Dephosphorylation Agents Depress Gap Junctional Communication between Rat Cardiac Cells without Modifying the Connexin43 Phosphorylation Degree

F. Duthe[1], E. Dupont[3], F. Verrecchia[1], I. Plaisance[1], N. J. Severs[3], D. Sarrouilhe[2], J. C. Hervé[1]

1 *Physiologie Cellulaire, UMR CNRS 6558, Poitiers, France*
2 *IBMIG, ESA CNRS 6031, Poitiers, France*
3 *Imperial College, Royal Brompton Hospital, London, UK*

Abstract. The functional state of gap junctional channels and the phosphorylation status of Connexine43 (Cx43), the major gap junctional protein in rat heart, were evaluated in primary cultures of neonatal rat cardiomyocytes. H7, able to inhibit a range of serine/threonine protein kinases, progressively reduced gap junctional conductance to approximately 13 % of its initial value within 10 min except when protein phosphatase inhibitors were also present. The dephosphorylating agent 2,3-Butanedione monoxime (BDM) produced both a quick and reversible interruption of cell-to-cell communication as well as a parallel slow inhibition of junctional currents. The introduction of a non-hydrolysable ATP analogue (ATPγS) in the cytosol delayed the second component, suggesting that it was the consequence of protein dephosphorylation.

Western blot analysis reveals 2 forms of Cx43 with different electrophoretic mobilities which correspond to its known phosphorylated and dephosphorylated forms. After exposure of the cells to H7 (1 mmol/l, 1h) or BDM (15 mmol/l, 15 min), no modification in the level of Cx43 phosphorylation was observed. The lack of direct correlation between the inhibition of cell-to-cell communication and changes in the phosphorylation status of Cx43 suggest that the functional state of junctional channels might rather be determined by regulatory proteins associated to Cx43.

Key words: Gap junction — Connexin43 — Protein phosphorylation — Rat cardiomyocytes — H7

Introduction

The coordination of contraction in the heart depends on the rapid, orderly propaga-

tion of action potentials *via* specialised cell-to-cell channels clustered in structures called gap junctions. These channels directly link the cytoplasms of neighbouring cells and mediate the reciprocal exchange of ions and low molecular-weight molecules (< 1000 Da), including second messengers (e.g. cAMP, inositol triphosphate and Ca^{2+}) between contacting cells. They differ from other membrane channels since they exist between two cells, they are relatively non-specific, and the movement of ions and molecules through them occurs by passive diffusion.

Structural studies have demonstrated that gap junctional channels are comprised of hemi-channels known as connexons, each formed by the oligomerization of six protein subunits (connexins) which delineate an aqueous pore. Connexins are homologous proteins encoded by a multigene family and are named according to their predicted molecular weight. Connexin43 (Cx43), widely distributed in different cell types, is the main gap junction protein expressed in ventricular myocytes, although Cx40 and Cx45 have been reported to be expressed as well (see Coppen et al. 1998). Cx43 is a phosphoprotein, and different phosphorylated forms can be electrophoretically detected (Musil et al. 1990). In neonatal heart cells in primary culture, Cx43 is predominantly phosphorylated and changes in the connexin phosphorylation state could modulate the degree of cell-to-cell coupling (for a review, see for example Sáez et al. 1993).

A 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 phosphorylation level of protein constituents on membrane channel functions, including the functional state of several membrane channels. BDM interrupted gap junctional intercellular communication (GJIC), and a part of its action seemed to result from a dephosphorylation process (Verrecchia and Hervé 1997). When gap junctional conductance is measured in whole cell conditions, channel activity rapidly declines if high energy phosphates (as ATP) are missing, unless the activity of endogenous protein phosphatase (PP) is inhibited (Verrecchia et al. 1999). These results suggest that phosphorylation at some sites is critical for the functional state of intercellular channels. The aim of the present study was to examine the consequence of dephosphorylating treatments inducing junctional channel closure on the level of phosphorylation of Cx43.

Materials and Methods

Ventricular cardiomyocytes were obtained from 1–2 day old rats and cultured for 2 or 3 days as recently described (Verrecchia et al. 1999). Briefly, evaluations of junctional conductance were made at room temperature (22–24 °C), after replacing the culture medium with a Tyrode's solution containing, in mmol/l: NaCl, 144; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 2.5; NaH_2PO_4, 0.3; HEPES 5 and glucose, 5.6 (pH 7.4). Macroscopic gap junctional conductance (Gj) was measured in voltage clamp conditions in myocyte cell pairs, using a dual whole cell patch clamp technique, with pipette filled with a solution containing (in mmol/l): KCl, 140; MgATP, 5; EGTA,

5; glucose, 10; GTP, 0.1; HEPES, 10 (pH 7.2). Both cells were at first clamped at a common holding potential (Vh, close to -70 mV), then a pulse was applied to one cell while the other was maintained at Vh to generate a transjunctional voltage difference (Vj). Therefore, when cells in contact were connected by open junctional channels, a junctional current (Ij) flowed through them from one cell to its neighbour, and Gj was calculated by dividing Ij by the amplitude of the Vj pulse. The gap junction permeability for the fluorescent dye 6-carboxyfluorescein (6-CF) was measured by analyzing the fluorescence recovery after photobleaching (gap FRAP) of one cell in a pair or in a small group of cells as previously described (Pluciennik et al. 1996).

For western blot analysis, the cells were washed in PBS and lysed in a solution containing 20 % SDS (SB20; Dupont et al. 1988). Protein estimation was performed using the DC protein assay kit supplied by Bio-Rad. One percent 2-mercaptoethanol was then added to the lysate, which was left 30 min at room temperature to reduce disulfide bonds. Five μg of total protein per lane was run on 12.5 % SDS polyacrylamide gels and electrophoretically transferred to PVDF membrane (Immobilon-P, Millipore). The resulting replica was incubated with an anti-connexin43 antibody supplied by Chemicon, incubated with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG; Pierce) and the enzymatic activity was revealed using NBT and BCIP substrate solution (Promega).

Results

Characteristics of the intercellular coupling in control conditions

Enzymatically dispersed cardiomyocytes seeded in culture dishes progressively establish cell-to-cell contacts and communication, allowing cell-to-cell diffusion of ions and of fluorescent dyes. The macroscopic junctional conductance of cardiac myocyte pairs, at least in a limited transjunctional voltage range (-15 to $+22.5$ mV), remained stable during the voltage steps and ranged from 0.5 to 119 nS and averaged 35.5 ± 25.8 nS (mean $\pm$ SD, $n = 600$) without compensation for the pipette series resistance.

The opening state of gap junctional channels is governed by protein phosphorylation/dephosphorylation processes

G_j measured in whole cell conditions rapidly fades when pipettes are filled with ATP-free solutions, suggesting that the functional state of intercellular channels is constantly under the influence of protein phosphorylation/dephosphorylation processes (Verrecchia et al. 1999). The effects of a broad spectrum inhibitor of serine/threonine protein kinases (PK), H7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine, Hidaka et al. 1984) have been examined at relatively high concentration (1 mmol/l) with intent to inhibit a range of PKs. H7, although structurally unrelated to ATP, is considered to compete with ATP for free enzymes. In presence of H7, the junctional conductance progressively declined before reaching a plateau
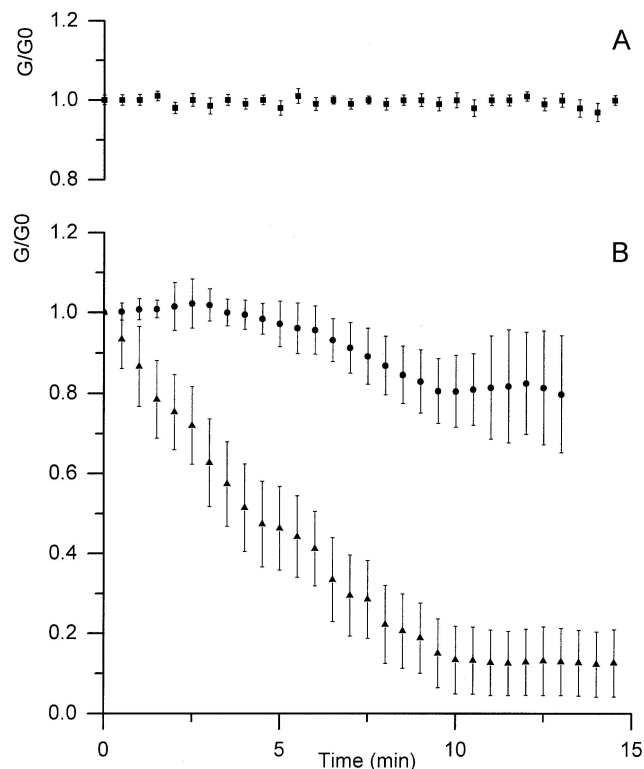
Figure 1. Cell-to-cell conductance between ventricular myocytes was progressively reduced by H7 unless endogenous phosphatase activities were simultaneously hindered. In whole-cell conditions, both cells were clamped at -70 mV and one of them was stepped to -80 mV every 30 sec while the second cell was maintained at -70 mV. Due 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. Junctional conductances are presented in units of their original value (means $\pm$ S.E.M.). **A.** as long as ATP 5 mmol/l was present in the patch pipette solution, intercellular electrical coupling was well preserved ($\blacksquare$, $n = 25$). **B.** 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 ($\blacktriangle$, $n = 6$). In contrast, when H7 was co-added with heparin (100 μg/ml) a substantial part of the cell-to-cell communication was preserved ($\bullet$, $n = 7$).

corresponding to approximately 13 % of the initial value within 10 min (Fig. 1). The reversibility of H7 action was examined by measuring the rate constant of cell-to-cell diffusion of a fluorescent dye by means of the gapFRAP method. The diffusion rate constant was reduced to 5 ± 3 % ($n = 13$) of its initial value after a 1h exposure to H7, then reached 60 ± 8 % 30 min after H7 removal (data not illustrated). In order to ascertain protein dephosphorylation influence of H7, to rule out other

possible mechanisms of action (*e.g.* a deleterious effect on membranes), this compound was added together with endogenous PP inhibitors. The efficiency of various PP inhibitors (including okadaic acid, calyculin A, PP inhibitor I2, cyclosporin A, KF, orthovanadate and heparin) to preserve cell-to-cell communication when PK activities are interrupted has been examined in ATP-deprived conditions. One of the most efficient, heparin (100 μg/ml), was also able to preserve a substantial cell-to-cell communication in spite of the presence of H7 (Fig. 1).

H7 appears able to inactive the main part of the basal activity of serine/threonine PK(s) responsible for the maintenance of junctional channels in an open state. In contrast, a tyrosine PK inhibitor (genistein) had no effect on intercellular dye diffusion between cardiac myocytes (Verrecchia et al. 1998). These results suggest that protein phosphorylation at some sites is critical for the normal activity of junctional channels to occur and that protein dephosphorylation by endogenous PP(s) is responsible for the decline of the junctional current.

Delayed BDM effects on gap junctional communication occur through protein dephosphorylation

2,3 butanedione monoxime (BDM) is often used as a tool for investigating the effects of changes in phosphorylation level of protein constituents on membrane channel functions, including several voltage-activated or ligand-activated ionic channels as well as junctional channels (see Verrecchia and Hervé 1997). BDM produced a rapid, dose-dependent and reversible abolition of the cytosolic continuity existing between cells *via* gap junctional channels even when ATP had been replaced in the pipette solution by a non-hydrolysable ATP analogue (adenosine 5′-O-(thiotriphosphate), ATPγS, 3 mmol/l) 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 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 (Fig. 2), suggesting that this nucleotide may contribute stable thiophosphate groups to cellular proteins and render them resistant to dephosphorylation gradually occurring during the oxime treatment. These results are consistent with the model of 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.

Uncoupling treatments considered to act through protein dephosphorylation do not alter connexin43 phosphorylation pattern

Immunoblot analysis of cultured neonatal rat ventricular myocytes revealed that Cx43 appears as two main electrophoretically separable forms (Fig 3). A more slowly migrating bands represented the highly phosphorylated forms of Cx43 (Hi P in Fig. 3), and a 41-kDA band representing the non-phosphorylated form of Cx43 (No P in Fig. 3). As seen on the picture, H7 or BDM treatments do not result in any obvious change in the ratio Hi P to No P.
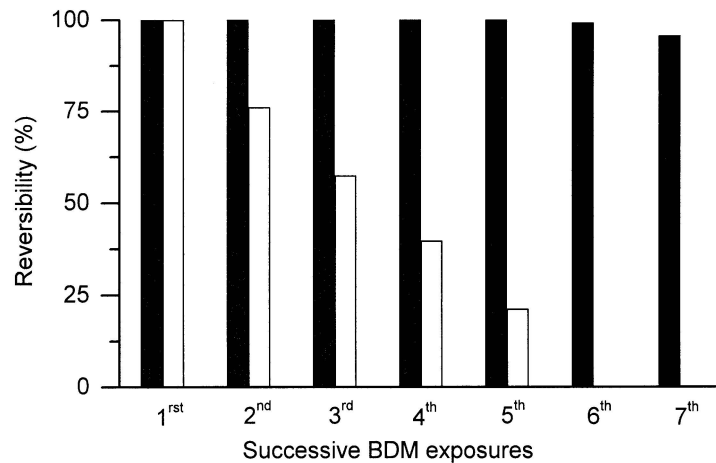
Figure 2. Comparison of the effects of BDM on junctional conductance in presence of ATP or of its non-hydrolysable analogue ATPγS. The introduction of BDM (15 mmol/l) in the close vicinity of the cell pair lead in both cases to a rapid decrease of cell-to-cell communication followed, after BDM washing by Tyrode's solution, by a variable reversibility. When ATP was used in the pipette-filling solution, the partial reversibility after successive exposures progressively decreased (open bars), whereas it was virtually complete, even after repetitive exposures in presence of ATPγS (filled bars).
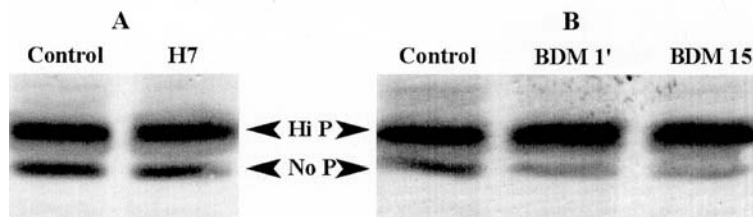

Figure 3. The reduction of cell-to-cell communication by dephosphorylating treatments occurred without concomitant change in Cx43 phosphorylation. **A.** H7 exposures for 1h (lane H7) did not significantly affect the level of each Cx43 species as compared to control (lane Control). **B.** Similarly, treatments with BDM for 1 and 15 min (lanes BDM 1′ and BDM 15′) did not appreciably change the ratio Hi P to No P as compared to control (lane Control).

Discussion

In cardiac tissue or vascular smooth muscle, the cytosolic continuity of cells *via* intercellular junctional channels integrates connecting individual cells into functional syncytia. As the intercellular propagation of the action potential is reduced (i.e. when the resistance to propagation from cell to cell is increased), the likeli-

hood of reentrant arrhythmias, heart block and vasospasm is greatly enhanced. The channel-forming proteins, connexins, are, except for the smallest one (Cx26), phosphoproteins or contain potential phosphorylation sites. Cx43, one of the major Cx in the body, present in many organs and cell types would predominantly exist under phosphorylated forms. Treatment of cardiac myocytes with H7, an inhibitor of several classes of PKs, including cAMP-dependent PKA, PKC, cGMP-dependent PKG, myosin light chain kinase (MLCK), casein kinases I and II, calmodulin-dependent PK II, considerably reduced the GJIC degree unless the activity of endogenous PPs was simultaneously inhibited, suggesting that junctional channel closure results from the inactivation of the main part of the basal activity of serine/threonine PK(s). 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 concentration 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.

BDM had a dual action on cell-to-cell communication and produced both a quick, dose-dependent and reversible closure of junctional channels as well as a parallel slow inhibition of junctional currents. The introduction of a non-hydrolysable ATP analogue, ATPγS, in the cytosol markedly delayed the second component, suggesting that the slow suppressant effect of BDM was due to protein dephosphorylation. However, the H7- or BDM-induced impairments of cell-to-cell coupling occurred without noticeable change in phosphorylation status of Cx43, leading to think that the dephosphorylated substrate might be an associated regulatory protein rather than Cx43 itself.

Decreases in the degree of GJIC have in many cases been correlated with changes in Cx43 phosphorylation status, supporting the notion that the state of phosphorylation of Cx43 is related to the functional state of Cx43 junctional channels (see for example Sáez et al. 1993, 1997). On the other hand, several decreases in the level of GJIC have also been ascribed to the activation of PKs, among which cGMP-activated PK (PKG), able to reduce gap junctional conductance in SKHep1 cells transfected with cardiac rat Cx43 (Kwak et al. 1995) or mitogen-activated protein (MAP) kinases, found for example involved in the disruption of gap junctional communication caused by some growth factors as epidermal growth factor (EGF; for a review, see Lau et al. 1996) or platelet-derived growth factor (PDGF; Hossain et al. 1998). These observations suggest that different pathways involving protein phosphorylations and dephosphorylations might be entailed in the regulation of GJIC, which may be dependent upon the extent and specificity of phosphorylation. Moreover, it was also shown that some compounds may inhibit junctional communication without altering Cx43 band pattern (see e.g. Mikalsen and Kaalhus 1997; Cruciani et al. 1999)

The present results give support to the view that dephosphorylating treatments may reduce the degree of cell-to-cell communication without altering the state of Cx43 phosphorylation. It has already been suggested that accessory proteins, possible targets for protein dephosphorylation, might be involved in the reg-

ulation of GJIC (Mikalsen and Kaalhus 1997). Several cytoskeleton proteins play a major role in the regulation of a variety of membrane receptors, ion channels and signalling proteins; in the case of junctional channels, direct associations of the cytoplasmic C-terminal tail of Cx43 with for example zonula occludens-1 (ZO-1; Toyofuku et al. 1998) and v-Src (Kanemitsu et al. 1997) have been observed. In conclusion, phosphorylation processes are essential for the activity of the junctional channels to be maintained but not necessarily directly through a change in phosphorylation status of the gap junction protein, connexin43. The identification of protein partners for Cx43 raises new possibilities about how this channel is regulated in cardiac tissues.

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