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Regulation of gap junctions by protein phosphorylation

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Abstract

Gap junctions are constituted by intercellular channels and provide a pathway for transfer of ions and small molecules between adjacent cells of most tissues. The degree of intercellular coupling mediated by gap junctions depends on the number of gap junction channels and their activity may be a function of the state of phosphorylation of connexins, the structural subunit of gap junction channels. Protein phosphorylation has been proposed to control intercellular gap junctional communication at several steps from gene expression to protein degradation, including translational and post-translational modification of connexins (i.e., phosphorylation of the assembled channel acting as a gating mechanism) and assembly into and removal from the plasma membrane. Several connexins contain sites for phosphorylation for more than one protein kinase. These consensus sites vary between connexins and have been preferentially identified in the C-terminus. Changes in intercellular communication mediated by protein phosphorylation are believed to control various physiological tissue and cell functions as well as to be altered under pathological conditions.

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Introduction

Most cells, with the exception of few types, such as spermatozoids, red blood cells, and skeletal muscle of adult vertebrates, can communicate to adjacent cells by gap junctions. These membrane specializations, also referred to as nexuses or macula communicans, contain intercellular channels which mediate movement of ions and small molecules (<1.2 kDa) between contacting cells. Each channel is formed by two hemichannels or connexons and each one of them is contributed by one of the two adjacent cells. A connexon is an oligomeric assembly of six polypeptide subunits termed connexins (Cxs) which are highly homologous and are encoded by a gene family (1,2). While a particular Cx (e.g., Cx43) can be expressed by a wide spectrum of tissues and cell types (3), the expression of some other Cxs (e.g., Cx33, Cx50 and Cx30.1, found in testis, lenses and skin, respectively) is apparently much more restricted (4,5).

Although Cx33 is unable to form functional homotypic channels when its tran-

script is translated in Xenopus oocytes, usually the expression of a single Cx type is sufficient to establish intercellular gap junctional communication (2). Frequently, a single cell can express more than one Cx, which can be localized in the same (6,7) or in different gap junction plaques (8). Moreover, functional gap junctions can be established in an exogenous expression system (4,5,9-17) where the role of protein phosphorylation in channels formed by a particular Cx can be analyzed at the functional level.

The extent to which cells are functionally coupled by gap junction channels depends on a multiplicity of control mechanisms, including gene transcription, message stability, translational and post-translational modification of the protein, and assembly into and removal from the membrane. In addition, a number of factors affect gating of assembled channels (1). Analysis of mechanism and regulation of each of these steps has become a key element in the study of gap junctions.

Gap junction regulatory mechanisms can lead to an increase or reduction of intercellular coupling with a wide spectrum of time courses (from milliseconds to hours) (18). The turnover of Cx26, Cx32 and Cx43 is between 2 and 5 h (7,19-25). Hence, changes in intercellular coupling that occur within a time course of a few hours in cell types expressing these Cxs could involve any of the steps in Cx biosynthesis from transcription to degradation. It has been shown that changes in Cx mRNA transcription rate and mRNA stability can be affected by the activation of intracellular second messenger pathways and affect intercellular gap junctional communication within a few hours. Although these changes could involve protein phosphorylation they will not be presented in this article and readers are referred to reviews published elsewhere (1,18).

Formation of gap junctions also requires appropriate cell adhesion mediated by either Ca^{2+}-dependent (NCAMs) or Ca^{2+}-independent (cadherins) cell adhesion molecules (23,26-30). Cell lines deficient in cell adhesion molecules do not assemble gap junctions and Cx43 localizes in a perinuclear cytoplasmic compartment (23,29), where it is found preferentially in its unphosphorylated form (23). Transfection with cDNA encoding a cell adhesion molecule induces at least two changes in Cx43: it promotes i) the insertion of Cx43 into the plasma membrane and assembly into gap junctions (23,29) and ii) phosphorylation of Cx43 (23). Although phosphorylation might play a role regulating the insertion and/or assembly of a phospho-Cx into the plasma membrane (23), this might not be an absolute requirement. For example, in exogenous expression systems the expression of mRNAs encoding Cx43 or Cx32 mutants with a shortened carboxyl terminal (devoid of all phosphorylatable seryl residues) is able to induce intercellular coupling (12,17). In addition, it has been reported that in an MDCK cell line, while formation of gap junctions depends on cell contact, phosphorylation of Cx43 by a protein kinase C-dependent pathway can occur in the absence of Ca^{2+}-dependent cell adhesion activity (30) and does not correlate with expression of intercellular coupling.

Phosphorylation of Cxs and possible functional roles

Studies on the membrane topology of Cxs have shown that both the amino and carboxyl termini of the Cxs are located inside the cell (31-33). The carboxy terminal region is one of the most divergent regions among Cxs and is where most of the phosphorylation sites have been identified (34-36). Thus, this region is likely to account for many Cx-specific properties, including responses to phosphorylation. Nonetheless, phosphorylation of amino acid residues located in the cytoplasmic loop of Cx56 has been recently shown to occur (36).

Cx26 has been reported not to be phosphorylated in rat hepatocytes (7) or in isolated mouse gap junctions treated with cAMP-dependent kinase (cAMP-dPK) (7,34), protein kinase C (PKC) or Ca^{2+}/calmodulin-dependent kinase II (Ca^{2+}-CM-dPK II) (34). Presently, nine Cxs are known to be phosphoproteins and their state of phosphorylation can be affected by different protein kinases (Table 1). Nonetheless, only few phosphorylation sites and protein kinases that phosphorylate them have been identified.

Various phospho-forms of Cx43 can be resolved by immunoblotting, making it a useful technique to evaluate its state of phosphorylation (13-15,20,25,29,30,33,35,37,41, 42,45-56). Nonetheless, not all changes in the state of phosphorylation of Cx43 alter the electrophoretic mobility of the protein (42). In addition, immunoblots do not allow further analysis of the different Cx43 phospho-forms (i.e., amino acid analysis and two-dimensional tryptic maps). Therefore, analysis of the state of phosphorylation of Cxs, including Cx43, requires complementary studies with metabolic radiolabelling followed by immunoprecipitation.

Cyclic nucleotides, diacylglycerol, lysophosphatidic acid, tumor promoter phorbol esters, growth factors, and oncogene products ($pp60^{v\text{-}src}$, $p130^{gag\text{-}fps}$ and ras oncogen) alter intercellular gap junctional communication and effect of some these agents depends on cell and Cx type (13,16, 20,30,34-36,38,41-47,51-71). It has also been suggested that phosphorylation of Cx43 in seryl residues might be involved in a number of different processes including its insertion into the plasma membrane (56), increase in its degradation, changes in unitary conductance of single gap junction channels and closure of gap junction channels (13,16, 45,57,61,62). In addition, phosphorylation of tyrosine residues has been associated with reduction in intercellular coupling (10,20).

Effect of Cx32 phosphorylation on intercellular gap junctional communication

Cx32 was one of the first channel-forming proteins shown to be phosphorylated in intact cells (63). Activation of either cAMP-dPK or PKC increases the state of phosphorylation of Cx32 (34,63,70,72) and at least the effect of cAMP-dPK is temporally correlated with an increased junctional conductance (g_j). Moreover, Chanson et al. (71) reported that an increase in intracellular [cAMP] in a human colonic T84 cell line induces a rapid (<20 min) increase in intercellular gap junctional communication mediated by Cx32 gap junctions, that is directly related to an increase in fluid secretion.

Table 1 - Connexins known to be phosphoproteins.

Phosphorylated amino acid residues identified in the intracellular domains of connexins are indicated. Isolated gap junctions, fusion proteins or synthetic peptides were used as substrates for *in vitro* phosphorylation assays. Purified protein kinases used in these assays are also indicated. ND: Not determined; Ser: serine; Thr: threonine; Tyr: tyrosine; PK: protein kinase; EGFR-Tyr K: epidermal growth factor receptor Tyr kinase; cAMP-dPK: cAMP-dependent protein kinase; Ca^{2+}-CM-dPK II: Ca^{2+}/calmodulin-dependent protein kinase type II; $pp60^{v\text{-}src}$: Tyr kinase encoded by the oncogene v-src; MAP K: mitogen-activated protein kinase.

Connexin	Phosphorylated amino acid residue	Protein kinase	Reference
Cx31	ND	ND	37
Cx32	Ser 233	cAMP-dPK, PKC	34
	Thr ND	Ca^{2+}-CM-dPK II	34
	Ser ND	cAMP-dPK, PKC	34
	Tyr ND	EGFR-Tyr K	38
Cx40	ND	ND	40
Cx43	Ser 266, 279, 282	MAP K	35
	Ser 368, 372	PKC	42
	Tyr ND	$pp60^{v\text{-}src}$	41
Cx45	ND	ND	43
Cx45.6	ND	Mg^{2+}-dependent PK	39
Cx46	ND	ND	44
Cx50	ND	Mg^{2+}-dependent PK	39
	ND	cAMP-dPK	
Cx56	Ser 118, 493	PKC, PKA	36

cAMP increases the amount of metabolically labelled Cx32 in primary cell cultures of fetal hepatocytes (70), implying either an increased rate of synthesis or a reduced rate of degradation. The stoichiometry of phosphorylation of Cx32 in vitro is low; by PKC it approaches 1 mol/mol and by cAMP-dPK it is about 0.1 mol of P_i/mol of protein (34,63,72). Nevertheless, the stoichiometry of phosphorylation in vivo and the cell compartment in which Cx32 phosphorylation occurs have not been determined. Moreover, in isolated liver gap junctions previously phosphorylated by PKC, treatment with cAMP-dPK does not increase the incorporation of ^{32}P into Cx32. On the other hand, if gap junctions are first phosphorylated by cAMP-dPK, treatment with PKC increases the incorporation of ^{32}P into Cx32, suggesting that PKC can phosphorylate other amino acyl residues besides those phosphorylated by cAMP-dPK (72). Using synthetic peptides corresponding to regions of the C-terminus of Cx32, it has been demonstrated that Ser 233 is phosphorylated by both cAMP-dPK and PKC (34). Other sites have not yet been identified.

A Cx32 mutant, with serine residues 233 and 240 replaced by asparagine residues, forms gap junctions in Xenopus oocytes with macroscopic gating properties (voltage dependence and pH sensitivity) that are indistinguishable from those formed by the wild-type Cx (12). These findings suggest that phosphorylation of those serine residues is not required for channel opening or closing by these conditions. Nevertheless, modulation of g_j due to changes in the assembly or retrieval of channels into or from the plasma membrane has not been studied. Phosphorylated Cx32 by PKC but not by cAMP-dPK is less sensitive to degradation by m and mμ-calpains (73), suggesting an alternative mechanism for the regulation of intercellular coupling. Thus, the extent to which intercellular coupling between Cx32-containing cells is modulated by phosphorylation requires a more exhaustive exploration.

Although Cx32 in isolated rat liver gap junctions is also a moderate substrate for Ca^{2+}-CM-dPK II, resulting in serine and threonine phosphorylation (34), the state of phosphorylation of Cx32 has not been exhaustively studied in cells treated with agents that selectively activate this kinase. Cx32 is not phosphorylated by $pp60^{v\text{-}src}$ in isolated rat liver gap junctions (Sáez JC, Nairn AC and Hertzberg EL, unpublished observation) or in Xenopus oocytes (10). Nevertheless, tyrosyl phosphorylation of Cx32 can occur in isolated liver gap junctions treated with the epidermal growth factor receptor tyrosine kinase (38). The functional consequence of Cx32 tyrosyl phosphorylation remains unraveled.

Effect of Cx43 phosphorylation on intercellular gap junctional communication

In cell lines and in neonatal (25,42) cardiac myocytes (33,50), two phosphorylated forms of Cx43 with slower electrophoretic mobilities (43-47 kDa) than the unphosphorylated form (41 kDa) can be identified either by immunoblotting or in immunoprecipitated Cx43 from ^{32}P-labelled cells. Immunoblot analysis of Cx43 in various rat tissues shows different, although tissue-specific ratios of the different forms of Cx43 (49). Variations in levels of the different Cx43 phospho-forms have also been detected during the ontogeny of the pineal gland (46). In primary culture of neonatal rat cardiocytes, Cx43 is predominantly phosphorylated in seryl residues and to a lesser extent in threonyl residues (25,42,50). Pulse-chase studies indicate that phosphorylation occurs soon after synthesis (20,23-25) and, at least in fibroblasts, dephosphorylation occurs thereafter (20). Inhibition of protein trafficking with monensin or brefeldin A reveals that Cx43 is partially phosphorylated before its exit from the Golgi apparatus (21).

In Rous sarcoma virus-transformed fibroblasts, where coupling is low, all forms of Cx43 are phosphorylated in both seryl and tyrosyl residues, and threonine phosphorylation of the least mobile species is also noted (20). A mutation replacing tyrosine 265 of Cx43 with phenylalanine does not prevent gap junction formation in Xenopus oocytes but it completely abolishes the inhibition of intercellular gap junctional communication and the tyrosyl phosphorylation induced by $pp60^{v-src}$; gap junctions formed by wild-type Cx43 and then exposed to src are phosphorylated in tyrosine residues and intercellular gap junctional communication is completely inhibited (10).

Activation of cAMP-dPK or PKC leads to various effects on cells of different types, even when cells expressing the same junctional proteins are compared (18). Frequently, in cells where the unphosphorylated form of Cx43 (Cx43-NP) predominates under basal conditions, stimulation of PKC with a tumor promoter phorbol ester (e.g., TPA) leads to rapid cell uncoupling and shifts the electrophoretic mobility of Cx43 forms (30,45,52, 53,55). By contrast, in cells where the phosphorylated forms of Cx43 predominate (e.g., rat cardiocytes) TPA promotes intercellular communication with no detectable changes in the state of phosphorylation, as evaluated by Western blots (42). Nevertheless, there are exceptions: in rat leptomeningeal cells (8), and in a rat liver epithelial cell line (IRA 20) (54) the phosphorylated forms of Cx43 predominate and TPA induces uncoupling without detectable changes in the state of Cx43 phosphorylation detected by Western blots. In IRA 20 cells TPA did not change the levels of Cx43 or of its mRNA but did result in the loss of Cx43 immunoreactivity by indirect immunofluorescence (54), suggesting that the inhibition in intercellular gap junctional communication might involve a post-translational modification that perhaps cannot be detected in denaturing gels. Therefore, Western blot analysis might not be the appropriate approach to study the correlation between all changes in phosphorylation. For example, the state of Cx43 phosphorylation studied by Western blot in rat heart myocytes does not change in response to agents that affect the activity of protein kinases or phosphoprotein phosphatases, although treatment with some of these agents does affect the incorporation of ^{32}P into Cx43 (42). The stimulation of cAMP- or cGMP-dPK or PKC does not significantly increase the incorporation of ^{32}P presumably because Cx43 is maximally phosphorylated under basal conditions. However, the incorporation of ^{32}P is greatly reduced after inhibition of protein kinase activities with staurosporine, and can then be stimulated by TPA (42). In neonatal rat cardiac myocytes Cx43 is predominantly phosphorylated (25,42,50) and it is localized in the plasma membrane (33). Agents that affect the incorporation of ^{32}P into Cx43 do not affect the distribution of the protein tested immunocytochemically in rat myocytes, suggesting that changes in the rate of phosphorylation detected with $^{32}P_i$ occur within the plasma membrane compartment. In all the systems mentioned above it seems that PKC mediates the effects of TPA. In support of this, after PKC is down-regulated cells are coupled and both coupling and state of phosphorylation of Cx43 become insensitive to TPA and phosphorylated forms of Cx43 are still detected as major components (45). Using synthetic peptides corresponding to the deduced sequences in the C-terminal region or recombinant fusion protein of the C-terminus of Cx43 it has been shown that residues 368 and 372 are phosphorylated by PKC but not by cAMP- or cGMP-dPK or Ca^{2+}-CM-dPK II (42) (Table 1). Both sites have been found mutated in visceroatrial heterotaxia and therefore implicated in the pathogenesis of this disease (74).

Cx43 can be phosphorylated by mitogen-activated protein (MAP) kinase in seryl residues 266, 279 and 282 (35) which

presumably are the same residues phosphorylated by cdc2 kinase, since a fusion protein of the carboxy terminal of Cx43 phosphorylated either by MAP or cdc2 kinase shows identical tryptic fingerprints (42) (Table 1). Furthermore, EGF-induced cell uncoupling is mediated by MAP kinase Cx43 phosphorylation in seryl residues of proline rich regions (35). A similar mechanism may operate in the PDGF-induced cell uncoupling which is PKC independent (68).

A membrane permeable derivative of cGMP reduces g_j in neonatal rat myocytes (61) and in SKHep1 cells transfected with rat Cx43 but not in SKHep1 cells transfected with human Cx43 (14). The state of phosphorylation of Cx43 expressed by SKHep1 cells stably transfected with rat Cx43 cDNA is increased by 8Br-cGMP but the site of phosphorylation is unknown as also is its interaction with other sites of phosphorylation by other protein kinases. In rat but not in human Cx43 the seryl residue 257 located in the carboxy terminal is flanked by proline and lysine, making it a possible site for phosphorylation by cGMP-dependent protein kinase. Although Cx43 does not present an obvious consensus site for cAMP-dPK phosphorylation, an increase in intracellular [cAMP] increases g_j in neonatal rat myocytes (57) and induces phosphorylation of Cx43 in the ovary (47).

Finally, indications that phosphoprotein phosphatases 1 and 2A participate in dephosphorylation of Cx43 have been obtained. In MDCK cells, treatment with okadaic acid, an inhibitor of these phosphatases, potentiates the increase in the relative amount of phosphorylated Cx43 induced by activation of a PKC-dependent pathway (30).

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