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Gap junction channel gating modulated through protein phosphorylation

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Abstract

As a ubiquitous post-translation modification process, protein phosphorylation has proven to be a key mechanism in regulating the function of several membrane proteins, including transporters and channels. Connexins, pannexins, and innexins are protein families that form gap junction channels essential for intercellular communication. Connexins have been intensely studied, and most of their isoforms are known to be phosphorylated by protein kinases that lead to modifications in tyrosine, serine, and threonine residues, which have been reported to affect, in one way or another, intercellular communication. Despite the abundant reports on changes in intercellular communication due to the activation or inactivation of numerous kinases, the molecular mechanisms by which phosphorylation alters channel gating properties have not been elucidated completely. Hence, this chapter will cover some of the current, relevant research that attempt to explain how phosphorylation triggers and/or modulates gap junction channel gating.

Keywords

gap junction; gating; protein phosphorylation; cell to cell coupling; tyrosine protein kinase; serine protein kinase

Overview

Gap junction channels allow intercellular communication between contiguous cells in a tissue and half gap junction channels, or hemichannels, may permit direct communication between the cytoplasm and extracellular milieu. Gap junction channels can be formed by one or more of the various protein isoforms named connexins (Cx). Gating properties of new families of proteins involved in the formation of intercellular channels are emerging, including those termed pannexins and innexins (Bruzzone *et al.*, 2003;Landesman *et al.*, 1999). It is essential to determine the abundance and physiological relevance of these new families in tissues as well as their regulation through phosphorylation.

Understanding the regulation of gap junction gating and permeability has been relatively complicated because many tissues express multiple connexin isoforms, and most of the time, these channels are not formed exclusively of one connexin subunit (homotypic), but consist of multiple distinct connexin isoforms yielding what are known as heteromeric channels (Wang

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and Peracchia, 1998). Some of these gap junction channels are highly selective for small ions or molecules and their gating mechanisms appear to involve a direct interaction with intracellular messengers (Ca^{2+}, pH, arachidonic acid, etc.), indicating that gating is required to preserve essential functions. This has been reinforced by recent remarkable discoveries of natural or induced connexin mutations that appear to be the root of several human diseases (Abrams and Bennett, 2000).

The ability of connexins to create heteromeric connexons, enormously increases the complexity of channel regulation. As has been demonstrated, each connexin provide channels with unique gating as well as perm-selectivity properties; hence, some connexin combinations can yield intermediate or new conductive or gating properties. These new properties have the potential to alter communication properties between cells and its regulation (Elenes *et al.*, 1999;Moreno *et al.*, 2004).

Changes in cell-to-cell communication have been observed after activation of protein kinases (Cottrell *et al.*, 2003;Duncan and Fletcher, 2002;Shi *et al.*, 2001) and protein phosphatases (John *et al.*, 2003). From the initial reports (Kanno *et al.*, 1984;Azarnia *et al.*, 1988), it has been reasonable to speculate that phosphorylation induces channel gating. Nonetheless, the mechanism is barely understood, especially because activation of distinct phosphorylation pathways has been shown to participate in many other cellular mechanisms not related to gating, such as the rapid turnover of channels, which, in turn, can alter cell-to-cell communication (Laird, 2005;Lampe and Lau, 2004;Solan and Lampe, 2005a). Therefore, it is essential to distinguish whether or not changes in intercellular communication are related to a mechanism inducing *bona fide* channel gating, or one targeting channels for removal or integration into the cell membrane.

A) Gating

The term “gating” is often used loosely to refer to any and all channel molecular transitions leading to channel opening or closing, meaning that during gating, a conductive pathway becomes either physically available, or unavailable. Gating has been defined as the mechanism by which the movement of ionic or non-ionic species becomes physically restricted due to the alteration of the molecular structure of the channel itself (Hille, 1992). This definition is appropriate for our purposes, with the additional concept that gating can induce a conformational change of a channel protein that involves a fast and reversible change in conductive properties (see reference 18). Gating can also include a reversible process where a complete or relative closure or opening of a channel occurs. It has been demonstrated that connexins build membrane channels that can rapidly gate (Veenstra and DeHaan, 1986).

In terms of gating types, they are referred mostly by 1) the molecular domain involved (like COOH gating or e-loop gating), or the gating inducer (like voltage or pH). For connexin-formed channels, those domains involved in gating are the carboxyl and amino terminus, as well as the extracellular domains. The inducers of gating can be categorized as electrical or chemical agents. When compared to excitable membrane channels, gap junction channels are also sensitive to transmembrane voltages, although their gating transitions are slower and often less sensitive to trans-junction or trans-membrane voltage (Moreno *et al.*, 2002a;Bukauskas and Weingart, 1994). Chemical gating includes changes in intracellular pH, intracellular and extracellular Ca^{2+}, and protein phosphorylation.

As for the presence of a sensor and a gate, distinct connexin domains operate as voltage sensors (Purnick *et al.*, 2000b) and others as gates (Trexler *et al.*, 1999;Stergiopoulos *et al.*, 1999) and these domains are also involved in the process of channel gating. The carboxyl-terminal tail, which is located in the cytoplasm, contains multiple phosphorylatable serine, threonine, and tyrosine residues, and has been considered as an intrinsic part of the voltage and low pH gate

for various gap junction channels, including connexin43 and connexin45. Therefore, if this domain is involved in the mechanism by which phosphorylation drives channels to gate, it is valuable to consider that phosphorylation-induced gating may be intrinsically linked to changes in the channel's molecular structure that reversibly affects permeability to ions or uncharged molecules. Moreover, alterations in the net charge of connexin's COOH terminal domain, induced by phosphorylation, could also modulate the kinetics of either voltage- or low pH-regulated gating. Hence, the diverse actions of phosphorylation may induce a great variety of modulation and gating possibilities in cells. For example, Cx43 channels are known to have more than one conductive state and phosphorylation drives each channel to a different conductive state, as occurs during phosphorylation by PKC (Moreno *et al.*, 1992). Channels can be driven to a non-conductive state, probably through a different mechanism as exemplified by the phosphorylation of connexin43 by the activated Src tyrosine protein kinase (Lin *et al.*, 2001).

B) Gating parameters

The level of communication between cells coupled through gap junctions is measured electrically by determination of the total conductance of a junction (g_j), which is directly related to the product of three parameters: the total number of channels (N), the unitary conductance (γ_j), and the open probability (P_o) of each channel ($gj=N \times \gamma_j \times P_o$). Only the last two of these three parameters are considered to be directly correlated to channel gating.

Number of channels (N)—N, or the number of channels present in the membrane, can be affected by the dynamics of insertion or removal of channels from the junctional membrane. Junctional conductance changes are expected during the formation of a junctional plaque where hemichannels from contiguous cells dock to form new functional channels or when a group of gap junction channels in a plaque are removed as an annular ring (Laird, 1996;Marquart, 1977). In either case, it is certainly not a gating phenomenon *per se*, since it's not reversible and the structure of the connexin proteins may not be modified.

Connexon incorporation into the junctional membrane could hardly be considered itself as a gating process, nonetheless, phosphorylation as well as de-phosphorylation have been shown to be involved in the mechanisms that modify the rate of incorporation of vesicles into plasma membranes (Jordan *et al.*, 1999;Solan and Lampe, 2005b). After incorporation of connexons into the membrane, the first opening of the channel or activation, has been described electrically (Bukauskas and Weingart, 1994). Activation could be considered a gating process where connexons dock and become fully conductive (Bukauskas *et al.*, 1995). Little is currently known about how phosphorylation can accelerate or retard this process (but see Paulson *et al.*, 2001).

Unitary conductance (γ_j)—Unitary conductance, γ_j, can also be affected by alterations in connexin protein phosphorylation, which has provided the first evidence demonstrating that the molecular modification of a gap junction channel (connexin43) can lead to a change in a channel's conductance (Moreno *et al.*, 1992). Gap junction channels are distinguished by their unitary conductance which depends on the composition of connexins making up the channels. For example, channels formed by connexin43 can gate between three different conductive states of approximately 30, 60, and 100 pS (Takens Kwak and Jongsma, 1992;Moreno *et al.*, 1992), and connexin43 phosphorylation favors transitions to the intermediate conductive state. From all the connexins studied, gap junction channels exhibit conductances that vary between 5 and 370 pS and under high transjunctional voltage, they all revert to a residual state, whose conductance could be between 10-25% of the conductance of their main open state, which indicates that most of these channels do not close completely under voltage clamp conditions. It is important to mention that under physiological conditions, gap junction channels are

believed to remain functional at their main conductive state and voltage gating may occur under extreme circumstances in low conductive pathways (Lin *et al.*, 2003). Moreover, even under these circumstances, the reduction in conductance due to voltage is temporal and not expected to affect junctional permeability of metabolites.

Open Probability (P_o)—The open probability (P_o) is the third parameter that can be altered to change junctional conductance. It is a stochastic index that tells us what percentage of the active life of channels is spent in the open state. In some instances, it can change in response to the activation of certain kinases, as occurs with connexin45 channels in HeLa cells, where P_o is reduced upon activation of cAMP-dependent protein kinases (van Veen *et al.*, 2000). Channel open-state probability (P_o) could be obtained as the fraction of the total time the channel occupies the open state. These values are computed from the activity of a single or multiple channels in a voltage clamp configuration during the application of a specific voltage or driving force across the channel studied. For connexins' intercellular channels, this driving force (mV) needs to be applied across the junction. Therefore it requires a dual whole cell voltage clamp configuration, which makes the acquisition of P_o significantly difficult even for specialized cell electrophysiology laboratories. Moreover, when recordings contain more than one channel, (which normally occurs during gap junction channel recording) the detection and automatic calculation of open and closed times need to be computed for each successive single channel level, which makes the final interpretation of the data even more difficult.

C) Regulation of gap junction channel gating through serine phosphorylation

Connexins have long been reported by many investigators to be regulated by numerous protein kinases, including those that phosphorylate serine and threonine residues (Lampe and Lau, 2004;Warn-Cramer and Lau, 2004). In many respects, connexin43 has been the most intensely studied connexin. Several consensus sites for serine/threonine phosphorylation by protein kinases A, C, and G have been identified at the end of the carboxyl-terminus tail (Sáez *et al.*, 1993;Kwak and Jongsma, 1996). More recently, consensus sites for MAP kinases have been identified (Lau *et al.*, 1996) and phosphorylation of other serines have been reported (Lampe *et al.*, 2006), and in many cases, serine phosphorylation appears to lead to channel gating.

Since the initial report from Burt and Spray (Burt and Spray, 1988), it has been clear that the use of cyclic AMP or GMP could modulate junctional conductance, and at that time, it was suspected that channel gating could contribute to these modulations. Elucidation of this mechanism started with the use of tumor cells expressing rat connexin43 and rat neonatal myocytes. Single channel recordings from these cells (Moreno *et al.*, 1992;Takens Kwak and Jongsma, 1992) indicated that under control conditions, three conductive levels could be detected (Moreno *et al.*, 1992) and that the intermediate state was preferred when PKC and PKG kinases were activated. With intracellular solutions based on $CsCl_2$ (Moreno *et al.*, 1993), the intermediate state resulted in 60 pS sub-level. Interestingly, this response was species-dependent, as human connexin43 did not respond to PKG, which has been attributed to a difference at residue 257 where rat connexin43 presents a serine and human connexin43 contains an alanine (Kwak *et al.*, 1995b). More recently, elegant site-directed point mutation experiments presented by Lampe et al. (2000) showed that serine368 in connexin43 is strictly necessary for the shift in unitary conductance induced by PKC.

Since there is a reduction in the distribution of unitary conductances due to connexin43 PKC phosphorylation, it is expected that the total junctional conduction would be reduced ($g_j = N \times \gamma_j \times P_o$), as it was reported for neonatal myocytes (Munster and Weingart, 1993). Nonetheless, double whole cell voltage experiment in transfected cells using the perforated patch clamp technique indicated that there was an increase in total conductance of about 45% (Kwak *et al.*, 1995c). These differences could be attributed in part to differences in cellular

phosphorylation pathways between cardiomyocytes and transfected cells. But for transfected cells, an increase in junctional conductance was an indication that either a large number of channels were incorporated and activated, or that the open probability of the already active channels increased during kinase activation. The latter option seems not possible because voltage gating protocols indicated that the open probability of connexin43 channels is above 90%. Nonetheless, it is important to consider that all assumptions on open probability of gap junction channels have been obtained from cell pairs where connexin expression is low and few channels are functional. Therefore, little is currently known on the behavior of channels as they are forming large gap junction plaques. Additional results are required from complementary techniques that could reasonably approximate the number of channels (N) in a gap junction plaque, such as freeze fracture or atomic force microscopy.

Studies of changes in biophysical properties due to the phosphorylation of connexin40 have been scarce (Jongsma *et al.*, 2000). Activation of PKA in SKHep1 cells expressing connexin40 increased both junction permeability and conductance by about 50%. When single channel recordings were compared after the use of halothane, it was clear that a shift in the unitary conductance had occurred. In control conditions, the main conductance peaks were 80 and 120 pS (using intracellular ions with lower mobility, as compared to other laboratories) (Elenes *et al.*, 1999;Haubrich *et al.*, 1996). After cells were treated with 8-bromo cAMP, the peak at 80 pS was significantly reduced, indicating that phosphorylated channels resided longer at higher conductance states, which resulted in increased total conductance of the junction (van Rijen *et al.*, 2000).

In a similar fashion, phosphorylation of connexin32 by cAMP during PKA activation is correlated with an increase in conductance in hepatocytes (Sáez *et al.*, 1986) or T84 cells (Chanson *et al.*, 1996). Unfortunately, single channel data are unavailable for these cell types to establish if there is a change in unitary conductance or open probability. What seems clear is that the activation of PKC through TPA appears not to have any influence on the gating of connexin32 channels expressed in SKHep1 cells (Kwak *et al.*, 1995a), although connexin26 channels responded with a decrease in permeability to Lucifer yellow and a reduction in the distribution of unitary conductance. The latter observation is quite intriguing because connexin26 is not a phosphoprotein (Sáez *et al.*, 1990;Traub *et al.*, 1989); however Locke *et al.*, (2006) have suggested that accessory molecules could affect connexin26 channel function (Herve *et al.*, 2004).

In contrast to connexin43, connexin45 channel gating to other substates has not been observed after different treatments, including cGMP, cAMP, PMA, and pervanadate, tyrosine phosphatase inhibitor (van Rijen *et al.*, 2000). In these cases, the distribution of the main unitary conductive peaks at 22 and 42 pS was not significantly modified. The activation of PKC by TPA specifically increased connexin43 junctional conductance to more than 50%. cGMP alone had no effect and cAMP induced a modest reduction in junctional conductance of approximately 20% from the initial pretreatment value. Since changes in conductance occurred within a few minutes, it was expected that changes in channel insertion had not occurred, thus the authors concluded that the observed increase in junctional conductance following TPA-treatment was more likely due to a change in open probability of the channels (see regulation by the v-src tyrosine kinase below). It's important to note that connexin45 forms channels strongly influenced by voltage gating, and in particular, to transmembrane voltage gating (V_m). Although the authors did not examine this question, it would be interesting to determine if phosphorylation is capable of modulating V_m. This interest is reinforced by the fact that for connexin45 channels, the gates for V_m and chemical gating appear to be the same or located in similar channel regions (Bukauskas and Verselis, 2004). It would be interesting to determine if other gap junction channels with strong V_m sensitivity are modulated in the same way by phosphorylation.

For lens equivalent mammalian Cx46 and chick Cx56 channels, activation of PKC enhances connexin phosphorylation and induces a reduction in junctional communication. Furthermore, sheep Cx49 (that corresponds to Cx50 in other mammals) can be phosphorylated by casein kinase I (Cheng and Louis, 2001) and its inhibition increases intercellular communication. Currently, there is a lack of single channel data that can help determine the mechanism of gating for channels formed by these connexins. In the case of connexin46 hemichannels, activating PKC (using phorbol esters) or blocking phosphatases reduced the total gating currents to 30-40% of their control value. Single channel recording of these hemichannels may clarify a mechanism for channel closure, but considering the fact that the reduction of the junctional currents was incomplete, these data strongly suggested that transitions to a residual or smaller substates may play a role in the closure of connexin46 hemichannels.

D) Regulation of connexin43 channel gating by the Src tyrosine protein kinase

Tyrosine protein kinases have long been reported by many investigators to disrupt gap junction function (Lampe and Lau, 2004;Warn-Cramer and Lau, 2004). The previous sections were devoted to the regulation of gap junction channels formed by various connexins by protein kinases targeting serine and/or threonine sites. This section will focus largely on the disruption of connexin43-mediated gap junctions by the viral (v) Src tyrosine kinase, the product of the *v-src* oncogene, with a special emphasis on published data involving changes in “gating” characteristics.

A brief discussion of the effects of v-Src on connexin43 gap junctional communication follows, as this topic has been covered extensively in recently published reviews (Lampe and Lau, 2004;Warn-Cramer and Lau, 2004). In most mammalian cells expressing high levels of constitutively kinase-active v-Src, gap junctional communication, typically measured by the intercellular transfer of a fluorescent dye, is markedly diminished compared to cells lacking v-Src (Crow *et al.*, 1990;Zhou *et al.*, 1999). By the combined use of *in vitro* phosphorylation of connexin43 using purified, activated Src kinase and site-directed connexin43 mutants, phosphorylation of connexin43 was discovered to occur at two primary sites, Y247 and Y265 (Lin *et al.*, 2001). Phosphorylation of these sites was likely a direct effect of v-Src because 1) purified Src was able to phosphorylate connexin43 in *in vitro* kinase reactions (Loo *et al.*, 1995), and 2) Src interacted with connexin43 *in vitro* and *in vivo*, which was mediated by the SH3 and SH2 domains of Src and a proline-rich region and phosphotyrosine sites, respectively, located in the carboxyl-terminus tail of connexin43 (Kanemitsu *et al.*, 1997;Loo *et al.*, 1999). This work, together with an analysis of the phosphorylation of Y247 and Y265 site mutants (Lin *et al.*, 2001), has suggested a working model of a mechanism for the phosphorylation of connexin43 by v-Src. This model proposes an initial SH3-mediated interaction between v-Src and connexin43, followed by the phosphorylation of Y265, SH2 domain interaction with the phosphorylated Y265, phosphorylation of Y247, and channel closure (Warn-Cramer and Lau, 2004;Lin *et al.*, 2001). In contrast, Zhou et al. (Zhou *et al.*, 1999), presented a different mechanism for the regulation of connexin43 by v-Src, which was dependent upon the activation of MAP kinase and the phosphorylation of one or more of the three identified MAP kinase sites in connexin43 (S255, S279, S282). The fundamental reasons for these experimental differences are currently unclear, but may relate to possible differences in the activation of MAP kinase in cells constitutively-expressing kinase-active v-Src versus cells in which v-Src is acutely-activated as in the case of cells containing temperature-sensitive v-Src upon shift to the permissive temperature.

The effects of the Src tyrosine kinase on connexin43 channel “gating” is one of the better studied model systems that has included investigations of the changes in the electrophysiological characteristics of connexin43 channels induced by the v-Src kinase. In these studies (Cottrell *et al.*, 2003), the effects of v-Src on the macroscopic electrical

conductances (g_j) and single channel unitary conductances (γ_j) of connexin43 channels were examined. Connexin43 knockout mouse cell lines expressing exogenous wild type connexin43 (Cx43wt) or connexin43 tyrosine site mutants (Cx43-Y247F, Cx43-Y265F, or Cx43-Y247F,Y265F) in the absence or presence of v-Src were employed in these studies (Cottrell *et al.*, 2003;Lin *et al.*, 2001). G_j was significantly diminished in cell lines co-expressing Cx43wt and v-Src, compared to the control Cx43wt alone value (Cottrell *et al.*, 2003). Cells co-expressing v-Src and the Cx43-Y247F,Y265F double mutant, showed no significant change in g_j compared to Cx43wt. These data indicated that v-Src reduces connexin43 macroscopic junctional conductances in cells stably expressing the activated tyrosine kinase, just as v-Src reduces junctional permeability measured by dye transfer (Crow *et al.*, 1990;Filson *et al.*, 1990). In addition, the observed resistance of the Cx43-Y247F,Y265F double mutant to v-Src indicated that phosphorylation of these two tyrosine sites was sufficient to cause electrical uncoupling in these cells.

To identify the basis for the reduction in g_j by v-Src, changes in single channel unitary conductance (γ_j) in these cells were first examined. Cells expressing Cx43wt exhibited single channel γ_j main state values of approximately 94 pS, in addition to substate conductances of 47 pS (Cottrell *et al.*, 2003). Cells co-expressing Cx43wt and v-Src, showed unitary conductances of 87 and 115 pS with substate values at 48 and 66 pS. Cells expressing the Cx43-Y247F,Y265F double mutant and v-Src showed a predominant 89 pS main state γ_j conductance. The connexin43 main state and substate γ_j values of v-Src cells were found to be statistically similar to those of cells lacking v-Src. Thus, based upon the predominance of γ_j values from 80 to 120 pS and little significant differences in either the size or frequency of the 30-70 pS substate, it was concluded that the reduction in macroscopic electrical conductance observed in v-Src containing cells was not likely due to a reduction in γ_j (Cottrell *et al.*, 2003). These data contrasted distinctly with an earlier finding that the disruption of gap junctional communication by TPA-activated protein kinase C was attributable, in large part, to a marked shift from the main state γ_j of ~100 pS to the ~60 pS substate, although concomitant changes in P_0 or N may have also occurred (Lampe *et al.*, 2000).

Could the v-Src-induced reduction in g_j result from a decrease in N, the number of gap junction channels? Although the report of Cottrell et al. (2003), did not examine this possibility directly, based upon earlier qualitative immunofluorescence microscopy work, gap junction plaques were detected at the plasma membrane of cells stably expressing Cx43wt and v-Src in a manner similar to cells expressing Cx43wt alone (Lin *et al.*, 2001). In addition, comparable levels of connexin43 were expressed in these cell lines. Thus, it was concluded that a major loss of gap junction plaques resulting in a decrease in N was an unlikely explanation for the v-Src-induced decrease in g_j. These results were consistent with the data of Atkinson et al. (1986), using the more sensitive approach of freeze fracture analysis of gap junction plaques. These investigators discovered that shifting cells containing temperature-sensitive v-Src kinase to the permissive temperature at which v-Src becomes activated did not produce changes in gap junctional area at cell interfaces at times when decreases in gap junction communication were evident. Thus, these combined data suggested that the v-Src-induced reduction in g_j was not likely the result of major changes in N, the number of gap junction channels at the plasma membrane. The elimination of γ_j and N as explanatory factors left a decrease in P_0 as the remaining factor that may explain the decreased g_j resulting from the actions of v-Src. However, P_0 cannot be reliably measured directly in all cell pairs that typically express gap junction plaques containing many gap junction channels and with each channel exhibiting multiple conductance states. Accurate measurements require cell pairs with low connexin expression.

Cottrell et al. (2003) also examined the possibility that v-Src phosphorylation may affect the selectivity of connexin43 channels to different dyes. This aspect of the study was approached by measuring the ability of v-Src cells to transfer two different dyes: Lucifer yellow (net charge

2^-, 457 Da) and NBD-TMA (net charge 1^+, 280 Da). Comparisons to the electrical coupling data showed that besides a decrease in P_0, channel, selectivity also appeared to be perturbed by v-Src phosphorylation with Lucifer yellow dye coupling being reduced to a greater degree than coupling measured with the NBD-TMA dye. Coupling measured with the latter dye was reduced greater than predicted based upon the changes in electrical conductances. Thus, v-Src acts directly to phosphorylate connexin43 gap junction channels at the Y247 and Y265 sites in the carboxyl-terminal region, which is associated with the reduction in g_j and dye transfer that are likely the consequence of diminished P_0 and possible alterations in channel permselectivity, but with little apparent effect on γ_j. This is a good example where alterations in channel permselectivity appear not to correlate with changes in unitary conductance. Since the pore of Cx43 gap junction channels is relatively large, small ions carrying an electrical current that determines the channel's unitary conductance are not selected when passing through the junction. On the other hand, the flux of larger molecules, such as fluorescent dyes, could interact more with the channel pore regions, for which there is a molecular size-related selection, and hence its transmission could be significantly modified and observed experimentally.

Effects of other tyrosine protein kinases on channel gating established by other connexins—Much less is known about the tyrosine kinase regulation of the gating of gap junction channels established by other connexins (Warn-Cramer and Lau, 2004). However, one study offers relevant and intriguing insights into the tyrosine kinase regulation of the gating established by mouse connexin45 (van Veen *et al.*, 2000). In this work, Hela cells, expressing exogenous mouse connexin45, were treated with pervanadate, a tyrosine phosphatase inhibitor, and the effects on g_j, N, γ_j, and connexin45 phosphorylation were observed. Pervanadate at 0.1 mM strongly reduced g_j from 10.5 to 4.5 nS, a reduction of approximately 57%. This reduction in macroscopic conductance was accompanied by an apparent increase (142%) in the phosphorylation of connexin45 as measured indirectly by the increase in the relative intensity of the 48 kDa connexin45 band, which represents a phosphorylated isoform based upon its sensitivity to calf intestine phosphatase. However, the tyrosine-specific phosphorylation of the 48 kDa band induced by pervanadate was not demonstrated directly, but inferred by exclusion of MAP kinase activated by pervanadate using the PD98059 inhibitor and by the demonstration that epidermal growth factor, which can activate MAP kinase, did not increase connexin45 phosphorylation. Measurement of single channel conductances following halothane treatment showed control transitions averaging 21.9 pS and 39.0 pS (van Veen *et al.*, 2000), which were not significantly different from the transitions observed following pervanadate-treatment. Thus, pervanadate did not appear to affect γ_j, in a fashion similar to v-Src. N, the channel number, was unlikely affected because the pervanadate-induced changes in g_j occurred over 1-2 minutes, which was judged to be too short a time period for substantial changes in channel numbers to occur. In addition, the authors stated that immunocytochemical studies did not show any differences in the subcellular localization of connexin45. Thus, like the case for v-Src, but unlike that for PKC, pervanadate-induced phosphorylation of connexin45 was associated with a marked reduction in g_j, which appeared to result from changes in open channel probability.

An important, yet difficult question to resolve concerns the identity of the tyrosine protein kinase(s), whose actions on connexin45 were revealed by pervanadate treatment. It is indeed possible that pervanadate may activate c-Src, which could phosphorylate connexin45 directly. To this point, c-Src and Src family members have been reported to be activated by pervanadate in a diverse variety of cells (Boulven *et al.*, 2002;Fan *et al.*, 2003;Takahashi *et al.*, 2004;Heiska and Carpen, 2005). On the other hand, pervanadate inhibition of phosphatases may merely permit the detection of low level, constitutive tyrosine phosphorylation caused by currently unknown tyrosine kinases.

E) Molecular mechanisms

The mechanism by which phosphorylation modifies the unitary conductance of the channels is unknown, but phosphorylation of the connexin carboxyl terminal region is required, and the resulting charge alteration may induce changes in the affinity of the carboxyl-terminus tail to complementary sites located in other intracellular domains, possibly the cytoplasmic loop region, which may modulate the channel to the partially open state. In the case of v-Src-induced phosphorylation of connexin43, another intriguing possibility is the creation of a unique phosphotyrosine residue, which may serve as a docking site for a putative SH2 (or PTB) domain-containing protein. This SH2-containing protein may in turn participate in the mechanism contributing to the closure of connexin43 channels. Thus, in the case of Src-induced phosphorylation of connexin43, either the tail gates and closes the channel in a manner similar as for chemical gating (both gates could be located in a distinct molecular domain) or perhaps phosphorylation at the tail leads to the interaction of novel proteins which contribute to channel closure. These distinct mechanisms remain to be elucidated in the future.

As we have presented in these previous sections, it is surprising that distinct phosphorylation pathways, which phosphorylate neighboring residues of the carboxyl-terminus tail, can induce such a variety of responses: from a total closure of channels, evidenced by a reduction in channel open probabilities to partial closure by shifts in channel unitary conductances. These observations cause one to consider the high specificity that is required to produce these distinct responses of a channel, especially when the phosphorylated residues are localized nearby one another in the COOH terminus.

F) Phosphorylation and channel perm-selectivity

A change in permeability towards large molecules would be associated intuitively with a change in channel pore dimensions. In phosphorylation-induced gating, smaller or larger channel pores will lead to a reduction or increase in permeability towards large molecules (Kwak *et al.*, 1995c;Kwak and Jongsma, 1996). Nonetheless, it's also reasonable to consider that a change in surface charge at the pore region, caused by negatively-charged phosphorylated residues, might also induce alterations in a channel's permeability and selectivity (Martinez *et al.*, 2002;Veenstra *et al.*, 1995), even though this phenomenon could not be directly related to pure gating. In this case, the channel's unitary conductance is not the only property changing, but also the permeability and selectivity of the pore to larger molecules. Here, N and open probability (Po), will still be part of the equation responsible for determining junction conductance changes, but permeability becomes an important issue, especially when unidirectional fluxes are considered (Suchyna *et al.*, 2000;Zhong *et al.*, 2003). Since gap junction channels are formed by connexins expressed in two distinct cells, channels can be assembled in heterotypic configurations (for example, Cx26/Cx32 or Cx45/Cx43). Since the perm-selectivity properties of each connexon can be different, these heterotypic channels can facilitate the flux in one direction, but not in the other. In electrical terms this is considered a rectifying element, and rectification to small ions or molecules seems to be less conspicuous than to large metabolites. Physiologically, in a particular tissue, the movement of metabolites could be favored in one direction, inducing a heterogeneity of molecule transmission that could be relevant to embryological differentiation processes.

This issue is important because many investigators use fluorescent dye permeability as a measure of changes in junctional conductance. In most cases, there is a clear correspondence between the gating of connexin channels and their reduction in permeability to the most common fluorescent dyes, such as Lucifer yellow or 6-carboxyfluorescein (Kwak *et al.*, 1995c;Veenstra *et al.*, 1995;Veenstra, 2000). However, Kwak et al. (1995c) reported little correlation between dye permeability and total conductance in experiments performed using cells expressing connexin43. Initial studies using neonatal rat myocytes indicated that activated

PKC caused a significant increase in junctional conductance, whereas permeability to Lucifer yellow was robustly reduced. This paradoxical result suggested that gating to a reduced conductive substate may occur, concomitantly with an increase in open probability. Therefore, data obtained through only dye coupling measurements should be considered cautiously. Some of the data reported may not be related directly to channel gating, but to changes in channel selectivity or permeability. This possibility should be considered seriously in future experiments that attempt to determine the mechanisms underlying gating by distinct protein kinases and intermediaries. For instance, the conductance of connexin45 junctions was substantially diminished upon TPA-induced phosphorylation, which was accompanied by a reduction in permeability to Lucifer Yellow, but not to neurobiotin (Martinez *et al.*, 2002). In another example, recent data suggested that activation of PKC can reduce connexin43 permeation to TBS, a low molecular weight dye, suggesting the possibility of a constriction in pore size that reduces permeability to large molecules (Ek-Vitorin *et al.*, 2006). And lastly, as discussed previously, v-Src phosphorylation also appears to induce changes in connexin43 dye selectivity, in addition to changes in P_0.

G) Modulation of other gating through phosphorylation

Gating by transjunctional voltage appears to be a phenomenon not related to a single region of the connexins channel. In connexin43, the carboxyl terminus has been associated with transjunctional and pH gating, meaning that this part of the molecule can move and occlude the pore of the channel, restricting the passage of ions or other solutes (Anumonwo *et al.*, 2001;Moreno *et al.*, 2002b;Revilla *et al.*, 2000). In a report by Moreno et al. (1993), the dialysis of alkaline phosphatases inside a cell induced a rapid decrease (50%) in junctional conductance of SKHep1 cells expressing connexin43. Concomitant with this reduction in conductance, there was a robust reduction in the time constant of voltage gating from 1.5 to 0.5 sec at 60 mV. Since the carboxyl-terminus tail is involved in the voltage gating process of connexin43 channels, it was proposed that dephosphorylation may reduce the energy necessary to induce voltage gating.

The intracellular termini of other connexins have been related to voltage gating, as exemplified with connexin32 and connexin26 (Purnick *et al.*, 2000a;Purnick *et al.*, 2000b). Amazingly enough, the phosphorylatable residues in the COOH terminus of Cx32 have been correlated with the modulation of channel assembly (Barrio *et al.*, 1999), indicating that what occurs on the intracellular side of the channel, in this case the COOH terminus, could be reflected in the extracellular side (e-loops). Other structures like the e-loops appear to participate in loop gating of hemichannels and may also be responsible for chemical gating of full channels (Bukauskas and Verselis, 2004). It would be interesting to determine if gating of this domain is also modulated by protein phosphorylation.

I) Effects of protein phosphorylation on heteromeric connexin channels

The co-expression of connexin45 and connexin43 produces bi-heteromeric channels with reduced sensitivity to TPA stimulation (Martinez *et al.*, 2002). Homotypic Cx45 and Cx43 channels are both inhibited by as much as 70% after TPA stimulation. When bi-heteromeric channels become phosphorylated during TPA treatment, this reduction is less than 50%. Since not all channels are expected to be identical, the average smaller reduction seems to indicate that some channel combinations respond less to activated PKC. The mechanism involved in this desensitization is unknown and needs to be thoroughly analyzed to determine the phosphorylation characteristics and interaction between connexins, although it has been determined that hyper-phosphorylated connexin43 is found in cells co-expressing connexin43 and connexin45 and this appears to induce the reduction of the unitary conductance of the mono-heteromeric channels (Zhong *et al.*, 2003). The effects of phosphorylation on other cardiac connexin combinations have not yet been studied.

J) Final thoughts

Until now, our knowledge of the mechanism of gating of gap junctional channels during protein kinase activation has been mainly restricted to homomeric channels, a few hemichannels, and much less to heteromeric combinations, although the temporal and regional co-expression of distinct connexins in mammalian tissues strongly suggests the formation and importance of hetero-multimeric channels. The demonstration that connexins can be phosphorylated and that their conductive and perm-selectivity properties are quite distinct, indicates the need to illuminate the mechanism(s) underlying the regulation of their gating. The limited number of gates that have been demonstrated in gap junction channels strongly suggests that most of the voltage-dependent and chemical gating kinetics would be affected by connexin phosphorylation. Moreover, despite the data suggesting that direct phosphorylation affects gap junction channels, it is important to recall that connexins forming the channels interact with other proteins localized adjacent to the plasma membrane. Such molecular complexes may also be affected by phosphorylation, which in turn may also induce changes in the permeability of channel gating. Thus, it is critical to understand how these distinct phosphorylation pathways affect homotypic or heterotypic channels and connexin-associated proteins, in order to understand and fully appreciate the influence of this pivotal regulatory mechanism on the modulation of the gating of intercellular gap junction channels.

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