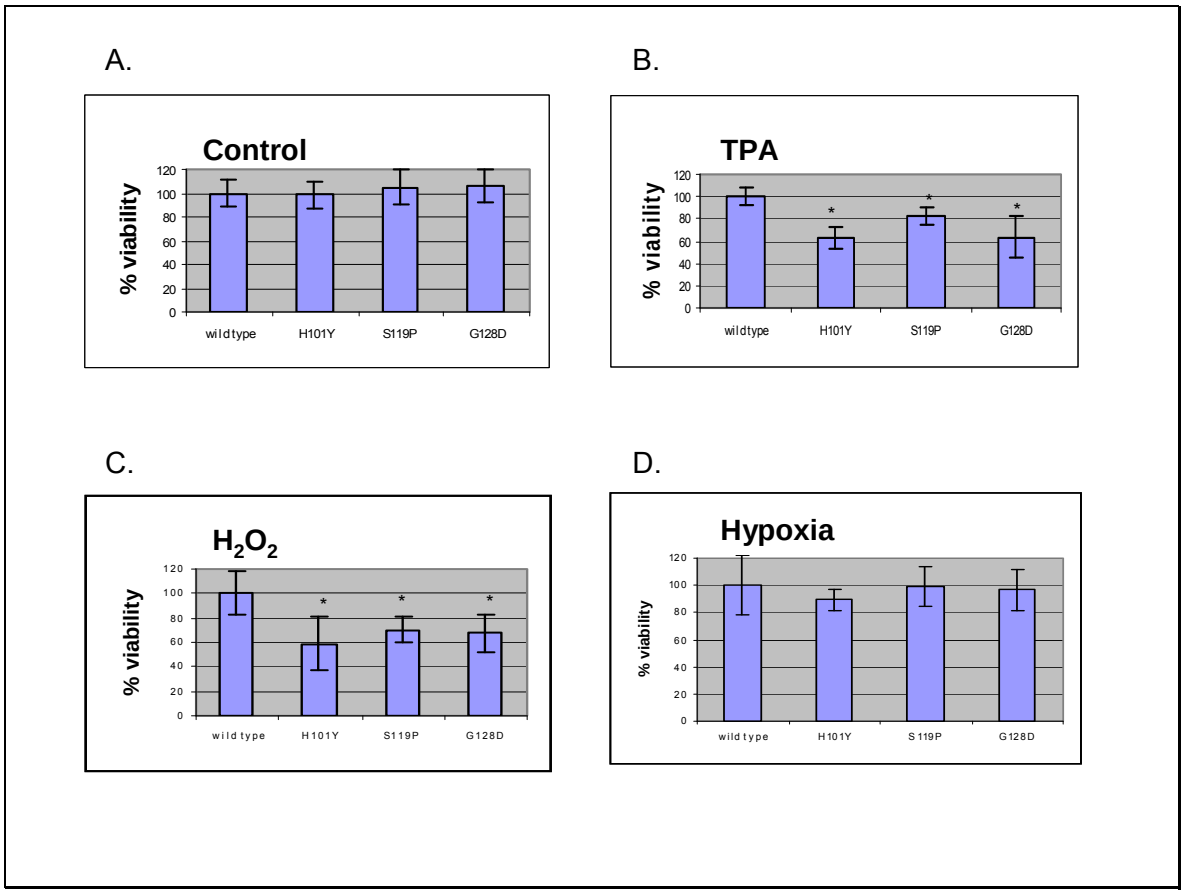
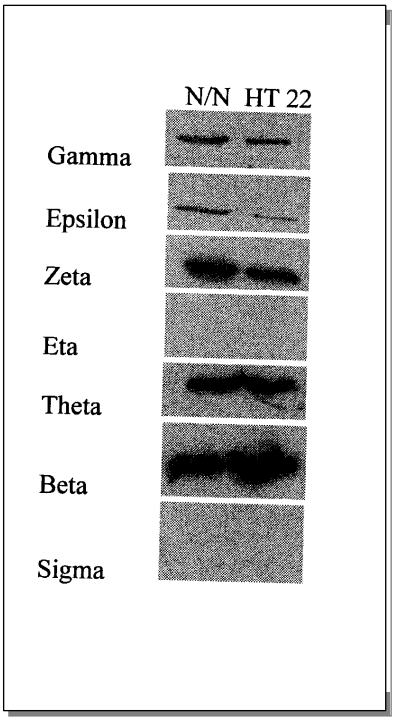


Figure 14 Cell viability

HT22 cells were transiently transfected with SCA14-type PKCγ and treated with 200 nM TPA (1 hr), 100 μM H_2O_2 (1 hr) or hypoxia (24 hr). A cell viability assay demonstrated the transfection with SCA14-type PKCγ increased cellular sensitivity to TPA and H_2O_2 in HT22 cells. (*) indicates statistical significance of $\rho \leq 0.05$.

CHAPTER 4 - Discussion

In this thesis, I have provided evidence that Cx57 is regulated by PKCγ. As stated in the introduction, several reports have suggested mechanisms for the molecular pathology of SCA14 including loss of function, gain of function and cytotoxicity. These reports implicate perturbations in calcium homeostasis, induction of E.R. stress, gap junction misregulation and sensitivity to oxidative stress. Only a few of these reports have suggested ataxia-related or cerebellum specific PKCγ substrates that may be specifically misregulated targets in SCA14. While many connexins have been shown to be substrates for PKCγ, few are neuronal specific. Here, I have shown that general activators of PKC as well as PKCγ specific activators cause the internalization of Cx57 gap junction plaques. This internalization is largely thought to be PKCγ specific based on the large degree of internalization induced by hydrogen peroxide and the lack of internalization in response to PKCε stimulation. Furthermore, extended activation of PKC and PKCγ induced apparent post-translational modification. However, because Cx57 down regulation was caused by TPA and not H_2O_2 within a one hour time span, it is likely that other isoforms of PKC have a regulatory role in the Cx57 life cycle. Researcher in the Takemoto lab have thoroughly characterized the HT22 cell line and have demonstrated that these cells express several PKC isoforms including PKCγ, PKCε, PKCζ, PKCθ and PKCβ, all of which are activated in the presence of TPA.



The dominant role of PKCγ among PKC isoforms as a regulator of Cx57 was further shown by analyses of the phosphorylation of Cx57 in response to PKC and PKCγ activators. Phosphorylation on serine residues was demonstrated in response to the PKC and PKCγ specific activator, whereas the PKCε specific activator, hypoxia, did not significantly increase serine phosphorylation. This treatment, however, caused a significant decrease in the level of threonine and serine phosphorylation. It is possible that the activation of PKCε or other hypoxia-activated enzymes leads to the activation of phosphatase enzymes that target to PKCγ substrates.

Likewise, PKCγ and PKCε could compete for the same phosphorylation sites on Cx57. If this is the case, it would contribute to explaining why H_2O_2 demonstrates a more dramatic effect on Cx57 internalization and serine phosphorylation. In the H101Y transgenic animal, the expression patterns for PKCγ and Cx57 in the cerebellum closely correlate. When these tissues were treated with H_2O_2, serine phosphorylation of Cx57 was not demonstrated. In fact, the level of serine phosphorylation on Cx57 in wild type tissue was larger than that found in the H_2O_2 treated, transgenic cerebellar tissue (Zhang et al., 2009). These observations further suggest that PKCγ targets Cx57.

PKCγ activity was shown to be critical for cell viability, because the dominant negative effect of SCA14-type PKCγ increased sensitivity to TPA and oxidative stress and decreased overall viability. This data aligns well with studies on protein aggregation, PERK activation and caspase activation done by other members of the Takemoto lab (Lin and Takemoto, 2007; Zhang et al., 2009). This indicates that Cx57 gap junction plaques are negatively regulated in response to oxidative stress in order to control apoptotic signaling. This pathway is interrupted by the expression of SCA14 mutants (Lin and Takemoto, 2007).

It is not the opinion of this author that Cx57 is the sole misregulated substrate of SCA14-type PKCγ. In fact, due to the genetic heterogeneity of this disease, it is likely that both loss-of-function and gain-of-function effects could be demonstrated by various mutant forms of PKCγ, impying that not only could normal physiological substrate targeting be prevented, mutant PKCγ may also target inappropriate substrates. Currently, the complete pathomechanism for SCA14 remains unclear. Studies on kinase activity, protein aggregation, E.R. stress and Purkinge cell development vary between cell lines and mutant identities, and for this reason identifying the normal physiological substrates of PKCγ is necessary for an overall understanding of this disease. While researchers have determined a number of PKCγ substrates, few have identified ataxia- or cerebellum-specific ones. Further research is required to synthesize the varied experimental evidence into an overall explanation of this disease.

The research done in this thesis invites the question: How much does the "bystander effect" contribute to overall disease etiology. This question will remain elusive until direct and specific gap junction inhibitors are available. Furthermore, the individual contributions of each connexin isoform to this disease are unknown. Finally, the complete connexin profile for Purkinje cells remains uninvestigated.

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