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Post-translational modifications in connexins and pannexins

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Abstract

Post-translational modifications are a common cellular process that is used by cells to ensure a particular protein function. This can happen in a variety of ways, for example from the addition of phosphates or sugar residues to a particular amino acid ensuring proper protein life cycle and function. In this review, we assess evidence for ubiquitination, glycosylation, phosphorylation, S-nitrosylation as well as other modifications on connexins and pannexin proteins. Based on the literature, we find that post-translational modifications are an important component of connexin and pannexin regulation.

Keywords

post-translational modifications; connexins; pannexin

Introduction

Post-translational modifications are a common method by which proteins can be modulated by intrinsic or extrinsic factors to potentiate or initiate a specific function. It is now well accepted that these modifications are a key way in which a protein can become useful in the context of the cellular physiology. This is not different when it comes to the connexin and pannexin family of proteins, where a fairly broad range of modifications has now been described that can alter the function of these proteins, from the dramatic (e.g., opening or closing of the channel/gap junction/hemichannel) to the subtle (e.g., insertion into specialized lipid-rafts).

Connexin and pannexin proteins are transmembrane proteins that allow for the passive diffusion of signaling molecules through their pores. Connexins are the key components to gap junctions linking the cytoplasm of two opposing cells allowing for rapid electrical or chemical integration among cells in a tissue. These proteins, when composed as a gap junction, allow for several functions, including electrical coordination of cardiac myocytes or ciliary beat frequency between tracheal epithelium, with several mutations in connexins associated with disease states (Boitano & Evans, 2000; Johnson & Koval, 2009; Kelsell, Di & Houseman, 2001; Lai et al., 2006; Palatinus & Gourdie, 2007). Undocked connexin hemichannels have also been hypothesized to play a more paracrine role for cellular communication in more pathological states (De Vuyst et al., 2007; Pearson et al., 2005).

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While membrane topology is similar to connexins, pannexins share no sequence homologies with connexins and are a more recently identified class of transmembrane proteins (Panchin et al., 2000). Pannexins can be found in three different isoforms (Pannx1, Pannx2 and Pannx3) encoded by three different genes with Pannx1 and Pannx3 sharing more similarities to each other than to Pannx2 (Penuela et al., 2007). In the past decade, a growing body of literature has revealed that pannexins play multiple roles as they have been shown to release ATP, participate in calcium wave propagation and are likely a component to the inflammasome (Dando & Roper, 2009; Locovei, Bao & Dahl, 2006; Silverman et al., 2009; Sridharan et al., 2010). Thus, both connexins and pannexins are critical for cells to coordinate direct and paracrine communication. In this review, we aim to bring together data focusing on the roles for glycosylation, phosphorylation, S-nitrosylation, ubiquitination, and other post-translational modifications in regulating the functions of both connexin and pannexin proteins.

Glycosylation

Glycosylation is a form of post-translational modification which consists of the enzymatic addition of glycans to form glycosylated proteins. The enzyme glycosyltransferase catalyzes the attachment of glycans to a nitrogen of an asparagine (N-linked glycosylation) or to a hydroxyl oxygen of threonine or serine (O-linked glycosylation) residues (Freeze & Sharma, 2010; Reis et al., 2010). Glycosylation mostly occurs in the endoplasmic reticulum or golgi apparatus and can affect protein folding and stability, influence protein trafficking and interfere with protein function (Pinho et al., 2011; Reis et al., 2010; Roth et al., 2010).

Connexin proteins are not glycosylated despite identification of N-glycosylation consensus sequence in Cx32 (Martin & Evans, 2004; Rahman, Carlile & Evans, 1993; Saez et al., 2003). However, it should be noted that a few reports have shown that inhibition of protein glycosylation in cells transfected with Cx43 can increase trafficking of Cx43 to the plasma membrane, its phosphorylation and its opening indirectly via a cAMP pathway (Wang & Mehta, 1995; Wang, Mehta & Rose, 1995; Wang & Rose, 1995).

Currently, the primary identified post-translational modification in pannexins is glycosylation. Glycosylation of the Pannx1 isoforms leads to a migration shift on SDS gel with bands representing three different glycosylation states whereas the multiple band pattern of Cx43 has been demonstrated to represent different phosphorylated states (Boassa et al., 2007; Boassa et al., 2008; Penuela et al., 2007; Penuela et al., 2009; Solan & Lampe, 2009). The different glycosylation states of Pannx1 results in a multiple banding pattern on a western blot with the Gly2 species migrating slower, then Gly1 as an intermediate band and the Gly0 species being the fastest migrating form (Boassa et al., 2007; Boassa et al., 2008; Penuela et al., 2007; Penuela et al., 2009). Of the three pannexin isoforms, Pannx1 and Pannx3 have been shown to be glycosylated and their glycosylation sites have been defined whereas the glycosylation site of Pannx2 has only been predicted (Boassa et al., 2007; Boassa et al., 2008; Penuela et al., 2007; Penuela et al., 2009; Penuela, Gehi & Laird, 2012).

Pannx1 and Pannx3 are N-glycosylated on the amino acids asparagine (N) 254 and N71 respectively and can be found in three different states: a core unglycosylated protein (Gly0), a high mannose-glycosylated protein (Gly1) and an extensively glycosylated species (Gly2) (Boassa et al., 2007; Penuela et al., 2007; Penuela et al., 2009). The role of N-glycosylation of Pannx1 was explored using site-directed mutagenesis of N254 targeted by N-glycosylation (Pannx1^{N254Q}; (Boassa et al., 2007; Boassa et al., 2008; Penuela et al., 2007; Penuela et al., 2008). Similarly, the role of Pannx3 N-glycosylation was investigated using the mutant Pannx3^{N71Q} (Penuela et al., 2007; Penuela et al., 2008). These glycosylation mutants exhibit reduced trafficking to the plasma membrane when transfected in different cell lines,

suggesting an important role for N-glycosylation in Panx1 and Panx3 trafficking (Boassa et al., 2007; Boassa et al., 2008; Penuela et al., 2007; Penuela et al., 2008). However, despite the low amount of Panx1^{N254Q} and Panx3^{N71Q} at the plasma membrane, the mutated proteins still form channels as evidenced by dye uptake measurements (Penuela et al., 2007; Penuela et al., 2009). Additionally, when wild type Panx1 and Panx1^{N254Q} are co-transfected, the trafficking of the glycosylation mutant to the plasma membrane is rescued, showing that they co-oligomerize and suggesting that N-glycosylation does not play a role in oligomerization of Panx1 (Boassa et al., 2008). Altogether, these studies demonstrate that the Gly0 species appears primarily after protein synthesis, whereas Gly1 is associated within the endoplasmic reticulum and Gly2 is modified in the Golgi apparatus prior to plasma membrane insertion. However, it should be noted that all Panx1 glycosylated species are capable of trafficking to the plasma membrane, but the Gly2 form is preferentially trafficked (Gehi, Shao & Laird, 2011).

Phosphorylation

Post-translational modification through phosphorylation acts as a major regulatory pathway in normal protein life cycles and can be further affected in pathological states by upregulation of kinase pathways. These pathways regulate protein functions through addition of a phosphate group by kinases to serine (S), threonine (T) and tyrosine (Y) sites which may lead to alterations in the hydrophobicity, charge and potentially structural re-organization of proteins, either promoting or inhibiting normal functions (Davis, 2011; Huttlin et al., 2010; Nishi, Hashimoto & Panchenko, 2011).

Phosphorylation of connexins has been extensively demonstrated and reviewed (particularly for Cx43) and is integral in their life cycle altering protein oligomerization, trafficking, membrane insertion and aggregation, gap junction communication, and internalization from the membrane (Laird, 2005; Laird, Castillo & Kasprzak, 1995; Marquez-Rosado et al., 2011; Saez et al., 1998; Saez et al., 1990; Solan & Lampe, 2005; Solan & Lampe, 2009). Phosphorylation has been demonstrated for multiple S/T/Y sites through c-/v-SRC; mitogen activated protein kinase (MAPK), protein kinase C (PKC), protein kinase A (PKA), P34^{cdc} (CDC2), casein kinase 1 (CK1) and calmodulin-dependant protein kinase (CAMK) pathways (Lampe & Lau, 2004; Saez et al., 1998; Solan & Lampe, 2005; Solan & Lampe, 2009).

Post-translational modifications through phosphorylation of cytoplasmic residues, primarily occurs within the connexin carboxyl terminus. Additionally, phosphorylation sites in Cx43 amino terminus (S5) and the Cx56 intracellular loop have been identified as phosphorylated (Berthoud et al., 1997; Chen et al., 2012; Wisniewski et al., 2010). Cx43 does not contain serines within its intracellular loop and is therefore not modified directly by phosphorylation within this region (Solan & Lampe, 2005). As a result of a short 19 amino acid carboxyl terminus containing only 2 serines, Cx26 is the only isoform that currently considered not to be post-translationally modified through phosphorylation (Traub et al., 1989). Conversely, Cx43 contains a total of 66 S/T/Y sites (32 in the carboxyl terminus) and has been demonstrated as highly regulated through phosphorylation primarily at serine residues (Chen et al., 2012; Crow et al., 1990; Marquez-Rosado et al., 2011).

Phosphoproteomic analyses used to determine the incidence of protein phosphorylation in vitro and across multiple tissues ranging from brain, kidney, lung, spleen (and more) derived from mice, rat and human have demonstrated that Cx43 as well as Cx47, Cx32 and Cx29 are phosphorylated at multiple sites at their carboxyl terminus as well as the amino terminus (Brill et al., 2009; Cooper et al., 2000; Huttlin et al., 2010; Rikova et al., 2007; Wisniewski et al., 2010). These studies and others further suggest that connexins are regulated by

complex multi-site phosphorylation which plays an important role in the rapid generation and turnover of connexin proteins in a multitude of tissues and organ systems (Chen et al., 2012; Johnson & Vaillancourt, 1994). This area of study will become increasingly important in terms of understanding how multiple phosphorylation events can “tip the balance” one way or another towards a particular protein function.

Connexins are post-translationally oligomerized to hexameric hemichannels prior to membrane insertion either within the endoplasmic reticulum (e.g. Cx26, Cx32) (Falk et al., 1997; Falk & Gilula, 1998) or in the trans golgi network (e.g. Cx43, Cx46) (Das Sarma et al., 2001; del Castillo et al., 2009; Koval, 2006; Koval et al., 1997; Musil & Goodenough, 1993; Puranam, Laird & Revel, 1993). A role for connexin phosphorylation in channel oligomerization has not currently been demonstrated (Solan & Lampe, 2005). Pulse chase studies have shown that newly synthesized Cx43 can be rapidly phosphorylated and de-phosphorylated (within 15–30 mins) (Crow et al., 1990) in the ER and golgi compartments (Laird et al., 1995). In addition, monomeric Cx43 phospho-isoforms have been identified which suggests that Cx43 is phosphorylated prior to insertion into the plasma membrane (Musil & Goodenough, 1993). Further, increases in PKA activity can promote Cx43 integration to the membrane and are associated with Cx43-S364 phosphorylation, but Cx43 is a poor substrate for PKA and does not appear to directly increase Cx43 phosphorylation, suggesting other intermediary pathways mediate the response (Paulson et al., 2000; TenBroek et al., 2001). Data demonstrating non-phosphorylated isoforms of connexins at the membrane suggests that they can be inserted to the membrane without phosphorylation (although transient phosphorylations could occur prior to entry to the membrane; (Musil et al., 1990; Musil & Goodenough, 1991; Solan & Lampe, 2005). This is also evident with truncated mutations of Cx43 (Δ 252) oligomerizing and trafficking to the plasma membrane. These truncated channels still form functional channels, suggesting that phosphorylation on the carboxyl tail is not a critical requirement (Johnstone et al., 2009; Martinez et al., 2003). Regardless, deletion mutagenesis studies have demonstrated that a portion of the Cx43 carboxyl terminus is required for trafficking. By creating a carboxyl terminus deletion at amino acid 236, Cx43 cannot traffic to the membrane, while deletions after amino acid 239 (Δ 243/ Δ 239) traffic to the membrane and form gap junctions (De Vuyst et al., 2007; Wayakanon et al., 2012). As with Cx43, Cx45 requires its carboxyl terminus to target the membrane. Phosphorylation does not significantly alter Cx45 trafficking to the membrane but may affect protein half-life (Hertlein et al., 1998). Current data therefore suggest that phosphorylation of connexins is not a critical determinant of oligomerization and membrane insertion, but could be involved in efficient gap junction assembly and trafficking.

The open and closed state of connexin hemichannels exists as a balance between inhibition and stimulation of carboxyl terminal residues. Mutagenesis studies of Cx43 have shown that mutations after amino acid 239 in Cx43 can still form functional gap junctions but inhibits hemichannel opening, suggesting that regulatory sites within the carboxyl terminus are required for hemichannel opening (De Vuyst et al., 2007). In general, connexin phosphorylation through PKC (e.g. S368) and MAPK activity maintains a closed hemichannel state (Bao, Locovei & Dahl, 2004a; Bao et al., 2007; Chandrasekhar & Bera, 2012; Contreras et al., 2002; Ek-Vitorin et al., 2006; Kwak & Jongsma, 1996; Schulz & Heusch, 2004; Srisakuldee et al., 2009). Indeed it has been shown that direct phosphorylation of Cx43-S368 by PKC reduces hemichannel opening (Bao, Reuss & Altenberg, 2004b). Similarly, treatments with lipopolysaccharide (LPS) and basic fibroblast growth factor (bFGF) decrease hemichannel activity in Cx32, Cx43 and Cx26 expressing HeLa cells (De Vuyst et al., 2007). However, the same treatments enhance hemichannel opening in C6-Cx43 cells (De Vuyst et al., 2007). This diversity in hemichannel signaling has been attributed to a balance between channel inhibition (phosphorylation) and

stimulation (de-phosphorylation) modifications on the connexin carboxyl terminus (Contreras et al., 2002; De Vuyst et al., 2007).

As described above, the carboxyl terminus of connexins (particularly Cx43) may act to promote efficient gap junction assembly from hemichannels. Once inserted to the membrane, hemichannels aggregate in “formation plaques” prior to incorporation into the gap junctional plaque (Johnson et al., 2012; Segretain & Falk, 2004). Truncation mutants of Cx43 result in reduced hemichannel aggregation and gap junction plaque formation, suggesting that phosphorylation of carboxyl terminal residues may be involved in efficient delivery of hemichannels to the gap junction plaque (Johnson et al., 2012; Palatinus, Rhett & Gourdie, 2011a). Increased formation and stability of gap junctions has been associated with PKA and CK1 activity. Increases in intracellular cAMP levels promote PKA activity which promotes (indirectly) Cx43-S364 phosphorylation and increases in GJ assembly and coupling (TenBroek et al., 2001). Casein Kinase 1 phosphorylation of Cx43-S325/S328/S330 also appears to promote gap junction assembly and stability (Cooper & Lampe, 2002). Gap junction aggregation occurs through directed trafficking by chaperone proteins e.g. zonula occludens-1/2 (ZO-1/ZO-2) (Rhett, Jourdan & Gourdie, 2011; Singh et al., 2005). Associations between Cx43 carboxyl terminus and ZO-1 promote targeting to gap junction plaques (Hunter et al., 2005; Jin et al., 2004; Segretain et al., 2004; Toyofuku et al., 1998). This can be disrupted through c-SRC phosphorylation of Cx43-Y265 which appears to be a critical site in the binding of ZO-1 (Toyofuku et al., 2001). Therefore phosphorylation may allow for efficient and directed movement of connexins within the membrane to sites of gap junction plaques.

In general for Cx43, CK1 (and PKA) pathways increase, and PKC, MAPK, V-SRC, and CDC2 reduce, gap junctional communication (Pahujaa, Anikin & Goldberg, 2007; Solan & Lampe, 2005; Solan & Lampe, 2008; Solan & Lampe, 2009). Phosphorylation-induced increases in gap junctional communication occur through CK1 phosphorylation of Cx43-S325/S328/S330 (Cooper & Lampe, 2002) and by PKA (Cx43-S364, indirectly) (TenBroek et al., 2001). However, as mentioned these sites are also associated with increased gap junction assembly, and it has been suggested that they may enhance plaque stability, leading to prolonged life cycle (Remo et al., 2011). In addition, phosphorylation of Cx40 by PKA pathways leads to altered (higher) conductance states (van Rijen et al., 2000).

Protein kinase C activity reduces Cx43 gap junctional communication. Reduced unitary conductance occurs following Cx43-S368 phosphorylation (Ek-Vitorin et al., 2006; Lampe et al., 2000) and site directed mutagenesis demonstrates that Cx43-S262 phosphorylation can reduce gap junctional communication which in turn may act to promote DNA synthesis and cell cycle progression (Doble et al., 2004). The impact of reduced communication through PKC phosphorylation of Cx43-S368 ranges from promoting wound closure (potentially by isolating cells from surrounding signals and promoting differentiation) (Richards et al., 2004), causing decreased cell-to-cell communication in the vascular wall (Straub et al., 2009) and reducing ischemia reperfusion injury in hearts (Palatinus, Rhett & Gourdie, 2011b). MAPK phosphorylation of Cx43-S255/S279/S282 leads to reductions in gap junctional communication by inhibiting the ability of the channels to open (Cottrell et al., 2003; Warn-Cramer et al., 1998). It should also be noted that several sites in the Cx43 tail may be phosphorylated through multiple pathways as is demonstrated for PKC induction by phorbol 12-myristate 13-acetate (PMA) which promotes Cx43 S279/S282 phosphorylation (Solan & Lampe, 2007; Solan & Lampe, 2009). In addition to the classically accepted MAPK sites, Norris et al demonstrated that Cx43-S262 sites were phosphorylated (but not S368) through MAPK pathways which lead to reduced gap junctional communication and meiotic resumption in mouse oocytes (Norris et al., 2008). Increases in CDC2 promote phosphorylation of Cx43-S255 and reductions in gap junction

communication which are associated with progression through the cell cycle (Kanemitsu, Jiang & Eckhart, 1998; Lampe et al., 1998). Activation of v-SRC pathways leads to phosphorylation of Cx43-Y247 and Cx43-Y265, which appears to occur in formed gap junctions leading to a reduction in gap junction communication (Solan & Lampe, 2008). In addition to these sites active v-SRC can also cause phosphorylation at sites associated with PKC and MAPK in Cx43 (Solan & Lampe, 2008).

Less is known for the other connexins. Sequence predictions and homology suggest that Cx37 may be similarly phosphorylated through MAPK pathways potentially at Cx37-S275 and Cx37-S282 which may affect gap junction assembly and communication but this has not been directly demonstrated (Burt et al., 2008). Phosphorylation of Cx45 through PKC pathways alters the occupancy time and conductive state of the channel (Kwak et al., 1995; van Veen, van Rijen & Jongsma, 2000). Although Cx45 is primarily phosphorylated at serines, substitution of all carboxyl terminus serines does not alter gap junctional communication in HeLa cells (Hertlein et al., 1998).

Phosphorylation also plays a role in the internalization of connexins from the gap junctional plaque and has been shown to alter the protein turnover rates for several connexins (Laird, 2005). During cell cycle Cx43 appears to be dynamically regulated at each of the stages S/ G1/G2 and M phases all represented by alterations in Cx43 phosphorylation and internalization. As cell cycle progresses, Cx43 appears at more intracellular locations and is highly phosphorylated at CDC2, PKC and MAPK sites suggesting that phosphorylation may be involved in removal of the connexins from the membrane gap junction plaques. However, it has not been conclusively demonstrated whether this results from removal of connexins from gap junction plaques or by reduced assembly to plaques (Solan & Lampe, 2009). In mitosis, phosphorylation of Cx43 by CDC2 at the S255/S262 regions is associated with an internalization of the protein (Kanemitsu et al., 1998; Lampe et al., 1998; Xie et al., 1997). During cell cycle Cx43-S368 phosphorylation is also associated with a reduction in gap junction plaque size and protein internalization (Lampe et al., 2000; Solan et al., 2003). Conversely it has been noted that de-phosphorylation of Cx43 (S365) promotes an intracellular redistribution of Cx43 (Beardslee et al., 2000; Solan et al., 2007) or removal of Cx43 plaques (Laird et al., 1995). Substitution of Cx45-S381/S382/S384/S385 residues, but not the remaining serines from the carboxyl terminus of Cx45 significantly reduces the protein half-life by approximately 50% suggesting that these sites are involved in protein degradation (Hertlein et al., 1998). More recently, the MAPK phosphorylation of Cx43 at S255/262/279/282 was identified as critical to binding with cyclin E and promotion through cell cycle (Johnstone et al., 2012).

While phosphorylation of connexins is integral in their functionality, much less has been demonstrated for modulation of pannexin channels. Despite this, all mammalian forms of pannexins do contain multiple S/T/Y sites (Penuela et al., 2007). Currently, there is limited structural data for the pannexins and their carboxyl terminus regions (Ambrosi et al., 2010). The membrane topology of pannexins appears to resemble that of the connexins with cytoplasmic amino terminus, intracellular loop and carboxyl terminus which shows greatest sequence variability in the Panx2 isoform (Penuela et al., 2012). As with the connexins, the carboxyl terminus regions are rich in S/T/Y sites. Consensus sequences for S/Y/T phosphorylation have been predicted based on sequence and membrane topology for pannexins 1–3 to occur primarily in the carboxyl terminus with fewer intracellular loop motifs (Barbe, Monyer & Bruzzone, 2006; Penuela et al., 2007). It has been shown that Panx1 ATP release can be induced by the P2X₇R activation through tyrosine kinase pathways but it is not clear whether Panx1 is directly phosphorylated or regulated through intermediary pathways (Iglesias et al., 2009). While data on phosphorylation of pannexins is

currently in its infancy there is clearly the potential for this post-translational modification in the regulation of the protein life cycle or function.

Nitrosylation

Post-translational modification can also occur through S-nitrosylation in which nitric oxide (NO) can bind to a reactive cysteine (C) thiol producing an S-nitrosothiol (SNO) (Stamler et al., 1992). While proteins often contain multiple cysteine residues, the majority of the observed biological effects imparted by NO occur on single, or only a few, cysteine residues within a protein. In addition to direct modification of proteins by S-nitrosylation, this post-translational modification can indirectly regulate other protein modifications including acetylation, phosphorylation and ubiquitination (Hess & Stamler, 2012; Park et al., 2004; Whalen et al., 2007; Yasukawa et al., 2005).

The effects of NO on hemichannels and gap junctions have primarily been investigated in vascular cell types including vascular endothelial and smooth muscle cells. Of particular note, it was initially shown that exogenous application of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) to cultured human umbilical vein endothelial cells significantly decreased dye transfer between coupled cells in a Cx37-dependent manner (Kameritsch et al., 2005). The observed decrease in gap junction permeability was independent of the effects of NO on guanylate cyclase and cGMP levels, indicating a separate effect of NO on the channels. In a separate study, NO application significantly reduced electrical coupling between cultured human microvascular endothelial cells mediated by effects on gap junctions composed of Cx37 which was similarly independent of cGMP (McKinnon et al., 2009). Conducted vasoconstriction in cremasteric arterioles has been shown to be dependent on Cx37 gap junctions and, importantly, is impaired during sepsis, a pathology resulting in upregulation of nNOS and increased NO production (McKinnon et al., 2006).

While S-nitrosylation of gap junctions composed of Cx37 has not been directly observed, evidence has emerged indicating the ability of Cx43 to be S-nitrosylated and this modification has been suggested to regulate gap junction permeability at the myoendothelial junction (MEJ) in the blood vessel wall (Straub et al., 2011). In this study, Cx43 was found to be locally enriched along with eNOS at the MEJ. Importantly, the Cx43 was constitutively S-nitrosylated at C271. The S-nitrosylation of C271 on Cx43 was directly correlated with an open channel probability as denitrosylation significantly reduced heterocellular communication at the MEJ, namely the movement of inositol triphosphate from smooth muscle cells to endothelial cells through Cx43 gap junctions. In agreement, pharmacological inhibition or genetic depletion of the denitrosylase GSNOR, which indirectly affects the extent of S-nitrosylation in these cells by reducing denitrosylation of the NO donor S-nitrosogluathione (GSNO), significantly increased gap junctional communication at the MEJ. Altogether, this study suggests the potential for connexin proteins to become S-nitrosylated at specific cysteine residues with the modification affecting channel permeability.

S-nitrosylation has also been proposed to regulate the permeability of undocked Cx43 hemichannels at the plasma membrane. During prolonged periods of ischemia or hypoxia, oxygen deprivation induces the opening of Cx43 hemichannels in cortical astrocytes and cardiomyocytes leading to cell death (Contreras et al., 2002; John et al., 1999; Kondo et al., 2000). The mechanisms regulating Cx43 hemichannel permeability under these conditions have been suggested to involve dephosphorylation of Cx43 hemichannels (a known stimulus for hemichannel activation (Bao et al., 2004b; Kim et al., 1999)) or oxidative modification due to increased production of reactive oxygen species, including NO. A novel study by Retamal *et al* found that metabolic inhibition of cortical astrocytes resulted in both

dephosphorylation of Cx43 hemichannels as well as an increase in S-nitrosylation, ultimately leading to an increase in cellular permeability as determined by dye uptake (Retamal et al., 2006). Interestingly, application of reducing agents to these cells did not affect Cx43 phosphorylation but significantly reduced both cell permeability and Cx43 S-nitrosylation. In agreement with this, application of exogenous NO donors results in increased cell permeability. Together, these observations indicate a functional role for Cx43 S-nitrosylation in regulating hemichannel permeability at the plasma membrane of astrocytes.

The potential regulation of hemichannels composed of Cx46 by nitric oxide has also been suggested. When expressed in *Xenopus oocytes*, Cx46 hemichannels exhibited increased voltage sensitivity and current amplitude upon application of GSNO as assessed by patch clamp electrophysiology (Retamal et al., 2009). Treatment of these cells with the reducing agent dithiothreitol (DTT) reversed the effects of GSNO on hemichannel currents and mutation of the two endogenous carboxyl terminus cysteine residues inhibited the effect of GSNO application. These observations may suggest that critical cysteine residues in the carboxyl terminus of Cx46, like C271 in Cx43, may be modified by S-nitrosylation to regulate channel permeability.

Of the three pannexin isoforms characterized to date, Panx1 is the most widely expressed isoform and is highly expressed in vascular smooth muscle and endothelial cells in the arterial vasculature (Billaud et al., 2011; Godecke et al., 2012; Lohman et al., 2012) and in multiple cell types in the central and peripheral nervous system (Ray et al., 2005; Vogt, Hormuzdi & Monyer, 2005; Xia et al., 2012). Importantly, all these tissues have enriched NOS expression and actively utilize NO for signaling. As Panx1 contains multiple cysteine residues and has been shown to be expressed in tissues with enhanced NO production, it is possible that Panx1 channels in these cells may be regulated post-translationally by S-nitrosylation. Further, it has been shown that mutation of C346 in the carboxyl terminus of Panx1 results in a constitutively leaky channel (Bunse et al., 2010). Also, mutation of C40 in the pore-lining region of the first transmembrane domain of Panx1 produces a constitutively open channel (Bunse et al., 2011). These results indicate the importance of Panx1 cysteine residues in regulating channel gating and permeability. While there are currently no published reports indicating direct modulation of Panx1 channels by S-nitrosylation, studies have indicated a role for Panx1 (as well as Panx2) channels in ischemia-induced neuronal cell death, a condition in which NO production is markedly enhanced (Bargiotas et al., 2011). Similarly, oxygen glucose deprivation and metabolic inhibition, conditions similar to ischemia, both induced excessive NO production and neuronal cell death mediated by opening of Panx channels which was attenuated by pharmacological blockade of nNOS or reduction of oxidized cysteine thiols with the reducing agent DTT (Zhang et al., 2008). Importantly, inhibition of guanylate cyclase in these experiments had little effect on Panx channel permeability, suggesting potential effects of NO by S-nitrosylation of Panx channels in these cells. Future studies will be essential to directly evaluate the potential for pannexin proteins to be post-translationally modified by S-nitrosylation and how modification of Panx channels in this manner affects channel gating and permeability.

Ubiquitination

The ubiquitin proteasome system regulates the degradation of many proteins. This can occur at the absolute N-terminal primary amine of proteins to induce autophagy (e.g., (Lichtenstein et al., 2011)). Although this is a relatively new area of connexin research, it could be quite important in multiple disease states. More traditional ubiquitination is comprised of several components, including E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases, that together mediate the transfer of the 76

amino acid protein ubiquitin to specific lysine (K) residues on target proteins (Leithe & Rivedal, 2007; Willis et al., 2010). Multiple ubiquitins can be covalently linked via K48 to form poly-ubiquitin chains, which target the ubiquitinated protein for degradation by the multi-subunit 26S proteasome (Leithe & Rivedal, 2007; Voges, Zwickl & Baumeister, 1999; Willis et al., 2010). Both connexins and pannexins have multiple lysine residues to which ubiquitin may be conjugated. Connexins can be ubiquitinated on K9 and K303 (Wagner et al., 2011), while pannexins can have ubiquitin conjugated to K409 (Kim et al., 2011). Thus, the possible ubiquitination of connexins and pannexins imply that these proteins may be regulated by proteasomal degradation.

Several studies have suggested that connexins and pannexins are ubiquitinated and degraded by the proteasome. The proteasomal inhibitor N-acetyl-L-leucyl-L-leucinylnorleucinal (LLnL) increased levels of Cx43 (Laing & Beyer, 1995) and Cx31 (He et al., 2005) and prolonged Cx43 half-life. Furthermore, immunoprecipitation with antibodies against Cx43 and ubiquitin suggested that Cx43 might be polyubiquitinated (Laing & Beyer, 1995), indicating that disrupting the ubiquitin proteasome system could alter connexin turnover. The tumorigenic compound phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) initiated hyper-phosphorylation and ubiquitin-mediated degradation of Cx43, which was prevented by proteasomal inhibitors (Leithe & Rivedal, 2004), demonstrating the importance of phosphorylation in ubiquitin-mediated connexin degradation. Phosphorylation of connexins facilitates interaction with proteins of the ubiquitin proteasome system, such as the ubiquitin ligase Nedd4 (Leykauf et al., 2006). Disrupting the interaction between connexins and ubiquitin proteasome proteins via proteasomal inhibitors alters the interaction between Cx43 and ZO-1, leading to Cx43 accumulation into enlarged gap junctions at plasma membranes (Girao & Pereira, 2007). Therefore, it has been suggested that the ubiquitin proteasome system most likely contributes to the internalization and degradation of connexins.

Despite these findings, there is mounting evidence against ubiquitination and proteasomal degradation of connexins. Degradation of endoplasmic reticulum-localized Cx43 by the proteasome is partly controlled by Cx43 interactions with the ubiquitin-like protein CIP75, which binds to ubiquitinated substrates. However, immunoprecipitation studies demonstrated that the CIP75-bound Cx43 was not directly ubiquitinated (Su et al., 2010). In addition, a mutant Cx43 construct in which all lysine residues were converted to arginines mimicked the response of wild type Cx43 to proteasomal inhibitors (Dunn et al., 2012). Taken together, these results indicate that direct ubiquitination may not be required for proteasomal degradation of Cx43. Instead, ubiquitination of connexins may contribute to other pathways regulating turnover. Indeed, recent studies have linked Cx43 monoubiquitination to lysosomal degradation. Following internalization from the plasma membrane, Cx43 can associate with the ubiquitin-binding proteins Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), Tsg101 (tumor susceptibility gene 101) and the ubiquitin-binding endocytic adaptor protein Eps15, which shuttle Cx43 along the endocytic pathway for subsequent degradation in lysosomal compartments (Girao, Catarino & Pereira, 2009; Leithe et al., 2009). Thus, although ubiquitination mediates connexin trafficking and other degradative pathways it is not clear whether direct ubiquitination is an important step during proteasomal degradation. Connexin turnover by this pathway most likely involves interaction between ubiquitinated substrates, even if connexins themselves are not tagged with ubiquitin.

Perturbation of connexin degradation by the ubiquitin proteasome has physiological and pathological consequences. Connexins are dynamically regulated with a short half-life between 1–6 hours (He et al., 2005; Leithe & Rivedal, 2004; Musil et al., 2000; Su et al., 2010). Proper connexin turnover rate is vital to maintain normal physiological functions (Li

& Wang, 2010). Dysregulation of dynamic gap junction protein turnover via inhibition of the ubiquitin proteasome degradation pathway (e.g. from oxidative stress) increases gap junction plaque size and enhances gap junction communication (Girao & Pereira, 2007). Inhibition of proteasome-mediated degradation of gap junction proteins and enhanced gap junction communication is associated with several cardiovascular pathologies, such as ischemia-reperfusion injury, cardiac proteinopathy (i.e. irregular protein aggregation), post-myocardial infarct arrhythmias and heart failure (Beardslee et al., 1998; Li & Wang, 2010). Thus, the ubiquitin proteasome pathway may provide novel therapeutic targets to treat cardiovascular diseases by restoring proper turnover of cardiac proteins, including connexins.

Much less is known about the involvement of the ubiquitin proteasome system in pannexin degradation. Mass spectrometry screens have identified K409 as a probable site of ubiquitination (Kim et al., 2011). However, biochemical evidence for pannexin ubiquitination and proteasomal degradation is lacking. A recent study has shown that Panx1 internalization and lysosomal degradation is controlled by its carboxyl terminus region, but Panx1 does not appear to be degraded by traditional endocytic pathways (Gehi et al., 2011). Thus further studies are needed to investigate the role ubiquitination plays in pannexin turnover (Schalper et al., 2012).

Other modifications

SUMOylation

It has now been demonstrated that connexins (i.e. Cx43) can be targeted for post-translation modification by less characterized pathways e.g. SUMOylation. Small ubiquitin-like modifier (SUMO) family of proteins act in most cellular compartments to adapt proteins in multiple processes including transcription, translation, cellular transport, protein interactions, cell growth and programmed cell death (Geiss-Friedlander & Melchior, 2007). In Cx43, L144 (intracellular loop) and L237 (carboxyl terminus) can be SUMOylated leading to a reduction in protein expression and gap junction formation (Kjenseth et al., 2012). Consensus motifs for sumoylation have been identified in Panx2 the sequence, however studies of immunoprecipitated Panx2 from mouse neuronal stem cells differentiated to neurospheres showed a lack of detection of SUMO1/2/3 suggesting Panx2 is not SUMOylated (Swayne, Sorbara & Bennett, 2010). Given the diversity of activity of SUMO proteins, this post-translational modification could potentially play a significantly role in connexins and pannexin protein regulation and channel formation.

Lipid modification, e.g., palmitoylation

The correct insertion of transmembrane proteins into the plasma membrane is a delicate process that involves the coordination of protein modifications for the binding of specific phospholipids (termed palmitoylation). It has been well described that connexins are associated with lipid rafts, especially enriched with caveoli (e.g., (Langlois et al., 2008; Locke, Liu & Harris, 2005)). However, only the carboxyl terminus of Cx32 (amino acids 277–283) has been identified to have a palmitoylation site (Locke, Koreen & Harris, 2006). Regardless, this concept was further elucidated by a series of experiments designed to identify all the phospholipids that could be associated with either Cx26 or Cx32 gap junctions. The authors found that the lipids associated with the two homotypic gap junctions were dramatically different, indicating these connexins may reside in very distinct parts of the plasma membrane (Locke & Harris, 2009), possibly placed there by palmitoylation modifications.

Thus far, there is no indication that pannexin isoforms associate with caveolin (Gehi et al., 2011). However, it has been demonstrated that Panx2 has distinct de-palmitoylated and

palmitoylated species that migrate at different molecular weights (~60 KDa and ~85 KDa respectively) as determined by treatment of western blots with hydroxylamine or metabolically labeling cells with BODIPY FL hexadecanoic acid. Swayne et al further suggested that the palmitoylated form of Panx2 resides in golgi and/or endoplasmic reticulum whereas the de-palmitoylated form resides primarily at the plasma membrane and indicates these changes are associated with differentiation of neuronal cells (Swayne et al., 2010). Elucidation of the exact site where Panx2 may be palmitoylated could prove quite interesting.

Caspase cleavage

The post-translational modification of proteins is generally considered to be a reversible process. However, a new type of terminal post-translational modification was recently described where the Panx1 protein was cleaved by caspase 3 during apoptosis, inducing a constitutive open state of the channel for the release of ATP (Chekeni et al., 2010; Dunn et al., 2012). The caspase 3 cleavage site was identified in human Panx1 at amino acid 376–379 (DVVD (Chekeni et al., 2010)). The implications from the work were that the carboxyl terminus of Panx1 could regulate the pore open or closed state. This concept was recently demonstrated in a novel experimental model (Sandilos et al., 2012). In this work the authors inserted a TEV sequence into the Panx1 caspase cleavage site allowing for controlled enzymatic cleavage of the carboxyl terminus. Next, a disulfide bond between the carboxyl terminus (C426) and an engineered cysteine within in the pore of the Panx1 channel prevented removal of the carboxyl terminus from the pore (Sandilos et al., 2012). Finally cleavage of the carboxyl terminus by TEV protease failed to produce current, and only after disulfide bond removal was current through the Panx1 channel restored (Sandilos et al., 2012). When summed with the caspase cleavage, the data indicates a role for the carboxyl terminus in regulating an open or closed pore, and that cleavage of the carboxyl terminus as the specific caspase cleavage site induces a constitutive open channel during apoptosis. Although multiple connexins have been implicated throughout the apoptotic pathway e.g. (Andrade-Rozental et al., 2000; Minogue et al., 2009; Seul et al., 2004), there are no direct links or functional results like that described for the action of caspase 3 on Panx1.

Conclusion

Although much has been learned about how post-translational modifications can have dramatic or subtle effects on connexin and pannexin function, most of these discoveries have only happened in the last decade. The creation of more specific antibodies towards modified amino acids (e.g., (Solan & Lampe, 2008)) and accessibility of proteomic arrays to more researchers will enable an even greater study of post-translational modifications associated with connexins and pannexins and their functional effect.

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Figure 1. Schematic list of post-translational modification throughout the lifespan of connexins and pannexins

Numbers are the amino acids in the proteins as indicated in the references. Note only when amino acids or specific regions on the protein could be identified were they included in the figure, e.g., although palmitoylation has been indicated for Panx2 (Swaine et al., 2010), the exact region on the protein remains unknown. * indicates it was shown indirectly through PKA; # indicates that this cysteine is assumed to be S-nitrosylated in a hemichannel based on (Retamal et al., 2006), but has not been directly shown; + indicates that this data is now in dispute according to work by (Dunn et al., 2012). (1) (Paulson et al., 2000; TenBroek et al., 2001); (2) (Locke et al., 2006); (3) (Cooper & Lampe, 2002); (4) (Cottrell et al., 2003; Kanemitsu et al., 1998; Lampe et al., 1998; Norris et al., 2008; Warn-Cramer et al., 1998); (5) (Doble et al., 2004; Ek-Vitorin et al., 2006; Lampe et al., 2000); (6) (Solan & Lampe, 2008); (7) (Straub et al., 2011); (8) (Kjenseth et al., 2012); (9) (Bao et al., 2007; Bao et al., 2004b); (10) (Toyofuku et al., 2001); (11) (Retamal et al., 2009); (12) (Lampe et al., 2000; Solan et al., 2003); (13) (Kanemitsu et al., 1998; Lampe et al., 1998; Xie et al., 1997); (14) (Beardslee et al., 2000; Solan et al., 2007); (15) (Hertlein et al., 1998); (16) (Yin, Gu & Jiang, 2001; Yin, Liu & Jiang, 2008); (17) (Wagner et al., 2011); (18) (Boassa et al., 2007; Penuela et al., 2007); (19) (Penuela et al., 2007); (20) (Chekeni et al., 2010); (21) (Kim et al., 2011).