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REVIEW

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# Is the junctional uncoupling elicited in rat ventricular myocytes by some dephosphorylation treatments due to changes in the phosphorylation status of Cx43?

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**Abstract** Gap junctions, specialized membrane structures that mediate cell-to-cell communication in almost all animal tissues, are composed of channel-forming integral membrane proteins termed connexins. Most of them, particularly connexin43 (Cx43), the most ubiquitous connexin, the major connexin present in cardiac myocytes, are phosphoproteins. Connexin phosphorylation has been thought to regulate gap junctional protein trafficking, gap junction assembly, channel gating, and turnover. Some connexins, including Cx43, show mobility shifts in gel electrophoresis when cells are exposed to phosphorylating or dephosphorylating treatments. However, after exposure of rat cardiac myocytes to different uncoupling dephosphorylating agents such as H7 or butanedione monoxime, no modification in the Cx43 phosphorylation profile was generally observed. The lack of direct correlation between the inhibition of cell-to-cell communication and changes in the phosphorylation pattern of Cx43 or, conversely, modifications of the latter without modifications of the intercellular coupling degree, suggest that the functional state of junctional channels might rather be determined by regulatory proteins associated with Cx43. The modulation of the activity of junctional channels by protein phosphorylation/dephosphorylation processes very likely requires (as for several other membrane channels) the formation of a multiprotein complex, where pore-forming subunits bind to auxiliary proteins (e.g. scaffolding proteins, enzymes, cytoskeleton elements) that play essential roles in channel localization and activity. Such regulatory proteins, behaving as targets for phosphorylation/dephosphorylation catalysers, might in particular control the open probability of junctional channels. A schematic illustration of the regulation of Cx43-made channels by protein phosphorylation involving a partner phosphoprotein is proposed.

**Keywords** Connexin43 · H7 inhibitor · PhosphoBase · Protein phosphorylation · Rat cardiomyocytes

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## Introduction

Intercellular communication is of paramount importance in allowing individual cells to successfully accomplish their task in a coordinated manner in multicellular organisms and tissues. Gap junctional intercellular communication (GJIC) relies on the existence of intercellular protein channels that span the lipid bilayers of adjacent cells and allow the direct exchange of ions and low-molecular-weight molecules (< 1000 Da), including second messengers (e.g. cAMP, inositol triphosphate and $Ca^{2+}$), between contacting cells. Structural studies have demonstrated that gap junctional channels comprise hemi-channels known as connexons, each formed by the oligomerization of six protein subunits (connexins, abbreviated Cx followed by the molecular mass in kilodaltons) which delineate an aqueous pore.

GJIC was for a long time regarded as a relatively passive way of intercellular signalling through cell-to-cell tunnels before it finally appeared that the degree of intercellular coupling is finely regulated in three main aspects: the number of channels present in the membrane, their unitary permeability and their functional state (open

probability). These characteristics are, as for a number of other membrane channels, independently regulated by several factors, including protein phosphorylation and dephosphorylation processes, intracellular pH and calcium concentration, voltage, different chemicals, etc. (for recent review, see for example Sáez et al. 2003).

Connexin43, the most ubiquitous of the connexins, is a phosphoprotein, and different phosphorylated forms can be electrophoretically detected (Musil et al. 1990). It is the main gap junction protein expressed in ventricular myocytes, for example in new-born rat cardiac myocytes in primary culture, where it is predominantly phosphorylated. Changes in the connexin phosphorylation state have been shown to modulate the degree of cell-to-cell coupling (for reviews, see for example Sáez et al. 1998; Cooper et al. 2000; Lampe and Lau 2000, 2003).

Cx43 indeed appears to be a target of both protein kinases (PKs), for example PKC (Doble et al. 2000; Bowling et al. 2001), MAP (Warn-Cramer et al. 1996) or tyrosine (v-Src: Lau et al. 1996; Kanemitsu et al. 1997; c-Src: Giepmans et al. 2001; Toyofuku et al. 2001) PKs, and phosphatases (Li and Nagy 2000; Jeyaraman et al. 2003). Modulators of these enzymes may then change the state of connexin phosphorylation, affect the GJIC and are believed to control various physiological tissue and cell functions, as well as to be altered under pathological conditions. Consensus sites vary between connexins and have been preferentially identified in the C-terminal region.

However, in several reports, treatments supposed to modify the phosphorylation status of cellular proteins were found to induce changes in the degree of GJIC without noticeable modification in the immunoblot pattern of connexins, or, conversely, the latter was found changed without alteration of GJIC degree (see for example Mikalsen and Kaalhus 1997; Shiokawa-Sawada et al. 1997; TenBroek et al. 1998; Hossain et al. 1999a, 1999b; Duthe et al. 2000; van Veen et al. 2000). The observations summarized in the present article suggest that GJIC modulation involves, besides probable direct modifications in connexin phosphorylation status, the formation of a multiprotein complex, where pore-forming subunits bind to regulatory protein(s) that play essential roles in channel activity.

## Potential sites of phosphorylation of Cx43

The main sites of protein phosphorylation in eukaryotic cells are serine/threonine and, to a lesser extent, tyrosine residues. Since not all of them in a phosphoprotein are phosphorylated, PKs must display some degree of specificity. Many studies indicate that this specificity is particularly determined by the primary sequence surrounding the phosphorylable residue. However, most PKs studied are able to accept variations in the surrounding sequence to a smaller or larger degree and related PKs may display overlapping, yet different, specificities.

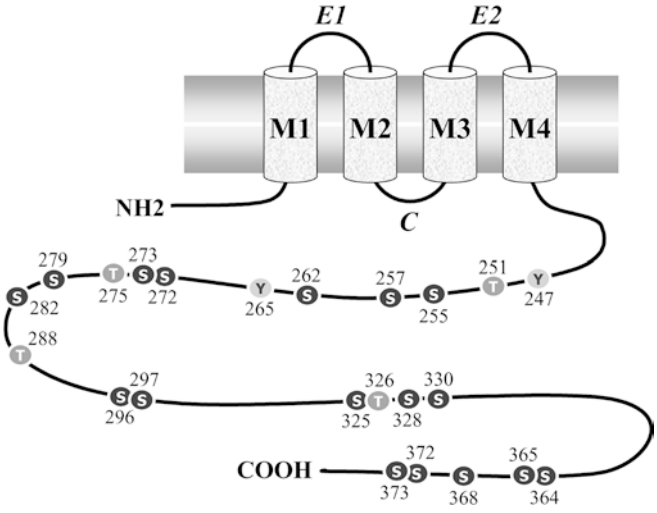


**Fig. 1** Locations of main potential phosphorylation motifs in the C-terminal domain of Cx43, based on the amino acid sequence proposed for Cx43 from rat cardiac myocytes (Beyer et al. 1987). The connexin proteins contain four transmembrane domains (*M1–4*) linked by one intracellular (*C*) and two extracellular (*E1–2*) loops, with the N- and C-terminal regions located intracellularly. The folding of Cx43 is arbitrarily represented

The large majority of connexins (the unique known exception being Cx26) are phosphoproteins and not only contain PK consensus phosphorylation sequences but have been shown to be phosphorylated. Up to now, all sites belong to the C-terminal region (Fig. 1), localized in the cytoplasm.

Biochemical and mutational approaches are used to identify the location of phosphorylated residues within the connexin sequence, whereas methods based on sets of rules governing the binding of a peptide substrate motif ("consensus site" surrounding the phospho-acceptor residue) to the kinase have been developed to suggest sites in proteins that may be phosphorylated by characterized kinases. Table 1 presents a comparison of the main reported phosphorylation sites (B columns) and of the potential motifs of phosphorylation by 10 major PKs, namely protein kinases A (PKA), C (PKC) and G (PKG), calcium calmodulin PK, type II ($Ca^{2+}$/CaM PK type II), casein kinases 1 (CK1) and 2 (CK2), myosin light chain kinase (MLCK), glycogen synthase kinase 3 (GSK3), $p70^{s6k}$ and $p34^{cdc2}$, found by means of PhosphoBase software (available at http://www.cbs.dtu.dk/databases/PhosphoBase/; see Blom et al. 1998) (C column). No consensus sequence was recognized in the C-terminal region for four of the above-mentioned enzymes (PKG, CK2, MLCK and $p70^{s6k}$). None of the other cytoplasmic regions of Cx43 (i.e. the N-terminal domain and the cytoplasmic loop) appear to contain consensus sequences for these PKs, but several other parts of the Cx43 sequence exhibit potential consensus sequences for phosphorylation, particularly the extracellular loops and transmembrane segments. However, these sites are probably inaccessible to intracellular enzymes.

**Table 1** Potential sites of phosphorylation in the protein sequence of the C-terminal tail of rat cardiomyocytes Cx43. The nature and location of the presumed phosphoacceptor residues appear in columns A, the main phosphorylation sites reported in the literature are listed in columns B, whereas the potential surrounding motifs of phosphorylation ("consensus sites") by 10 major protein kinases, predicted by use of PhosphoBase software, appear in column C

| A | | B | | C |
|---|---|---|---|---|
| Sites | | Already described | | Given by the program |
| Position | AA | Enzymes | Reference | Potential consensus sites for |
| 373 | S | Unidentified | Yogo et al. (2002) | PKA |
| | | | | $Ca^{2+}$/CaM PK type II |
| 372 | S | PKC | Saez et al. (1993) | PKC |
| 369 | S | Unidentified | Yogo et al. (2002) | |
| 368 | S | PKC | Saez et al. (1993) | CK1 |
| | | | Lampe et al. (2000) | |
| | | Unidentified | Yogo et al. (2002 | |
| 365 | S | Unidentified | Cruciani and Mikalsen (1999) | PKA |
| | | Unidentified | Yogo et al. (2002) | |
| 364 | S | Unidentified | TenBroek et al. (2001) | GSK3 |
| | | PKA | Shah et al. (2002) | PKC |
| 330 | S | CK1 | Cooper and Lampe (2002) | |
| 328 | S | CK1 | Cooper and Lampe (2002) | CK1 |
| 326 | T | | | GSK3 |
| 325 | S | CK1 | Cooper and Lampe (2002) | |
| 297 | S | | | PKC |
| 296 | S | | | PKA |
| | | | | $Ca^{2+}$/CaM PK type II |
| 288 | T | Unidentified | Goldberg and Lau (1993) | |
| 282 | S | MAPK | Kanemitsu and Lau (1993) | CK1 |
| | | | Warn-Cramer et al. (1996) | |
| 279 | S | MAPK | Kanemitsu and Lau (1993) | |
| | | | Warn-Cramer et al. (1996) | |
| 275 | T | | | CK1 |
| | | | | GSK3 |
| 273 | S | MAPK | Warn-Cramer et al. (1996) | |
| 272 | S | MAPK | Warn-Cramer et al. (1996) | |
| 265 | Y | $pp60^{v\text{-}Src}$ | Swenson et al. (1990) | |
| | | | Loo et al. (1995) | |
| | | | Lin et al. (2001) | |
| 262 | S | $p34^{cdc2}$ | Kanemitsu et al. (1998) | PKC |
| 257 | S | PKG | Kwak et al. (1995b) | |
| 255 | S | $p34^{cdc2}$ | Kanemitsu et al. (1998) | $p34^{cdc2}$ |
| | | | Lampe et al. (1998) | CK1 |
| | | MAPK | Kanemitsu and Lau (1993) | |
| | | | Warn-Cramer et al. (1996) | |
| 251 | T | | | GSK3 |
| 247 | Y | $pp60^{v\text{-}Src}$ | Lin et al. (2001) | |

Cx43 was estimated to be routinely phosphorylated on at least five sites in unstimulated rat 1 cells (Cooper et al. 2000). The critical importance of the phosphorylation of specific sites of the C-terminal domain of Cx43 is illustrated by the loss of gating properties of truncated proteins and the restoration of the ability of different agents to disrupt GJIC after co-expression of the separate C-terminal region of Cx43 (for review, see for example Lampe and Lau 2000)

## Dephosphorylating treatments may induce cell-to-cell uncoupling without affecting Cx43 phosphorylating patterns

### The GJIC level is governed by protein phosphorylation/dephosphorylation processes

The degree of cell-to-cell coupling, determined in pairs of cardiac myocytes by estimation of the macroscopic junctional conductance ($G_j$), remains stable with time in whole cell conditions when the pipette solution contains ATP at a minimal concentration of 2 mM but rapidly fades when pipettes are filled with ATP-free solutions (Sugiura et al. 1990; Vera et al. 1996; Verrecchia et al. 1999; Duthe et al. 2001). ATP is the cell's major energy currency, and its intracellular concentration (ranging from 3 to 7.5 mM in cardiac myocytes; Allen et al. 1985) rapidly increases with metabolite (e.g. glucose) utilization and decreases with exposure to metabolic inhibitors. The progressive run-down of channel activity was shown to be due to a shift in the protein phosphorylation/dephosphorylation balance (Verrecchia et al. 1999; Duthe et al. 2001). The functional state of intercellular channels then appears as constantly under the influence of protein phosphorylation/dephosphorylation processes.

H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (Hidaka et al. 1984)] is a broad spectrum inhibitor of several serine/threonine PKs, including PKA, PKC, PKG, MLCK, CK1 and 2, and $Ca^{2+}$/CaM PK II. When H7 was present at relatively high concentration (1 mmol/L) with intent to inhibit a range of PKs, the junctional

conductance between new-born rat ventricular myocytes progressively declined before reaching a plateau corresponding to approximately 13% of its initial value within 10 min (Fig. 2), unless the activity of the protein phosphatase (PP) responsible for the channel rundown in these cells (protein phosphatase 1, PP1; Duthe et al. 2001) was inhibited by heparin (100 μg/mL; Fig. 2).

H7 appears able to inactivate the main part of the basal activity of serine/threonine PK(s) responsible for the maintenance of junctional channels in an open state. A residual part of this activity might have subsisted because H7, considered as a competitive inhibitor with respect to ATP, was present in the intracellular medium at lower concentrations than ATP (e.g., in whole cell conditions, 1 versus 5 mmol/L) and/or because involved PK(s) might have relatively high H7 inhibition constants. In contrast, tyrosine PK inhibitors [genistein (Verrecchia et al. 1998) or 4-amino-7-*t*-butyl-5-(4-tolyl)pyrazolo[3,4-*d*] pyrimidine (Plaisance et al. 2003)] had no effect on intercellular dye diffusion between cardiac myocytes.

The nucleophilic agent 2,3-butanedione monoxime (BDM), considered to have a "chemical phosphatase" activity (Coulombe et al. 1990) or to enhance the activity of endogenous phosphatases (Zimmermann et al. 1996), is often used as a tool for investigating the effects of changes in the phosphorylation level of protein constituents on membrane channel functions, including the functional state of several membrane channels. BDM interrupts GJIC, and a part of its action seems to result from a dephosphorylation process (Verrecchia and Hervé 1997). This compound indeed produced a rapid, dose-dependent and reversible abolition of the cytosolic continuity existing between cells via gap junctional channels (Fig. 3A), even when ATP had been replaced in the pipette solution by a non-hydrolysable ATP analogue (adenosine 5′-*O*-(thiotriphosphate), ATPγS, 3 mmol/L, Fig. 3B) for at least 30 min to allow its diffusion into the cells and its incorporation in phosphoproteins, suggesting that this acute suppressant effect of BDM was not due to protein dephosphorylation. However, whereas the reversibility after BDM withdrawal rapidly declined when ATP was used, the presence of ATPγS allowed a complete re-establishment of GJIC, even after repetitive BDM exposures, suggesting that this nucleotide may contribute stable thiophosphate groups to cellular proteins and render them resistant to the dephosphorylation gradually occurring during the oxime treatment. These results are consistent with the model of a dual mechanism of BDM action proposed for some other membrane channels, consisting of a quick channel block and a parallel slow inhibition consecutive to protein dephosphorylation.

Both H7 and BDM effects illustrate the fact that the degree of gap junctional communication is governed by protein phosphorylation/dephosphorylation processes, that protein phosphorylation at some sites is critical for the normal activity of junctional channels, and that a permanent protein dephosphorylation by endogenous PP(s) is responsible for the decline of the junctional current when protein phosphorylation is impaired.

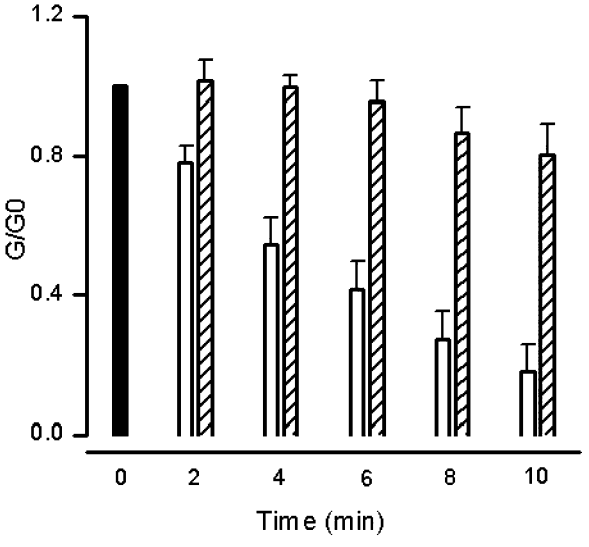


**Fig. 2** The cell-to-cell conductance between ventricular myocytes was progressively reduced by H7 unless endogenous phosphatase activities were simultaneously hindered. In whole-cell conditions, both cells were clamped at −70 mV and one of them was stepped to −80 mV every 30 s while the second cell was maintained at −70 mV. Owing to the transjunctional voltage difference, a current crossed the cell-to-cell junction, compensated by an opposite current supplied by the feedback amplifier connected to the cell maintained at −70 mV. Macroscopic junctional conductances are presented in units of their original value (means ± SEM). When ATP (5 mmol/L) was present in the patch pipette solution, the intercellular electrical coupling was well preserved (not represented), but the presence of H7 (1 mmol/L) elicited a progressive decline of junctional conductance, reaching after approximately 10 min a stable plateau equivalent to about 13% of its initial level (*open columns*, $n = 6$). In contrast, when H7 was co-added with heparin (100 μg/mL), the major part of the cell-to-cell communication was preserved (*hatched columns*, $n = 7$)

### Decreased GJIC can occur without concurrent modification of Cx43 phosphorylation pattern

Immunoblot analysis of cultured neonatal rat ventricular myocytes revealed that Cx43 usually appears as three main electrophoretically separable forms (Fig. 4): a more slowly migrating band representing the highly phosphorylated forms of Cx43 (44 kDa; P2), a middle phosphorylated form (42 kDa; P1) and a 40 kDA band representing the non-phosphorylated form of Cx43 (P0). As shown in these profiles, H7- or BDM-induced depression of GJIC generally did not result in any obvious change in the band pattern of Cx43; however, a shift was occasionally observed after H7 exposure.

## Connexin protein partners may be involved in the modulation of channel activity

In a majority of tissues, the cytosolic continuity between cells via intercellular junctional channels integrates

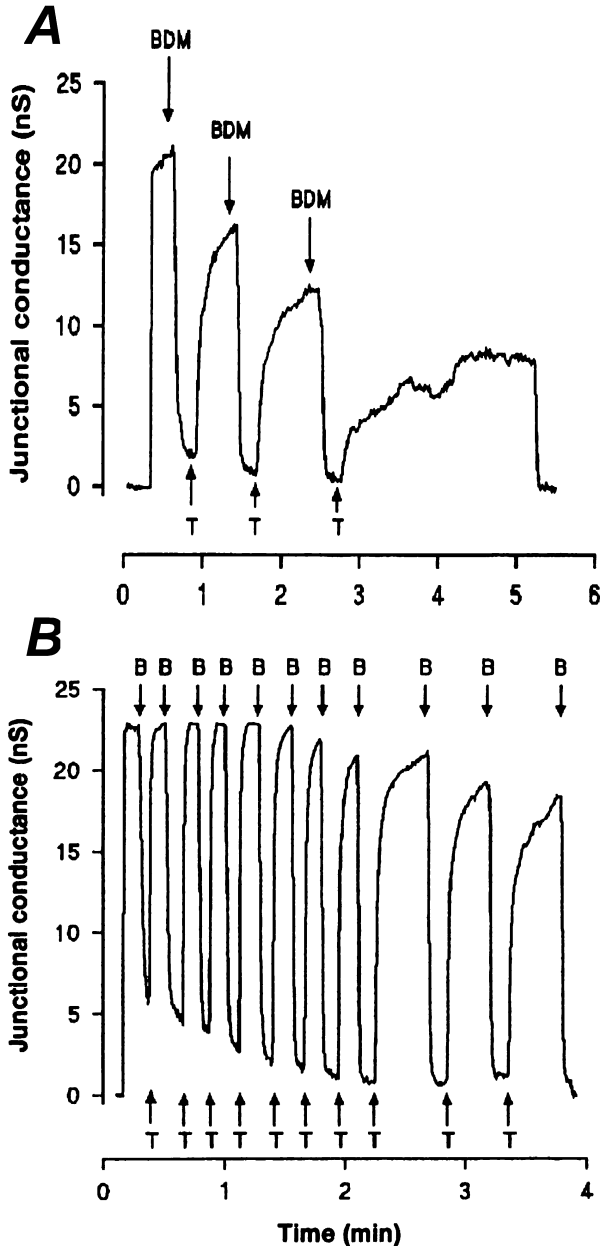


**Fig. 3A, B** Comparison of BDM effects on junctional conductance in the presence of ATP or of its non-hydrolysable analogue ATPγS. **A** When the pipette-filling solution contained ATP, the introduction of the drug (BDM, 15 mmol/L) in the close vicinity of the cell pair led to a rapid decrease of the cell-to-cell current, elicited by a transjunctional voltage difference, followed, after BDM washing by Tyrode's solution (*T*), by a progressively decreased reversibility. **B** When ATP was replaced by ATPγS, the $G_j$ decrease during BDM exposure (*B*) was followed by complete reversibility after its removal (*T*), even after repetitive exposures. (Reprinted with permission from Verrecchia and Hervé 1997)

connecting individual cells into functional syncytia. Connexins are, except the smallest one (Cx26), phosphoproteins or contain potential phosphorylation sites. Cx43, the most common connexin, present in many organs and cell types, predominantly exists in phosphorylated forms (Musil et al. 1990). In vivo, the connexin phosphorylation state was found to vary during physiological stages. Cx43 phosphorylation, for example, was found to increase during lactation in myoepithelial cells in rat mammary glands (Yamanaka et al. 1997). In developing rat brains, Cx43 levels in frontal cortex and brainstem increased with age whereas their phosphorylation status decreased (Hossain et al. 1994). A decrease in Cx43 phosphorylation status was also reported to occur in early apoptosis and early mitosis (see Wilson et al. 2000) and during ischemia (Beardslee et al. 2000). Connexin phosphorylation, implicated in the regulation of a broad variety of connexin processes (e.g. trafficking, assembly, disassembly and degradation, as well as channel gating; for recent reviews, see Lampe and Lau 2000; Lau et al. 2000; Cruciani and Mikalsen 2002), is of major importance in the regulation of gap junctional communication. Phosphorylation of connexins does not appear to be required for the formation of functional intercellular channels but rather to affect the channel characteristics. Indeed, Cx26, unphosphorylated, forms functional intercellular channels; for other connexins, the transfection of cells with C-terminal truncated connexins allows establishment of cell-to-cell coupling. However, channels formed in these conditions exhibit permeabilities and electrophysiological properties different from those made with wild-type connexins (Fishman et al. 1991; Dunham et al. 1992).

The fact that some dephosphorylating treatments, such as H7 and BDM exposures, are able to impair cell-to-cell coupling without noticeable change in the phosphorylation status of Cx43, leads us to think that the uncoupling mechanism is more complex than a direct connexin phosphorylation/dephosphorylation and plausibly involves regulatory proteins important for modulation of the channel activity.

## Emerging evidence for the importance of connexin partners

Although Cx26 cannot be phosphorylated (Traub et al. 1989), exposure of Cx26-SKHepl cells transfected to TPA reduced Lucifer yellow dye transfer and the junctional conductance and markedly decreased the amount of large channel conductances (140–150 pS) in favour of smaller conductances (40–70 pS; Kwak et al. 1995a). TPA-induced junctional uncoupling was seen, in Syrian hamster embryo (SHE) cells, to occur without change in the Cx43 band pattern (Rivedal et al. 1994). In T51B rat liver epithelial cells, the junctional uncoupling elicited by PDGF required participation of PKs but took place without a gross hyperphosphorylation of Cx43, suggesting that some, if not most, of Cx43 phosphorylations may not be directly required for GJIC blockade (Hossain et al. 1999a, 1999b). In Cx45-HeLa cells, TPA increased macroscopic junctional conductance without affecting Cx45 phosphorylation (van Veen et al. 2000). In immortalized ibroblasts derived from embryonic mice, PKA activation enhanced GJIC without increase of the

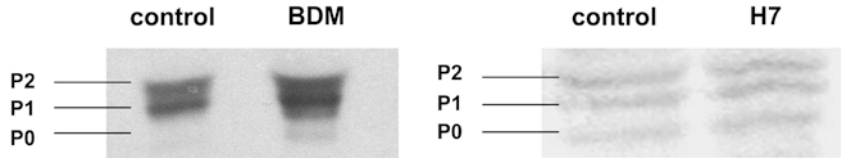


**Fig. 4** The reduction of cell-to-cell communication by dephosphorylating treatments occurred without concomitant change in Cx43 phosphorylation. Cells were washed in PBS and lysed in a solution containing 20% SDS (SB20; Dupont et al. 1988). 2-Mercaptoethanol (1%) was then added to the lysate, which was left 30 min at room temperature to reduce disulfide bonds. Then 10 μg of total protein per lane was run on respectively 10% (BDM) or 11% (H7) SDS polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Biorad). The resulting replica was incubated with a mouse anti-Cx43 antibody supplied by Chemicon, incubated with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG; Promega) and the enzymatic activity was revealed using NBT and BCIP substrate solution (Sigma). *Right*: H7 exposure for 30 min (*right lane*) did not significantly affect the level of each Cx43 species as compared to control (*left lane*). *Left*: similarly, treatment with BDM for 15 min (*right lane*) did not appreciably change the Cx43 band pattern as compared to control (*left lane*)

phosphorylation status of Cx43, suggesting that PKA-mediated phosphorylation of another protein able to interact with specific residues of the Cx43 C-terminus was required in this positive regulation (TenBroek et al. 1998). In the rat liver epithelial cell line IAR6.1, TPA was shown to block GJIC mainly by the direct action of PKC but also partly through cross-talk with the MAP kinase activity (Rivedal and Opsahl 2001). The latter authors suggested that PKC blocked GJIC either by inducing a type of Cx43 phosphorylation that causes no significant electrophoresis mobility shift or, alternatively, via phosphorylation of another substrate. Several mechanisms, for example through Cx43 channel gating (e.g. the channel open probability), trafficking of Cx43 or recruitment of hemichannels into gap junctions at the cell surface, were suggested to explain these effects (TenBroek et al. 2001). In SHE cells, the Cx43 phosphorylation status changed minimally after exposure to TPA or the hepatic peroxisome proliferators (Cruciani et al. 1997). In cultured astrocytes, containing predominantly phosphorylated forms of Cx43, hypoxia elicited an important reduction in the degree of GJIC and reduced Cx43 phosphorylation, but junctional uncoupling occurred prior to Cx43 dephosphorylation (Li and Nagy 2000). In rat cortical astrocytes, reoxygenation after hypoxia reduced dye coupling by approximately 70% without changes in the state of phosphorylation or levels of Cx43 (Martinez and Sáez 2000). However, limited changes in the degree of phosphorylation of Cx43, particularly on tyrosine residues, might occur without shift of the electrophoretic mobility of the protein.

Conversely, various treatments induced changes in the connexin phosphorylation status without alteration of the GJIC degree, in osteoblastic MC3T3-E1 cells for example, where short exposure ($< 5$ h) to basic fibroblast growth factor (bFGF) stimulated Cx43 phosphorylation without affecting junctional communication (Shiokawa-Sawada et al. 1997). SHE cells in primary culture exposed for 15–30 min to permolybdate, for example, underwent a strong change in Cx43 band pattern without a concurrent decrease in GJIC (Mikalsen and Kaalhus 1997). In fibroblasts expressing a Cx43 mutant, epidermal growth factor (EGF) treatment induced Cx43 phosphorylation (presumably at the alternate Ser272 and Ser273 MAP kinase sites, normally not phosphorylated by MAP kinase in vivo) without GJIC blockade (Warn-Cramer et al. 1998). EGF also induced Cx43 phosphorylation in the human kidney epithelial cell line K7, complete within 15 min, whereas enhanced GJIC only appeared 2–3 h later (Vikhamar et al. 1998). In MAC-T cells, a bovine mammary epithelial cell line, serum deprivation resulted in important reductioin Cx43 expression and a shift towards the unphosphorylated form, whereas cell-to-cell dye transfer was only moderately reduced (Sia et al. 1999). In V79 fibroblasts, TPA induced Cx43 phosphorylation and interrupted gap junctional communication (Cruciani et al. 1999), but the latter authors emphasized that these two parameters might in fact be dissociated, and that GJIC suppression might, for example, result from a TPA-induced binding of an accessory protein to Cx43.

Altogether, these results suggest that disruption of GJIC is more complex than direct connexin phosphorylation/dephosphorylation and requires the participation of additional regulatory components. Direct association of several proteins with Cx43 (for reviews, see Duffy et al. 2002; Thomas et al. 2002; Hervé et al. 2003) and probable involvement of protein–protein interactions in the regulation of Cx43 gap junctions are in accord with this hypothesis.

## Regulatory phosphoprotein hypothesis

The hypothetical association of Cx43-made channels and of a regulatory phosphoprotein is depicted in Fig. 5, where this partner protein is arbitrarily represented at the membrane level. Such a regulatory protein might control the open probability of the channels. Each of the factors which determines the macroscopic gap junctional conductance (the number of channels, their individual conductance and their open probability) is indeed individually controlled. Pharmacological agents classically used to resolve single-channel events when cells are not spontaneously very weakly coupled, are considered to reduce the open probability of the channels without modifying their other characteristics. In Cx45-HeLa cells, for example, protein phosphorylation did not affect

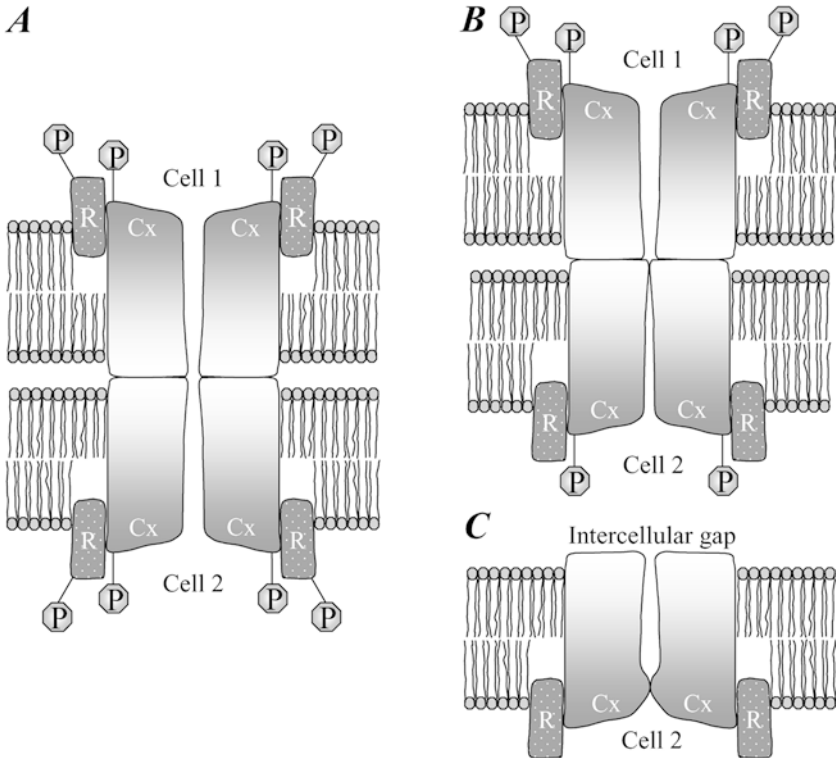


**Fig. 5A–C** Hypothetical view of the association of Cx43-made channels and of a regulatory phosphoprotein partner, arbitrarily represented at the membrane level. **A** The junctional channel (represented in its open state) is formed by connexins (*Cx*) assembly. The phosphorylation status of associated regulatory proteins (*R*) would control some of the channel characteristics (particularly its open probability). **B** The dephosphorylation of the regulatory protein (*R*) would be responsible for an interruption of the cell-to-cell communication. **C** A dephosphorylation of Cx43 itself may modify the pore aperture, with a resulting change in the single-channel conductance, even a complete channel closure. The dephosphorylating treatment might (as arbitrarily represented) or not also dephosphorylate the regulatory protein (only half of the junctional complex is represented)

single-channel conductance and the regulation was suggested most likely to act by modulation of the open probability of Cx45 junctional channels (van Veen et al. 2000).

Dephosphorylating treatments might dephosphorylate the regulatory phosphoprotein controlling the open probability of the channel, triggering a channel closure, without modification of the phosphorylation status of Cx43 (Fig. 5B). A complete junctional uncoupling was observed after removal of ATP from the pipette solution of only one of the cells of a pair (Verrecchia et al. 1999), showing that ATP deprivation in one of the contacting cells is sufficient to insulate this cell from its neighbour cell with normal ATP content, as imagined in Fig. 5B. A Cx43 dephosphorylation (as envisaged Fig. 5C) or hyper-phosphorylation may independently cause conformational changes in the protein, with subsequent modifications in the pore aperture and single-channel conductance. Changes in the phosphorylation status of appropriate sites in the Cx43 sequence may also be the cause of important conformational changes, as associations between discrete domains of Cx43 through intramolecular non-covalent interactions, in which the C-terminal domain acts as a gating particle that obstructs the pore (Hossain et al. 1999b; Zhou et al. 1999; Anumonwo et al. 2001). In transfected cell pairs, even in plaques large enough to mediate coupling, only a small fraction of channels would be open at the same time (Bukauskas et al. 2000). These authors emphasized the fact that the relationship between clustering and channel opening has not been established and suggested that other proteins, localized in areas of cell–cell contact, might promote connexin aggregation or clustering. Channel gathering was also found essential for the activity of some other channels, Kv1.4 potassium channels for example, for which channel clustering appears to be obligatory for suppressing internalization and requires the presence of a membrane-associated guanylate kinase (Jugloff et al. 2000).

## Perspectives

The data summarized here give support to the view that dephosphorylating treatments may reduce the degree of cell-to-cell communication without altering the state of Cx43 phosphorylation. The identification of protein partners for Cx43 (for reviews, see Duffy et al. 2002; Thomas et al. 2002; Hervé et al. 2003) raises new possibilities about how this channel is regulated in cardiac tissues, but, up to now, very little is known on the functional importance of such interactions. In rat ventricular myocytes, GJIC is tightly controlled by the adenine nucleotide concentration and involves, besides

Cx43 itself, additional regulatory components. The fact that GJIC remains stable in these cells in the presence of 2 mM ATP whereas a decrease to 1 mM induces a cell-to-cell uncoupling, a decrease that should hardly affect the activity of PK (since their $K_m$ for ATP is in the micromolar range), raises the question of why ATP needs to be present at such relatively high concentrations.

ATP serves as co-substrate for protein phosphorylation but might also be used for another phenomenon that helps to maintain the channels in an open configuration. Actin polymerization, for example, requires ATP and the regulation of actin filament dynamics is regulated by ATP hydrolysis (Carlier 1991). Millimolar exogenous ATP concentrations are required to promote F-actin stability and to attenuate the effects of cytochalasin D (Kamkin et al. 2001). The [ATP]/[ADP] ratio could also be involved in the regulation of GJIC. The percentage changes of [ADP] are much larger than those of [ATP] because of its low concentration in resting cells, but, up to now, the modulation of GJIC by ADP has not yet been thoroughly investigated.

The importance of the protein phosphorylation/dephosphorylation balance in the regulation of connexin trafficking, assembly and disassembly, as well as the modulation of the opening state of junctional channels, is progressively gaining support. Future studies will be required to establish the mechanisms by which protein phosphorylation, of connexin itself and plausibly of associated regulatory proteins, regulates the function and processing of connexins throughout their life cycle. Furthermore, the patho-physiological relationship between changes in phosphorylation and cell-to-cell coupling malfunctioning needs to be characterized.

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