Electrical conductance of mouse connexin45 gap junction channels is modulated by phosphorylation

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Received 27 September 1999; accepted 14 February 2000

Abstract

In this study we report about the modulation of connexin45 (Cx45) gap junction channel properties by phosphorylation of the connexin molecules through different protein kinases. Phosphorylation of Cx45 was studied in HeLa cells transfected with mouse Cx45 (mCx45). Using Western blotting (WB) and immunocytochemistry, these cells were found exclusively positive for Cx45 and the protein was separated as a doublet of bands with a calculated mass of 46 and 48 kD. After dephosphorylation using calf intestine phosphatase (CIP), the 48 kD band disappeared almost completely leaving a single band at 46 kD. This effect can be prevented by including phosphatase inhibitors during CIP treatment. These results indicate that the 48 kD signal represents a phosphorylated form of Cx45. To investigate the effects of (de)phosphorylation of Cx45 on the conductive properties of gap junction channels built of this connexin, cell pairs were subjected to dual voltage clamp experiments and coupling was determined before and after addition of PMA, 4α-PDD, cAMP, cGMP, and pervanadate to the superfusate. 100 nM of the PKC activating phorbol ester PMA increased normalized junctional conductance by 50.9±28%. 100 nM of the inactive phorbol ester 4α-PDD had no significant effect. Activation of PKA with 1 mM 8-Br-cAMP decreased coupling by 20.9±5.7% while 1 mM 8-Br-cGMP (PKG-activation) was ineffective. 100 μM pervanadate, a tyrosine phosphatase inhibitor, reduced coupling by 43.7±11.1%. Single channel measurements, under identical phosphorylating conditions, were not significantly different from each other and all frequency histograms exhibited two conductance peaks at approximately 20 and 40 pS. WB analysis revealed, as compared to control conditions, a relative increase of the 48 kD signal upon stimulation with pervanadate (142±42%) and 8-Br-cAMP (50±23%) whereas neither stimulation with PMA nor 8-Br-cGMP had a significant effect. These experiments show that electrical intercellular conductance via Cx45 gap junction channels is differentially regulated by phosphorylation.

Keywords: Gap junctions; Protein kinases; Protein phosphorylation; Cell communication

1. Introduction

Electrical activation of the heart is initiated in the sinoatrial node. From this locus, the action potential is conducted along the atria, through the AV-node, His bundle, bundle branches, and the purkinje system, finally resulting in a synchronous activation of the ventricles. The action potential is propagated from cell to cell by current flow through specialized membrane structures called gap junctions. Gap junctions are agglomerates of intercellular channels that directly connect the cytoplasm of adjacent cells. Individual gap junction channels are composed of 12 gap junction molecules (connexins, Cx), assembled from two hexameric hemichannels in adjacent cells, head to head aligned. Connexins belong to a family of highly related, but not identical proteins, of which up to now, in mammals, 15 members have been cloned, identified, and named after their theoretical molecular mass (for a review see Ref. [1]).

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In the heart, at least four connexin types are reported to be present, i.e. Cx37, Cx40, Cx43, and Cx45. Cx37 was detected in the endocardial layer of rabbit hearts [2]. In myocardium, Cx40 is preferentially expressed in the atria and the conduction system [3–9], Cx43 is mainly found in the working myocardium and parts of the conduction system [5,6,9].

Initially, Cx45 was reported to be ubiquitously expressed throughout the myocardium [7,8,10,11]. However, recently Coppen et al. [12], showed that this apparent generic expression of Cx45 was mainly caused by a non-specific cross reaction of the antibody with Cx43, which suggested that Cx45, like Cx43 is expressed throughout the heart. Coppen et al. [12] used a new, non-cross-reacting antibody to localize Cx45 in rat and mouse heart. They reported Cx45 expression to be regional and associated with the atroventricular node. His bundle and peripheral ventricular conduction system where it is located at the borderzone between the purkinje-fibers and the working ventricular myocardium [12,13]. In this position, Cx45 may play a significant role in the propagation of the action potential from the conduction system to the working myocardium. However, little is known about the regulation of Cx45 gap junction channels. Earlier studies have shown that Cx45 is a highly voltage sensitive connexin, that forms cation selective channels with low conductance [14–17]. It also appeared that the Cx45 molecule, like Cx40 and Cx43, contains multiple potential phosphorylation sites which allows for modulation of channel properties through (de)phosphorylation by protein kinases/ phosphatases. Several studies have shown that Cx45 can indeed exist in a phosphorylated configuration [18–21]. Kwak et al. [14], reported that the single channel conductance of Cx45 gap junction channels is changed by activation of protein kinase C (PKC) with 8-Br-cAMP or protein kinase G (PKG) with 8-Br-cGMP. Intercellular conduction of the cardiac action potential is maintained by many channels, and the electrical conductance of gap junctions is not only regulated by their single channel conductance, but also by the number of channels and their open probability. Until now, no studies have been published on the modulation of the total macroscopic conductance of gap junctions composed of Cx45. In this study we report that the macroscopic conductance of gap junctions formed of mCx45 is increased by stimulation with PMA, decreased by stimulation with 8-Br-cAMP and pervanadate (a tyrosine phosphatase inhibitor and activator of MAPkinase), but is not affected by stimulation with 8-Br-cGMP. In addition, WB analysis of isolated Cx45 protein reveals a doublet of bands with a calculated mass of 46 and 48 kD in which the 48 kD band represents a phosphorylated form. The relative intensity of the 48 kD band is increased upon stimulation with 8-Br-cAMP and pervanadate but not significantly changed upon stimulation with PMA and 8-Br-cGMP.

2. Materials and methods

2.1. Cells and culture conditions

Human cervix carcinoma HeLa cells, non-transfected (wild type) or stably transfected with mouse Cx45 (mCx45) cDNA, were kindly provided by Dr. K. Willecke. Cell culture conditions were as previously described [22].

2.2. Antibodies

For both immunocytochemistry (IC) and Western blotting (WB), the following antibodies and dilutions were used; a rabbit polyclonal antibody raised against a peptide, representing amino acids 285–298 of mCx45 (Alpha Diagnostics), 5 µg/ml (IC) and 2 µg/ml (WB), a rabbit polyclonal antibody (crude serum) raised against amino-acids 259–396 of mCx45, kindly provided by Dr. T.H. Steinberg, Washington University, St. Louis, 1:4000 (IC) and 1:2000 (WB), a rabbit polyclonal antibody raised against 19 amino acids in the carboxy terminus of mCx40 (Alpha Diagnostics), 0.5 µg/ml (IC) and 4 µg/ml (WB), a mouse monoclonal antibody raised against amino acids 236–382 of rat Cx43 (Transduction Laboratories), 1 µg/ml (IC) and 0.5 µg/ml (WB) and a rabbit polyclonal antibody raised against amino acids 4–17 of human PKG-1β (Stress Gen), 2 µg/ml (IC) and 0.5 µg/ml (WB).

Secondary antibodies were purchased from Jackson Laboratories; Texas Red conjugated goat–anti-mouse IgG (IH, 13 µg/ml), Fluorescein Isothiocyanate (FITC)-conjugated goat–anti-rabbit IgG (IH, 3 µg/ml), or from Biorad-Life Science; horse radish peroxidase (HRP) conjugated antibodies, raised in goat against rabbit (WB, 62.5 ng/ml), and mouse (WB, 112.5 ng/ml).

2.3. Electrophysiological measurements

For electrophysiology, cells were seeded in 35 mm petri dishes (Falcon 3001) at 1.5×10^5 cells/cm². Prior to each measurement, culture medium was replaced by modified Tyrode’s solution [23], containing (in mM) NaCl 140, KCl 5.4, MgCl₂ 6H₂O 1, CaCl₂ 2H₂O 1.8, HEPES 5, Glucose 5, pH 7.4. Patch electrodes were backfilled with 0.22 µm filtered solution containing (in mM): CsCl 135, MgCl₂ 1, CaCl₂ 0.5, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N’-tetraacetic acid (EGTA) 10, Na₂ATP 5, HEPES 10, pH 7.2 (adjusted with CsOH). Measurements of gap junctional conductance and single gap junction channel currents were performed as described before [24].

2.4. Phosphorylating conditions

For each experiment, fresh Tyrode’s solution containing 1 mM 8-Br-cAMP (Sigma) or 1 mM 8-Br-cGMP (Sigma) was prepared. Phorbol 12-myristate 13-acetate (PMA, Sigma) and 4α-phorbol 12,13-didecanoate (4α-PDD,
Sigma), were first dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 100 μM and diluted in Tyrode to a final concentration of 100 nM. Pervanadate was prepared freshly before each experiment as a 500× stock solution by mixing equal volumes of 0.1 M H2O2 and 0.1 M Na3VO4. In whole cell experiments, cells were exposed to a phosphorylating treatment by switching the superfusion (6 ml/min) from control Tyrode to one containing a phosphorylating substance after steady state conductance was reached. For single channel experiments, cells were pre-incubated for 10 min with normal tyrode or tyrode containing phosphorylating drugs. Dephosphorylation was achieved by addition of 1 IU/ml calf intestine phosphatase (CIP), an alkaline phosphatase, to the internal pipette solution.

2.5. Immunocytochemistry

Passaged cells were plated on 16-mm-diameter sterile glass coverslips and grown within 2 days to sub-confluency. Next, coverslips were rinsed in PBS, fixed in methanol (−20°C, 2 min) and washed three times with PBS. Fixed cells were permeabilized with 0.2% Triton X-100/PBS for 1 h, and prior to the first and second antibody incubation, non-specific binding was blocked with 2% bovine serum albumin (BSA) for 30 min. Incubation with primary antibodies raised against Cx40, Cx43, Cx45 or PKG-1β was performed overnight in PBS/10% normal goat serum (NGS), in a total volume of 50 μl/coverslip. Immunolabeling was performed using Texas Red- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies against either mouse or rabbit IgG. All incubation steps were performed at room temperature (20±2°C, 2 min) and washed three times with PBS (except after blocking with BSA). Sections were mounted in Vectashield (Vector Laboratories) and examined with a Nikon Optiphot-2 light microscope equipped for epi-fluorescence.

To test the specificity of the observed labeling patterns, coverslips were incubated with (1) a mixture of primary antibody and a 500-fold excess of the synthetic peptide to which the antibodies were raised, (2) 10% NGS and (3) the second antibody alone.

2.6. Protein isolation

Cells destined for protein isolation were grown to subconfluency in 10 cm diameter Costar tissue culture dishes. Prior to treatment with the various phosphorylating agents, cells were washed extensively with PBS and normal culture medium was replaced for 2 h by serum-free medium. In order to activate the various kinases, 1 mM 8-Br-cAMP, 1 mM 8-Br-cGMP, 100 nM PMA and 0.1 mM pervanadate were added to the cultures. 100 nM 4a-PDD, a non-activating analogue of PMA, was used as a negative control for PKC induced phosphorylation. Pre-incubation of the cells, (prior to stimulation with pervanadate), with 50 μM PD90859, a specific inhibitor of MAP kinase, was used to study the effect of pervanadate. After 10 min, cells were washed with PBS, collected in lysis buffer (400 μl/10 cm dish; 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM phenylmethylsulfonyl fluoride (PMSF), protease-inhibitor-cocktail) and sonicated on ice (3×10 s, 60 W, Branson Sonic Power). Cellular debris was concentrated by centrifugation (5 min, 14 000 rpm) and total cellular protein in the supernatant was determined using the Lowry protein assay [25].

2.7. Dephosphorylation of mCx45

A 250 μg amount of total cellular protein, isolated as described, was incubated for 3 h at 37°C in the presence of 2 IU of calf intestine phosphatase (CIP, Boehringer). Incubation was stopped by addition of 4× Laemmli buffer (200 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerin, 20% β-mercaptoethanol, 0.1% bromophenol blue) and boiling the samples for 5 min [26]. Control incubations were performed in the presence of phosphatase inhibitors (10 mM EDTA, 11 mM Na3VO4 and 50 mM NaF).

2.8. SDS–PAGE and Western blotting (WB)

Aliquots were diluted with 4× Laemmli buffer and boiled for 5 min. Equal amounts (50 μg/lane) of each sample were separated overnight (80 V) on a 10% SDS–polyacrylamide gel [26], (18×24 cm) and electrophoretically transferred (4°C, 3 h, 350 mA) to nitrocellulose membrane (0.45 μm, Biorad). Protein transfer was assessed by Ponceau S staining. Prior to primary antibody incubation with antibodies raised against Cx40, Cx43, Cx45 or PKG-1β (overnight, 4°C, in 0.1% BSA/0.1% Tween20/PBS), the nitrocellulose membrane was blocked with 5% dried milk powder (1 h, RT, in 0.1% Tween20/PBS). Next morning, the membrane was washed (3×5 min, 0.1% Tween20/PBS), incubated with horse radish peroxidase conjugated secondary antibody (1 h, 4°C, 0.1% BSA/0.1% Tween20/PBS), and washed again (6×5 min, 0.1% Tween20/PBS). Signals were visualized by 1 min development in Enhanced Chemo Luminiscence reagent (ECL, Amersham) and exposure to XB-1 film (Kodak).

2.9. Statistical analysis and quantification of Cx45 Western blotting

Macroscopic gap junctional conductance was measured and corrected for both series and membrane resistance as previously described [27]. Initial conductances and steady state values after exposure to the phosphorylating drug were determined and subjected to a paired Students t-test. Steps in single channel current were measured by hand from digitized I/j traces and converted into step amplitude.
histograms with a 4 pS bin width, using custom software (MacDAQ, kindly provided by A.C.G. van Ginneken, Department of Physiology, University of Amsterdam), running on a Macintosh microcomputer (Apple Inc.).

Mean single channel conductances ($\gamma$) of each individual experiment were determined by fitting a sum of two Gaussian distributions to step amplitude histograms, using KaleidaGraph data analysis and graphics presentation software (version 3.0.8, Adelbeck Software). All experiments contained at least 50 events. For each treatment, the number of events in each bin were averaged and plotted with the standard error for each bin.

WB signals were transferred to Adobe Photoshop software through high resolution scanning (Microtek ScanMaker 630) of the XB-1 films and digitized with ImageQuant software. From three independent experiments, for all different samples, the ratio between the 48 and 46 kD band was calculated. Within each separate experiment, ratio’s of the samples were compared to the ratio obtained from control cells which was set at 100%. Statistics were performed using SchoolStat 2.0.6 software. Significance between the different groups was tested with a one-way ANOVA and an two-sided independent $t$-test. Significance was accepted when $P \leq 0.05$.

3. Results

3.1. Immunocytochemistry

To ensure that HeLa/mCx45 transfected cells expressed Cx45 channels exclusively, cell cultures grown to sub-confluence (Fig. 1A), were immunocytochemically stained using antibodies raised against Cx40, Cx43, and Cx45. Cx45 transfected cells stained positive for Cx45 exclusively using two different antibodies raised against different epitopes (amino acids 285–298 and 259–396) in the carboxy terminus of mCx45. Staining was found as strong punctate intercellular labeling (Fig. 1B and C). Almost all of the fluorescent labeling was concentrated at the contact sides between cells while only little was localized in the cytoplasm of the cells. In particular, the Cx45 antibody provided by Dr. Steinberg revealed, as shown previously, some fine speckled non-specific labeling in the cytoplasm but this signal was clearly distinguishable from specific intercellular gap junction labeling [16]. Pre-incubation of the Cx45 antibody (raised against amino acids 285–298) with a 500-fold excess of the peptide to which it was raised prevented the punctate intercellular labeling (Fig. 1D).

Recently, it has been shown that the most commonly used Cx45 antibody raised against aminoacid residues 259–396 failed to stain these Cx43 transfected cells. Furthermore, mCx45 transfected HeLa cells were found to be completely negative both for Cx40 and Cx43 as identified in a double-labeling experiment using well characterized antibodies raised against mouse Cx40 and rat Cx43 (Fig. 1E). Altogether, these experiments justify the use of both Cx45 antibodies in our experiments since both recognize Cx45 equally well and our cells prove to express no Cx43. Finally, the untransfected HeLa cells were negative for Cx45 antibody staining (Fig. 1F) which implies that our cells solely express the transfected mCx45.

3.2. Modulation of macroscopic conductance

In general, HeLa/mCx45 cell pairs were well coupled (coupling after correction for series and membrane resistance varied between 2 and 100 nS). Fig. 2 shows typical examples of modulation of macroscopic conductance by phosphorylation. When 100 nM of the phorbol ester PMA was added, a strong increase in electrical conductance, in this case from 2 to 4.9 nS, was measured (Fig. 2A). Addition of 100 nM 4cP-PDD, the inactive phorbol ester had no noticeable effect (Fig. 2B).

When 1 mM 8-Br-cAMP was present in the bathing solution, coupling decreased from 27.3 to 18.6 nS (Fig. 2C). Administration of 1 mM 8-Br-cGMP had no effect (Fig. 2D), while stimulation with pervanadate reduced coupling from 10.5 to 4.5 nS (Fig. 2E). In most cases, the time from the onset of the effect to a new steady state level was about 1.5–2 min.

The statistical result from eight experiments for each treatment is shown in Fig. 3. PMA significantly increased junctional conductance by 50.9±28% (mean±S.E.M., $P \leq 0.033$), while the inactive phorbol ester 4c-PDD had no significant effect ($-5.9\pm8.1\% P \leq 0.26$). On average, 8-Br-cAMP decreased coupling significantly by 20.9±5.7% $P \leq 0.005$, while 8-Br-cGMP had no effect ($-0.3\pm7.0\%, P \leq 0.29$). Pervanadate, however, significantly reduced coupling by 43.7±11.1%, $P \leq 0.0006$. Although the initial macroscopic conductance within the treatment groups was rather variable, no significant differences in average baseline conductance between the treatments were detected, nor was there any correlation between the initial conductance and the observed effect (data not shown).

3.3. Modulation of microscopic conductance

Macroscopic gap junctional conductance is determined by the number of channels, the conductance of a single channel and the open probability. To assess whether single channel conductance ($\gamma$) is influenced by the phosphorylating treatments mentioned, we measured $\gamma$. We used 2 mM halothane to reduce junctional current flow to a level where single channel currents could be measured.
Fig. 1. Immunocytochemical staining of connexin expression in HeLa cells transfected with mCx45. Panel 1A: Phase-contrast view of transfected cells cultured to subconfluency. Cx45 expression is shown as clear punctate intercellular labeling in panel 1B and 1C after labeling with two different antibodies raised against mouse Cx45. Pre-incubation of the Cx45 antibodies (Alpha Diagnostics) with the peptide to which they were raised prevented intercellular labeling almost completely as depicted in panel 1D. Double labeling with antibodies raised against Cx40 and Cx43 did not result in any positive staining as shown in panel 1E. Finally, panel 1F shows that untransfected (wild type) HeLa cells are negative for labeling with Cx45 antibodies.

Under control conditions, two sizes of current transitions were detected (Fig. 4A, inset). One transition corresponding to an average conductance of 21.9 pS ($\gamma_{1,1}$), and a second transition corresponding to an average conductance of 39.0 pS ($\gamma_{2,1}$). Occasionally, independent of treatment and transjunctional voltage, slow closures of the Cx45 gap junction channel large conductance state ($\gamma_{2,2}$) were observed (Fig. 4B).

Fig. 5 shows the single channel histograms after pre-incubation with the different phosphorylating agents. In these experiments we have also added CIP (1 U/ml) to the pipette solution to dephosphorylate gap junctions (Fig. 5F). All histograms show slightly different, but similar single channel conductances. The ratio between the amplitudes of the two conductances is however somewhat variable. To verify whether these variations are statistically significant,
Fig. 2. Typical examples of the effect of phosphorylating treatment on macroscopic conductance between HeLa/mCx45 cell pairs. A. Exposure to 100 nM PMA increases conductance from 2.0 to 4.9 nS (145% increase). B. A 100 nM 4a-PDD concentration had no effect on the macroscopic conductance. C. A 1 mM cAMP concentration reduced conductance from 27.3 to 18.6 nS (32% decrease). D. A 1 mM cGMP concentration did not alter macroscopic conductance. E. A 0.1 mM pervanadate concentration strongly reduces gap junctional conductance from 10.5 to 4.5 nS (57% reduction).
the fraction of events under the gaussian of $\gamma_1$, was divided by the area under both gaussians for each experiment [$F(\gamma_1)$]. The values for $\gamma_1, \gamma_2$ and $F(\gamma_1)$ were subjected to analysis of variance (ANOVA) to check if the variance between the treatments was significantly different from the variance within the treatments. The results are presented in Fig. 6. No differences were detected for $\gamma_1$ (Fig. 6A, $P \leq 0.17$), $\gamma_2$ (Fig. 6B, $P \leq 0.77$), or for the values of $F(\gamma_1)$ (Fig. 6C, $P \leq 0.063$).

### 3.4. Western blotting of phosphorylated Cx45

WB analysis of samples prepared from untransfected HeLa cells confirmed the results obtained with immunocytochemistry: wild type HeLa cells do not express detectable levels of Cx40, Cx43 (not shown), or Cx45 protein (Fig. 8A, lane 1). However, total protein isolated from HeLa cells transfected with mCx45, was found positive for Cx45 (Fig. 7A, lane 3), but as expected negative for Cx40 or Cx43 (Fig. 7A, lanes 1 and 2, respectively). The Cx45 antibody (raised against amino-acids 259–396) revealed two bands with a calculated molecular mass of 46 and 48 kD of which the 46 kD band was most prominent. To clarify whether these bands represented phosphorylated forms of the Cx45 protein, an aliquot of isolated protein was treated with calf intestine phosphatase (CIP). After treatment with CIP, the 48 kD band, but not the 46 kD band, disappeared which could be prevented by including phosphatase inhibitors during CIP treatment (Fig. 7B). These results suggest that the 48 kD band is a phosphorylated form of Cx45 and that under normal culture conditions, a fraction of expressed Cx45 protein exists in this phosphorylated form. In our hands, the commercially available Cx45 antibody (raised against aminoacid residues 285–298 of mCx45) proved to be inadequate for use on WB since background signals were unacceptably high.

In order to test whether short term activation of various intracellular protein kinases induced differences in the degree of phosphorylation of Cx45, cells were stimulated for 10 min in serum-free medium with the previously mentioned activators. Fig. 8A depicts a representative WB stained for Cx45. As shown in lane 1, untransfected HeLa cells do not show positive labeling for Cx45. All protein samples isolated from differentially stimulated HeLa cells transfected with mCx45, show bands of 46 and 48 kD (Fig. 8A, lanes 2–7). Additionally to the specific labeling of Cx45 protein, all separated samples, both from wild type and transfected HeLa cells, show two non-specific bands of unknown nature with a calculated molecular mass of 55–60 kD. In all samples the unphosphorylated 46 kD band appeared to be most prominent whereas the intensity of the 48 kD band varied between the different treatments. Fig. 8B shows the relative differences of the ratio between the density of the 48 kD band and that of the 46 kD band as calculated for the different treatments (mean±S.D., $n=3$, $P<0.05$). The ratio of untreated (control) cells was set to 100%. As compared to control cells (lane 2), stimulation with 8-Br-cAMP (lane 3) and pervanadate (lane 7) significantly increased the relative intensity of the phosphorylated 48 kD band with 50±23 and 142±42%, respectively. Pre-incubation of the cells (prior to stimulation with pervanadate) with 50 μM PD90859, a specific inhibitor of MAPkinase, did not alter the 48 kD/46 kD ratio compared to the ratio of pervanadate treated cells (not shown). In contrast, stimulation with 8-Br-cGMP (lane 4) and PMA (lane 5) appeared to be ineffective in altering phosphorylation of Cx45 as the signal could not be
Fig. 4. Single channel characteristics under normal conditions using halothane to resolve single channels. A, inset. Current trace of non-stepped and stepped cell of a HeLa/mCx45 cell pair (upper and lower trace, respectively) at a transjunctional voltage of 20 mV. This trace shows a small current transition ($\gamma_{j,1}$) and a large current transition ($\gamma_{j,2}$). The histogram is an average of eight experiments (bars represent 4 pS bin averages ± S.E.M.). Gaussian curve fitting revealed two mean channel conductances of 21.9 ± 1.6 and 39.0 ± 1.6 pS (mean ± S.E.M.). The fraction of events belonging to $\gamma_{j,1}$, $F(\gamma_{j,1})$, was 0.3 (mean ± S.E.M.). B. Current trace of non-stepped and stepped cell (upper and lower trace, respectively) of cell pair under control conditions, showing slow closure of the Cx45 channel.
Fig. 5. Averaged single channel histograms of HeLa/mCx45 cell pairs after pretreatment with 100 nM PMA (A), 100 nM 4α-PDD (B), 1 mM 8-Br-cAMP (C), 1 mM 8-Br-cGMP (D), 0.1 mM pervanadate (E), 1 U/ml CIP (F).

**A**
100 nM PMA  
N=8, n=731  
γ₁=20.8±2.5 pS  
γ₂=39.4±2.3 pS  
F(γ₁,1)=0.32±0.07

**B**
100 nM 4α-PDD  
N=8, n=548  
γ₁=24.0±1.3 pS  
γ₂=40.6±1.8 pS  
F(γ₁,1)=0.37±0.9

**C**
1 mM 8-Br-cAMP  
N=6, n=757  
γ₁=19.5±1.9 pS  
γ₂=37.5±1.7 pS  
F(γ₁,1)=0.28±0.13

**D**
1 mM 8-Br-cGMP  
N=7, n=487  
γ₁=21.4±1.8 pS  
γ₂=38.0±2.6 pS  
F(γ₁,1)=0.42±0.11

**E**
0.1 mM pervanadate  
N=7, n=840  
γ₁=22.1±1.6 pS  
γ₂=43.0±1.6 pS  
F(γ₁,1)=0.39±0.13

**F**
1 U/ml CIP  
N=6, n=1182  
γ₁=22.4±3.1 pS  
γ₂=34.9±1.7 pS  
F(γ₁,1)=0.44±1.6
Fig. 6. Bar plots of smallest channel conductance (mean±S.E.M., A), largest channel conductance (mean±S.E.M., B), and the fraction of events belonging to $g_{ij}$, $F(g_{ij})$ (mean±S.E.M., C). To verify whether exposure to (de)phosphorylating agents altered the size or distribution of single channel conductances, these parameters were subjected to analysis of variance (ANOVA). No statistical differences between the treatments were detected. $P=0.17$, $P=0.77$, and $P=0.063$ for $g_{ij}$, $g_{ij}$, and $F(g_{ij})$, respectively.
4. Discussion

4.1. Expression and phosphorylation of Cx45

Until now, little was known about the effects of phosphorylation on channels built of Cx45. This study was performed to provide data concerning the phosphorylation of Cx45 protein in relation to the conductance of Cx45 gap junction channels.

Previous studies have shown that untransfected HeLa cells are sometimes weakly coupled by endogenous gap junction channels although they proved to be negative for several connexin mRNA transcripts, including Cx45 mRNA [22,28]. The biophysical properties of these channels, however, are rather similar to those of Cx45. Similar to other studies [18], we were not able to demonstrate Cx45 protein in our wild type cultures neither with immunocytochemical staining nor on WB. Using both techniques, we showed unambiguously that our HeLa cells transfected with mCx45 were positive exclusively for Cx45. Staining of the cells with two different Cx45 antibodies resulted in clear punctate intercellular labeling. Total protein samples prepared from HeLa/mCx45 cells were positive for Cx45 which was shown on WB as a doublet of bands with a calculated mass of 46 and 48 kD. Several authors have shown that Cx45 can be phosphorylated mainly at serine residues but also on threonine and tyrosine residues [18–21]. In our samples, upon treatment with calf intestine phosphatase, the 48 kD band, which was of minor intensity compared to the strong 46 kD band, disappeared indicating that this band represented a phosphorylated form of the Cx45 protein. Co-incubation during the phosphatase treatment with phosphatase inhibitors prevented disappearance of the 48 kD signal thereby underpinning this conclusion. Laing et al. [21] also have shown that the (proposed proteolytic) 46 kD band was of unphosphorylated nature but that the mobility of the 48 kD form was not affected by dephosphorylation. The electrophoretic mobility of the protein as described in other studies shows some discrepancy. Some authors have separated Cx45 as a single band of 45 kD [12,18,20,29], or of 48 kD [15,16,30]. Others claim that the protein exists of both a 46 and a 48 kD band of which the 46 kD band was proposed to be a proteolytic product of the full protein [19,21]. Possible explanations for these contradictions might be: differences in used Cx45 antibody, percentage of the SDS–PAGE gel, quality of the protein markers, processing of the protein sample and nature of the Cx45 protein.

Western blot analysis was performed to elucidate which types of protein kinases were able to phosphorylate Cx45. Upon exposure to cAMP and pervanadate, the relative intensity of the phosphorylated form of mCx45 was significantly increased. Sequence analysis learns that the carboxyl terminus of Cx45 contains putative sites for phosphorylation by PKA. Preliminary results in our laboratory obtained with SKhep1 cells which endogenously express low amounts of hCx45, have indicated that upon stimulation with 8-Br-cAMP phosphorylation of hCx45 channels is also increased.
Fig. 8. A. Representative WB showing the effects evoked by activation of various protein kinases on phosphorylation of Cx45. Untransfected HeLa cells serve as a negative control for Cx45 expression (HeLa). HeLa cells transfected with mCx45 were stimulated with cAMP, cGMP, PMA, 4α-PDD and pervanadate (per). Phosphorylation of Cx45 in treated cells was compared with phosphorylation of untreated control cells (con). B. Relative change in phosphorylation measured by the ratio 48 kD/46 kD and compared to the control conditions (100%) as calculated from three independent experiments. Pre-incubation of the cells with 50 μM PD90859 did not alter the ratio compared to that of pervanadate treated cells (not shown).

Although it has been shown that pervanadate strongly activates MAPkinase [31], we could not exclude that the pervanadate-induced increase in phosphorylation of Cx45 we measured, is partly mediated by secondary effects. First, pervanadate is also known as a potent inhibitor of tyrosine phosphatase activity [32], and it has been shown that Cx45 can be phosphorylated on tyrosine residues [20]. Secondly, the PLS/TP motif, the most optimal sequence recognition site for both Erk1 and Erk2 [33], is not included in the carboxyl terminus of mCx45.

To differentiate between these two mechanisms, we pre-inhibited the cells with 50 μM PD90859, a specific inhibitor of MAPkinase, prior to stimulation with pervanadate. Specific inhibition of MAPkinase did not alter the
increased phosphorylation induced by pervanadate. Furthermore, stimulation of the cells with epidermal growth factor (EGF, an activator of MAP kinase) failed to increase phosphorylation as compared to control. From this we have to conclude that the increase in phosphorylation of Cx45 upon stimulation of the cells with pervanadate is not mediated by activation of MAP kinase, but presumably by tyrosine phosphatase inhibition.

Incubation with PMA and cGMP did not significantly alter phosphorylation of Cx45 as compared to control. Even if exposure of the cells to PMA is prolonged to 15 or 25 min, phosphorylation is not altered (data not shown).

Because some evidence exists that expression of PKG in cultured cells may be lost during repeated passages of the cells [34], and because stimulation of this kinase seemed ineffective in altering Cx45 phosphorylation, we checked the expression of PKG in our HeLa cells. Both from WB analysis and immunocytochemical staining it can be concluded that PKG-1 is present in these cells (data not shown). Therefore, stimulation of this kinase does not affect Cx45 phosphorylation.

4.2. Cx45 macroscopic junctional conductance is differentially modulated

Macroscopic junctional conductance of Cx45 gap junction channels was significantly decreased after stimulation with cAMP and pervanadate, remained unaltered after stimulation with cGMP or using 4α-PDD (the non-phosphorylating structural analogue of PKC activator PMA) but was increased after stimulation with PMA. Comparison of the electrophysiological results with those of the Western blot experiments implies that exposure to cGMP or 4α-PDD does not affect phosphorylation and conductance of Cx45 gap junctions. Exposure to cAMP or pervanadate, which lead to increased phosphorylation of Cx45, both result in a decrease in junctional conductance. Perhaps surprisingly, PMA treatment which increased macroscopic conductance of Cx45 gap junctions, did not affect Cx45 phosphorylation. Phosphorylation by exposure to TPA or PMA was found for Cx32 [35], Cx40 [36], and Cx43 [37, 38]. However, PKC induced phosphorylation of connexins is presumably not the only mechanism by which phorbol esters influence gap junction channels. Although Cx26 cannot be phosphorylated [35, 39], exposure of SKHeP1 cells transfected with rat Cx26 to TPA, reduced lucifer yellow dye transfer and junctional conductance and markedly decreased the amount of large channel conductances (140–150 pS) in favor of the smaller conductances (40–70 pS) [14]. This action of phorbol esters of unknown mechanism might also be responsible for the increase in junctional conductance of mCx45 channels in our experiments.

Macroscopic gap junctional conductance is determined by the number of channels, the open probability, and the conductance of one single gap junction channel. The timecourse of the modulatory effect was 1–2 min, making a change in the number of channels highly unlikely, also because the half-life of Cx45 was reported to be 4.2 h [20]. This was confirmed by immunocytochemistry, showing no differences in subcellular localization, which would indicate rapid internalization of Cx45 protein (not shown). Reducing the open probability of the channels with halothane, allowed us to evaluate alterations in single channel conductance, but prohibited analysis of open probability itself.

4.3. Phosphorylation doesn’t affect single channel conductance

Under control conditions, two channel conductances were measured. One large state of 39.0 pS and one small state of 21.9 pS. These channel conductances compare well to those previously reported in the literature. Using identical pipette solutions, Kwak et al. [14] showed that hCx45, endogenously expressed in SKHeP1 cells, had only one channel conductance of 36 pS at V = 25–50 mV. However, at larger V, a second smaller conductance of 22 pS became apparent. Using the same cells, Moreno et al. [15] found only one conductance of 28 pS with pipette solutions similar to ours, and Hermans et al. [40] detected one conductance of 17.8 pS, but with a very dissimilar pipette solution. In N2A cells, single chick Cx45 (cCx45) gap junction channels had conductances of 32 and 35 pS, with 120 mM KCl and 140 mM CsCl as main pipette ion, respectively, at V < 50 mV [17]. At larger V, a substate of 20 pS was detected (120 mM KCl as main ion in the pipette). In our experiments, the smaller conductance and the larger conductance state were measured both at 20 and 50 mV. Although independent openings and closures of the ~22 pS conductance were frequently observed, it is not clear whether it is an independent population of channels, or a substate of the larger conductance. Pre-treatment of the cells with (de)phosphorylating drugs did not result in significant changes in the single channel conductances or distribution. The small hCx45 conductance of 22 pS was reported to shift towards 16 pS, after treatment with TPA, a phorbol ester very similar to PMA [14]. In our experiments, PMA did reduce the single channel conductance from 21.9 to 20.8 pS, although not significantly. Like in our experiments single channel distributions of hCx45 were insensitive to treatment with cGMP or cAMP [14]. By exclusion, since phosphorylation alters macroscopic conductance, and presumably not the number of channels or single channel conductance of Cx45 gap junction channels, we conclude that modulation occurs by changes in the open probability of mCx45.

In conclusion, this study demonstrates that phosphorylation of Cx45 is differentially regulated by various protein kinases and that the alterations evoked by phosphorylation differentially affect the electrical coupling between transfected cells expressing mCx45.
Immunocytochemistry has revealed that in the mouse heart, Cx45 gap junction channels are mainly located in the conduction system, and more specifically at the borderzone between the central core of the system and the working ventricular myocardium. In the conduction system, Cx45 is co-expressed with Cx40 and Cx43 [12,13], while in the working myocardium Cx45 is predominantly expressed (see Ref. [41] and references therein). From studies on transfected cells it is known that Cx45 is able to form functional gap junction channels with both Cx40 and Cx43 [22], while Cx40 and Cx43 fail to form functional channels [22,42,43]. In this position, Cx45 might function as an intermediate for impulse propagation between the conduction system and the myocardium.

Our results suggest that under (patho)physiological conditions, e.g. under ischemic conditions, when the propensity for arrhythmia’s increases and an increased cAMP level in the Purkinje system has been measured [44] modulation of Cx45 channel properties might play an important role thereby affecting transmission of the electrical impulse from the conduction system to the working ventricular myocardium.

Acknowledgements

A.A.B.v.V. and H.V.M.v.R. were financially supported by a project grant no. 97. 184 from the Netherlands Heart Foundation (to H.J.J.). The authors wish to thank Prof. Dr. K. Willecke (Institute for Genetics, Bonn, Germany) for providing the Hela cells used in this study and Dr. T.H. Steinberg (Institute for Infectious Diseases, St.Louis, USA) for providing the Cx45 antibody.

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