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Review

Connexin phosphorylation as a regulatory event linked to channel gating

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Abstract

The main proteins required for functional gap junction channels are known as connexins and most of their isoforms indicate that they can become phosphorylated. Connexin phosphorylation has been reported to participate in modifying junctional communication and the mechanisms involved apparently depend on which kinase becomes involved. Although multiple reports have suggested a strong influence of phosphorylation on channel gating, not enough physiological studies have been performed to determine precisely the gating mechanisms implicated. Moreover, gap junction channels follow other various gating mechanisms, including voltage gating and chemical gating, where phosphorylation could act as a modulator. The quest for this chapter has been to discriminate those instances where phosphorylation acts directly as a gating trigger and where it acts indirectly or only as a modulator. Despite recent efforts, the mechanisms involved in all these cases are barely understood.

Keywords: Gap junction; Gating; Protein phosphorylation; Cell to cell coupling

Contents

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1. Introduction

Among the multiple membrane channels, only gap junction channels are known to allow intercellular communication between the cytoplasms of contiguous cells in tissue, and in some configurations, these channels allow direct communication between the cytoplasm and the extracellular media. Gap junction channels can be formed of one or various protein isoforms named connexins, that constitute a family of highly homologous proteins. A new family of proteins termed pannexins is emerging, and it would become necessary to determine their abundance and physiological relevance in mammalian tissues [1].

Some of these gap junction channels are highly selective for small ions or molecules, and their gating mechanisms involve intracellular messengers; this indicates that their presence is required to maintain essential functions in many organs. Natural or induced mutations in these proteins cause the development of severe diseases to appear [2].

The permeability properties of cardiac gap junction channels have been studied for more than 35 years [3], and from those day until now, it has been a great challenge, mainly because the proteins (connexins) that form these channels display intrinsic isoform diversity, and because of their capacity to form heteromeric connexons in vivo [4]. Since each connexin has unique gating as well as permeability and selectivity properties, some combinations of connexins produce intermediate or new biophysical properties, which would definitively change the interconnecting properties between cells.

2. Regulation of gap junctional communication by phosphorylation

The activation of protein kinases [5–8] and protein phosphatases [9,10] have been correlated with a reduction or increase of junctional communication, therefore, since those first reports [11–13] it has been reasonable to speculate that phosphorylation induces channel gating. Nonetheless, to understand and control properly junctional communication through changes in phosphorylation, it has become necessary to determine the exact mechanisms of action, especially because the activation of distinct phosphorylation pathways have been shown to participate in many different mechanisms involved in the rapid turn over of channels, which in turn alters cell-to-cell communication [14]. In short, it is necessary to differentiate if a change in junctional communication is related to a pathway that induces bona fide channel gating or a pathway that targets the channels to be removed or incorporated into the cellular membrane.

3. Gating

It has been amply demonstrated that connexins form membrane channels that, in their connexon or full channel configuration, can open or close. If compared to channels from excitable channels, gap junction channels are often sensitive to molecules or transmembrane voltage and as for other membrane channels, under a stimulus, a sensor and a gate are involved in the process of channel opening or closing.

The word gating is often used quite loosely to refer to any and all channel molecular transitions on its way to opening or closing, broadly meaning by this, that during gating, a conductive pathway becomes either physically available or un-available. Gating has also 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 [15]. Therefore, we can intuitively consider gating for membrane channels, as the mechanism that closes or opens a membrane channel and this should not be different from what occurs in a gap junction channel.

For the purpose of this chapter and following Bertil Hille's definition [15], gating of membrane channels will be defined as the induced conformational change of a protein that involves a fast and reversible change in conductive properties. This should include a reversible process where a complete or relative closure or opening of a channel occurs. This way, at any sub-cellular level in which gap junction channels, connexons or hemichannels suffer a conformational change that alters reversibly their conductance can be considered a gating event. Note here that those events that occur when the connexons are at the cytoplasmic membrane are relatively easy to determine, and in turn, those are the ones that have been described in most of recent studies [16–19]. This is because most voltage gating techniques have been developed to determine the conductive properties of membrane channels, as the ones formed by connexons [10,20,21] but we should not forget those changes that occur at subcellular levels where the access to conductance measurements is difficult, as in Golgi or transport vesicles.

In contrast to changes in junctional conductance through channel gating, it is also easy to envision changes in intercellular communication without channel gating. Here, a group of gap junction channels in a plaque can be removed in an annular ring [22,23]. In this case, the reduction in the number of channels will bring an instantaneous large reduction in total conductance between the cells. Hence, to understand if phosphorylation intervenes in the mechanism that drives channels to gate, it would be necessary to consider the following ideas about gap junction channels' gating:

1) Gating is intrinsically linked to the molecular structure of the channel, and there are several different ways to modify the structure of the channel and induce gating [24].
2) There are activating and inactivating gating mechanisms that drive the channels to allow larger or smaller conduction, including the non-conductive state or closed state, for example, chemical gating by CO_2 [25].
3) Since most of the membrane channels are known to have more than one conductive state, gating also includes those

processed that drive each channel to a different conductive state, for example, phosphorylation of Cx43 by PKC [26].

4. Parameters involved in modulating GJC through phosphorylation

In order to change the electrical communication between cells, the total conductance γj will be directly related to product of the number of channels (*N*) times the unitary γj of each one of the channels in the junction times the open probability of each one of the channels. These three parameters are all correlated and only two of them will indicate if gating is involved:

N, or the number of channels present in the membrane, can be affected by inserting or removing connexons at different rates from the junctional membrane. This in turn will be directly affecting junctional communication, but certainly it is not per se a gating phenomenon. An example worth to mention to this regard corresponds to the rundown of channels that leads to a reduction in coupling, in particular where double whole cell voltage clamp experiments are performed and that could be partially prevented by preventing protein de-phosphorylation, although this change in phosphorylation is not associated with connexins directly, but probably to other regulatory molecules associated with connexins [27].

The unitary conductance γj of each channel can also be affected by changing the phosphorylation level of proteins in the cell. This has been the first evidence that there is a molecular modification of the gap junction channel (Cx43) that leads to a change in the conductance of a channel [28]. Apparently, the channel formed by Cx43 can gate between three different conductive states, and the phosphorylation of connexins favours or stabilizes the transitions into the intermediate state [26,29].

The open probability (Po) is the third parameter that can be altered to change junctional conductance, and this has been determined to change in some instances, including that of connexin45 expressed in HeLa cells after the activation of cAMP-dependent kinases [30].

A change in permeability to large molecules would be intuitively associated with a change in the pore dimensions of the channels. After gating due to phosphorylation, smaller or larger channel pores will lead to a reduction or increase in the permeability to large molecules [31,32]. It is also reasonable to think that a change in surface charge at the pore region, due to negatively-charged phosphorylated residues, could also determine changes in the permeability and selectivity of the channels [33,34], although this phenomenon could not be directly related to pure gating. In this case, the unitary conductance of the channel is not the only property changing but also the permeability and selectivity of the pore. Here, *N* and open probability (Po) will still be part of the equation that determines changes in junctional conductance, but the term permeability becomes an important issue, especially when unidirectional fluxes are considered [35].

5. Sub-cellular regions where phosphorylation can affect channel gating

As mentioned above, to regulate junctional communication by phosphorylation, the cells may follow three known mechanisms: changing the total number of channels in a junction, the permeability of these channels and/or the time they remain open. Moreover, any cellular mechanisms that affect these three parameters will affect directly not only the communication between cells but also the communication between the cytoplasm and those intracellular compartments where functional connexons become part of their membranes.

Protein phosphorylation is a broadly distributed phenomenon that could affect at many proteins and enzymes at different levels but just a few will be related to the process known as gating. For example, to maintain the intracellular milieu, it is expected that most connexons need to be in the closed state as they are inserted in the membrane. Therefore, it is feasible that those phosphorylation events that occur in cellular organelles [36] may correspond to a pre-gating process necessary to keep the connexons closed. This needs to be demonstrated, although some evidences indicate that these connexons are closed since vesicles that carry these connexons need to have a particular intra-vesicular pH and internal media regulated [37], therefore it is expected that while these channels become inserted in the membrane they are in the closed state.

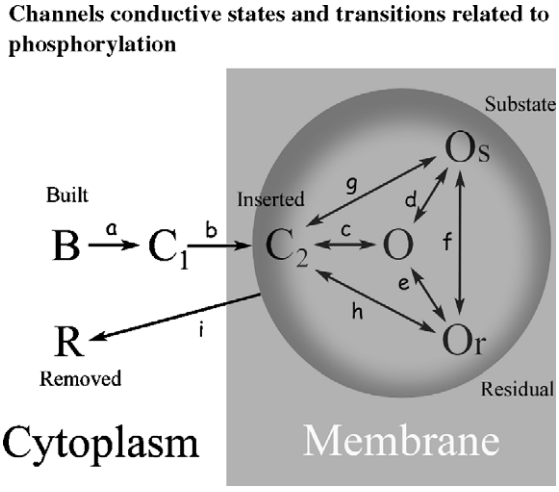

Fig. 1. Schematic representation of the distinct connexon/channel conductive states and the transitions to/or from one state to another. B and R correspond to the building and removed states. Note that all gating processes (except for a) occur at the cytoplasm membrane. Although the direction and preference may or may not be influenced by phosphorylation, they are included to try to represent all possibilities. The transition from the circle to R indicates that channels or hemichannels at any state may be removed from the cell membrane.

Channel states	Transitions
(C indicates a closed state, and O, an open state)	a. Gating: closing of connexons in vesicles
B. Building of the hexameric connexon	b. Incorporation into the membrane
C_1. Vesicle-related closed connexons	c. Docking activation of full channels and activation of hemichannels and complete closure of channels
C_2. Connexon incorporated into the membrane (hemichannels)	d. Gating between O and Os
O. Full open channel (or hemichannel)	e. Gating between O and Or
Or. Channel's residual conductance	f. Gating between Or and Os
Os. Channel's conductive sub-state	g. Gating between Os and C_2
R. Removed channel	h. Gating between Or and C_2
	i. Removal from the membrane

Another possibility is that this pre-closing is due to the way connexins are assembled, indicated that connexons stable structure as hemichannels is closed, and when they dock with other connexons they open. So probably some of the phosphorylation events that occur during channel trafficking are related to keep the channels closed. De-phosphorylation at the surface may allow them to reopen. Or maybe docking takes place and no de-phosphorylation is needed.

One interesting point is related to Cx26. Since this connexin has been shown not to be a phosphorylatable protein, it has been postulated that those connexins become inserted in the membrane before being oligomerized [38]. What about heteromeric connexons that are assembled through other pathways [39]? Are these connexons also closed in its natural state and become open when forming a complete channel? These differences need to be elucidated, especially for Cx26, since it is not a phosphorylatable protein and it seems to co-oligomerize with other connexins [40].

6. Pathways' analysis to determine whether phosphorylation may induce gating

6.1. Transition a. Connexon building (B to C_1)

The first gating process that a connexon may suffer is expected to occur after it has been built. Either the connexon has been built in a closed configuration, or it becomes necessary to keep it in the closed state since its conductance and low selectivity can prevent the membrane to become leaky as soon as the new connexons are inserted into the plasma membrane. Phosphorylation is a process that occurs even before this stage, when the channels are being assembled [67]. One of the functions of phosphorylation at this stage would be to increase the probability of closing of the channels, although apparently their COOH tail is not facing Golgi's intracellular media. As Golgi's vesicles require to control their internal milieu [37], connexons inserted in the membrane should remain closed. Then, the phosphorylation of connexins at this stage may represent a mechanism involved in keeping the connexons closed or with very low permeability or high selectivity. This has yet to be proven although it has been demonstrated that a non-phosphorylatable connexin (Cx26) has a separate traffic path to reach the membrane [41], suggesting the relevance on the phosphorylation sites for connexins' transport through Golgi.

6.2. Transition b. Membrane incorporation (C_1 to C_2)

This could hardly be considered itself as a gating process, unless there are demonstrated changes that show that the connectivity of the tissue becomes altered with this. 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 [42], therefore the balance between incorporation and removal should alter the communication level between cells. Nonetheless, some studies strongly suggest that the phosphorylation of the COOH terminal may not be necessary for the incorporation of the channels into the membrane [16,43,44].

6.3. Transition c. Activation of channels during docking (C_2 to O)

For full channels, this is definitively a gating process, where connexons dock and become a full conductive channel [45]. Little has been investigated on how phosphorylation can be involved (but see Ref. [46]) in this process although it may result extremely important to the generation of quick transitions that improve junctional communication through the addition of connexons to the membrane.

Although the interaction between connexin docking seems to be favoured by phosphorylation processes [46], this mechanism has not been clearly determined. This process of opening may be accelerated through the intervention of kinases.

6.3.1. Chemical/Vm gating (from O to C_2)

Through this same pathway, open channels may close completely through triggers that include low pHi, membrane anaesthetics, increase in cytosolic Ca and others [47,48]. In a gating process like this, phosphorylation can participate actively since the tagging by PO_4 residues may change the properties of the cytoplasmic terminals and favour the closure of the channels.

Here resides the bulk of the discussion for this chapter, since it has been proposed that v-Src reduces the open time of the channels to reduce conductance between cells; this has recently been strongly examined [5]. Inside the circle of Fig. 1, gating can occur between the different channel states, including the closing of any open state to the closed C2 state. If v-Src reduces the conductance only through channel gating, then the total number of channels should remain identical. According to the studies by Lin et al. [49], the

junctional area is not modified, indicating that if the number of channels does not change, then γj or Po changes could be responsible. In studies followed by Cottrell et al. [5], v-Src and MAPK expressing cells communicated through Cx43 channels show no change in the distribution of conductances, suggesting that the open probability is the parameter that becomes reduced to decrease junctional communication between the cells.

For Cx45, there are differential effects with regard to the type of kinase activated [50,51]. For mCx45 expressed in HeLa cells, PMA (an adenylate cyclase activator) increases the total conductance between the cells, and cAMP and pervanadate (activator of MAPK) reduce it. All these effects occur without a shift in the unitary conductance of the channels (under halothane) indicating that either the number of channels or their open probability is changing. According to van Veen's data, the effect of PMA on protein phosphorylation (as seen through Western blotting) is negligible; therefore, the gating of Cx45 could be due to un-specific pathways or changes in phosphorylation of other regulatory proteins associated to these channels. In the case of pervanadate, its effect appears to be mediated by tyrosine phosphatase inhibition, indicating that the possible decrease in conductance is consistent with v-Src inhibition found in Cx43 [5]. Phosphorylation by cAMP is the one consistent with a gating mechanism induced by phosphorylation that does not go to an intermediate conductive state and apparently does not affect the number of channels. cAMP, but not cGMP increases connexin phosphorylation and decreases the total conductance, indicating that the open probability of the channels is substantially reduced.

Cx45 is a connexin that forms channels strongly influenced by voltage gating, in particular to transmembrane voltage gating (Vm). Although the authors have not presented data that indicates the contrary, it appears as if phosphorylation is modulating Vm. This is reinforced by the fact that for this connexin channels, the gates for Vm, and chemical gating appear to be the same or located in the same channels regions [24]. cAMP is known to increase assembly, so in this case, it seems that this tow processes are going in opposite directions, so maybe the gating effect by phosphorylation is larger but the authors could not detect it.

Finally, gating at the cytoplasmic membrane will constitute one of the physiological ways to maintain coupling between cells during specific functions, like in the retina, where closing and opening can work as a real gating modulator for visual modulation [52].

Recent work has established that phosphorylation at Tyrosine Y265 resulted from a cascade of events in which ERK was a major participant [53]. This work clarified that the whole CT is required for the inhibition of conductance. Hence, ERK appears to gate Cx43 channels rapidly. The mechanism associated with this gate requires the presence of CT [43] where the presence of CT independently co-expressed with tailless channels recovered inhibition during phosphorylation [54]. This strongly suggest that CT is an integral part of the gating mechanism, although it has not been clarified yet how the closing of the channel occurs, hence reminds to determine if CT participates as a gate or as an effector that triggers other gating mechanisms in the channel. As it will been presented later, gating by phosphorylation not only performs closure of the channels, but also changes in its permeability to large molecules.

6.4. Transition d. Gating to a sub-state (O to Os)

This gating process was the first one related to the activation of kinases in mammalian cells [28,55]. In this case, Cx43 can be phosphorylated at the COOH terminus through PKC and this induces a change in the residence of the open state at a 60 pS sub-level. This process is clearly depending on the phosphorylation of residue 368 as was demonstrated by the use of Cx43 mutants [56]. Not all connexins do respond in the same way and it can be species dependent as murine and human Cx43 respond differently [57].

In the case of Cx46 hemichannels, the gating after activating PKC (PMA) or blocking phosphatases reduces their total currents to 40–30% of their original value. Single cha- nnel recording on these channels will clarify some the closing mechanism, but for now, and due to the fact that the reduction of the currents was not complete, it strongly suggests transitions to residual or sub-states (see transition e below).

Studies of the changes in biophysical properties due to the phosphorylation of Cx40 have been scarce. The activation of PKA reduces the time that Cx40 channels spend in sub-states, indicating that the total conductance of the junction probably increases [58]. In a similar fashion, the phosphorylation of Cx32 by cAMP is correlated with an increase in conductance, although no single channel data is available to corroborate if there is a change in unitary conductance or open probability. In contrast, this type of gating is not observed in Cx45 channels by different treatments including cGMP, cAMP, PMA and pervanadate. In all these cases, there is no shift in the distribution of unitary conductances but it appears to be more like a change in open probability of the channels (see O to C_2).

For lens Cx46 and Cx56 channels, the activation of PKC enhances protein phosphorylation and a reduction in junctional communication. Cx49 can become phosphorylated by casein kinase I [59] and its inhibition increases intercellular communication. At this point, there are no single channel data that can help to determine the mechanism of gating for channels formed by these connexins.

The mechanism by which phosphorylation modifies the unitary conductance of the channels remains unknown, but phosphorylation of CT is required, and probably the alteration of charge changes the affinity of the main channel, which, in turn, maintains the channel in a partially open state. A rapid reduction in junctional communication is also observed during the activation of ERK. This kinase

phosphorylates serine residues 255, 279 and 282. Although there are no single channel recordings, the rapid change in electrical communication indicates a gating mechanism and possibly a direct interaction [54]. Further studies would be required to determine the nature of these changes.

6.5. Transitions e, f. Gating to a residual or in between residual and sub-state (from O to Or or Os)

This gating is a known process that requires transjunctional voltage across a full channel, or transmembrane potential for hemichannels. In this case, and for transitions g and h, few experiments have been related to the participation of phosphorylation to regulate their kinetics.

6.6. Transition i. Removal from the membrane

Gap junction channel turn over is an active phenomenon that requires a few hours in some tissues to renew gap junction plaques. It has been recently confirmed [22,42,60] that the removal of gap junction channels from plaques requires the formation of annular junctions which can be considered as internalized vesicles, with a double membrane that contains several gap junction channels. During this process of vesicle internalization, channels may or may not stay at the open state. Although not demonstrated yet, these channels may remain open during the formation of vesicles and then gate as they are internalized and become digested by lysosomes or proteosomes [61]. Electrophysiologically, this could be represented as large, non-reversible conductance transitions that represent the closure of a vesicle that engulfs multiple channels. Although electrophysiologically this resembles the closing of multiple channels, this can hardly be considered a gating phenomenon dependent on protein phosphorylation.

7. Impact of phosphorylation on channel perm-selectivity

This issue is quite important, since many authors use fluorescent dye permeability to determine changes in junctional conductance. In most of the cases, there is a clear correspondence between the gating of connexin channels and their reduction in permeability to the most common fluorescent dyes, like Lucifer yellow and 6-Carboxyfluorescein [32].

As most of these cases, some data presented may not be related directly to channel gating but change in channel selectivity of permeability [32,34,62]. This should be considered seriously in future experiments to determine the mechanisms behind gating by distinct kinases and intermediaries. For instance, the conductance of the junctions formed by chicken Cx45 is substantially reduced when phosphorylated by TPA reducing also its permeability to LY, but not to Neurobiotin [33].

8. Voltage activated and inactivated channels have a sensor and then a gate that reduces their conductance

Gating by transjunctional voltage appears to be a phenomena not related to a single region of the connexins channel. In Cx43, the carboxyl terminus has been correlated 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 [16,44,63]. The amino terminus has been also related to voltage gating in Cx32 and Cx26 [64,65]. Amazingly enough, the phosphorylatable residues in the COOH terminus has been correlated with the modulation of channel assembly, indicating that what occurs in one side of the connexin molecule could be reflected in the opposite side. Besides, other structures like the e-loops appear to participate in loop gating of hemichannels and also to be responsible for chemical gating of full channels [24].

9. Heteromultimeric channels and phosphorylation

There are not abundant experiments focused on changes in junctional communication by phosphorylation in heteromultimeric channels. This should become one of the most exciting and difficult areas to explore, as it appears that the norm is the cellular expression of multiple connexins [2].

The co-expression of connexin45 and Cx43 produces bi-heteromeric channels with reduced sensitivity to activation by TPA [33]. Homotypic cCx45 and rCx43 have both been inhibited by as much as 70% after activation with TPA. When bi-heteromeric channels become phosphorylated during TPA activation, this reduction is less than 50%. Since not all channels are expected to be identical, the average lesser reduction seems to indicate that some channel combinations respond less to activation by PKC. The mechanism involved in this de-sensitization is unknown and needs to be thoroughly analyzed to determine the interaction between connexins, although it has been determined that hyperphosphorylated Cx43 is found in cells expressing both Cx43 and Cx45 and this appears to induce the reduction of the unitary conductance of the mono-heteromeric channels [66]. The effects of phosphorylation on other cardiac connexin combinations have not yet been studied.

10. Summary

Until now, our knowledge of the mechanism of gating of gap junctional channels during kinase activation has been mainly restricted to homomeric channels, a few hemichannels, and even less to heteromeric combinations, although the temporal and regional co-expression of distinct

connexins in mammalian tissue strongly suggests the formation of heteromultimeric channels.

The demonstration that connexins can be phosphorylated and that their conductive and perm-selectivity properties are quite distinct indicates the necessity to learn and describe their gating mechanism. Besides, apparently a limited number of gates are present in these gap junction channels which strongly suggest that most of the voltage-dependent and chemical gating kinetics would be affected by phosphorylation levels.

Despite all data suggesting that a direct phosphorylation affects gap junction channels, it is important to recall that these channels interact with other proteins close to the membrane and form part of conglomerates where protein–protein interactions may be affected by phosphorylation, which in turn may also induce changes in the permeability of channel gating.

Unless we understand how these distinct phosphorylating pathways affect either homotypic or heterotypic channels, or those proteins associated with gap junction channels, we will not be able to determine and control the influence of the most important metabolic pathways in the regulation of cellular intercommunication.

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