Human cardiac gap-junction coupling: effects of antiarrhythmic peptide AAP10

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Aims Ventricular arrhythmia is one of the most important causes of death in industrialized countries and often accompanies myocardial infarction and heart failure. In recent years modification of gap-junctional coupling has been proposed as a new antiarrhythmic principle. We wanted to examine whether the gap junction modulator (antiarrhythmic peptide) AAP10 exerts effects on human cardiac gap junctions, whether the effect might be enhanced in uncoupled cells, whether it affects electrical and metabolic coupling, and which of the cardiac connexin isoforms (Cx40, Cx43, Cx45) may be affected.

Methods and results We determined the influence of 50 nM AAP10 (H2N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH2) on macroscopic gap junction conductance by dual whole-cell voltage clamping in human and rat cardiomyocytes. Cells were partially uncoupled by CO2-mediated acidosis (pH 6.3) or kept at ‘normal’ conditions (pH 7.4, T 36°C). Furthermore, we investigated effects of AAP10 in HeLa cells stably transfected with connexin 40, 43, or 45 and on metabolic coupling determined by dye transfer (Lucifer yellow). AAP10 (50 nM)-enhanced gap-junctional intercellular coupling in human and rat cardiomyocytes, completely prevented CO2-acidosis-induced uncoupling and improved metabolic coupling. The coupling effect of AAP10 was significantly enhanced in previously uncoupled cells. Regarding the connexin isoforms, AAP10-enhanced electrical and metabolic coupling in HeLa cells expressing Cx43 or Cx45, but not in HeLa cells expressing Cx40.

Conclusion We conclude that the antiarrhythmic peptide AAP10, which improves gap-junctional intercellular coupling and prevents uncoupling by acidification in human cardiomyocytes, might be useful for antiarrhythmic strategies regarding arrhythmias caused by uncoupling of Cx43 and Cx45, but not Cx40.

KEYWORDS Antiarrhythmic peptide; AAP10; Gap junctions; Human cardiomyocytes; Dual whole-cell voltage clamp

1. Introduction

Gap junction channels form the basis of intercellular communication in many organs. They allow propagation of action potentials (electrical coupling) as well as transfer of small molecules (metabolic coupling). In the cardiovascular system, they play an important role in excitation spread in heart and maintaining a normal heart rhythm. Gap junction channels are formed by two hemichannels (connexons) each composed of six proteins called connexins. There are several connexin isoforms known; the most important one in the heart is Cx43, which is the predominant isoform in working myocardium. Cx40 is mainly found in the conduction system and atrium and Cx45 plays a role during development and is found in adult hearts in the conduction system and at the border between myocytes and fibroblasts (for review see Camelliti et al.1).

Arrhythmia is one of the most important final causes of death in industrialized countries and often accompanies myocardial infarction and heart failure. Gap-junctional uncoupling has been identified as an important (among others) contributing factor in the pathophysiology of reentrant arrhythmia.2,3 In particular, during cardiac ischaemia gap-junction uncoupling occurs after about 15 min and has been linked to the initiation of ventricular fibrillation (type IB, typically after 20–40 min of ischaemia).

In recent years a new antiarrhythmic principle has been proposed by modulation of gap-junctional coupling using antiarrhythmic peptides and their derivatives.4–7 The lead compound AAP10 (H2N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH2) was shown to enhance electrical coupling in rat and guinea pig cardiomyocytes6,8 and to prevent ischaemia-induced type IB ventricular fibrillation.9 Although these results could be reproduced by others and also with compounds chemically closely related to AAP10,7,10–14 there are, however, still several important open questions:

(i) Is the effect of the antiarrhythmic peptide AAP10 limited to certain cardiac connexin isoforms?
(ii) Is it effective on both electrical and metabolic coupling?
(iii) Will the effect be enhanced in partially uncoupled cells?
(iv) Finally the most important question: Does it work in human cardiomyocytes?

Therefore, we investigated the effect of AAP10 on human and rat cardiomyocytes with and without uncoupling by CO2-mediated acidosis and the influence of AAP10 on electrical and metabolic coupling in cardiomyocytes as well as in Cx40, Cx43, or Cx45 transfected HeLa cells.

2. Methods
(For a detailed description of methods see Supplementary material online.)

2.1 Cells
We determined the effect of AAP10 on intercellular coupling in freshly isolated human atrial cardiomyocytes, cultured neonatal rat cardiomyocytes, and HeLa cells stably transfected with genes encoding for Cx40, Cx43, or Cx45. Human right atrial biopsies (free atrial wall, undiseased atrium) were taken from patients connected to extracorporal perfusion for cardiac surgery. All patients gave written informed consent prior to the operation. The study was approved by the ethics committee of the University of Leipzig and was in accordance with the Declaration of Helsinki. All animal experiments were performed in accordance with the German Law on animal welfare and were approved by the local committee for animal welfare.

Procedures for isolation of pairs of human cardiomyocytes and culture of neonatal rat cardiomyocytes and transfected HeLa cells were described before14,15 and in Supplementary material online).

2.2 Dual whole-cell voltage clamp
Cells were transferred to an experimental chamber mounted on an inverted microscope and were continuously superfused with modified Tyrode’s solution (in mmol/L: NaCl 135, KCl 4, CaCl2 2, MgCl2 1, NaH2PO4 0.33, HEPES 10, glucose 10; pH 7.4, T 36°C). Two synchronized discontinuous patch clamp amplifier (SEC 05, npi electronic, Tamm, Germany),17 were used. Electrodes were pulled from borosilicate glass capillaries with inner filament having resistances of 4–6 MΩ. For perforated patch, 240 μmol amphotericin B18 were added to electrode solution (in mmol/L: NaCl 8, CaCl2 1, CsCl2 125, HEPES 10, EGTA 10, MgATP 3, Na2ATP 2, NaGTP 0.1, pH 7.2). Macroscopic gap-junctional conductance was determined by applying transjunctional voltage differences from −50 mV to +50 mV for 200 ms, thereby assessing the cord conductance. Junctional conductance (gj) was calculated as described previously.6,8 In addition, we applied transjunctional voltage differences of −120 mV to +120 mV for 2000 ms in order to assess the voltage-dependent inactivation. Data in the latter case were fitted to the two-state Boltzmann equation.19

To determine the time-course of gap junctional conductance, a transjunctional voltage difference of −10 mV for 200 ms was applied once a minute. Before wash-in AAP10 (50 nmol/L for 30 min), 0.05% BSA was added to extracellular solution to block unspecific binding sites in the perfusion system. To determine the effect of AAP10 pre-treatment in gap-junction uncoupling bicarbonate-buffered Tyrode’s solution (in mmol/L: NaCl 137, KCl 5, CaCl2 2, MgCl2 1, NaHCO3 24, NaH2PO4 0.4, glucose 10) was used and has been adjusted to pH 6.3 by insufflations of 100% CO2. Based on the concentration-response curves of earlier studies4,5,9 we have chosen 50 nM AAP10 as a test concentration, since in all test systems, the antiarrhythmic peptides and AAP10 were effective at concentrations of 1–100 nM.

In additional experiments on human cardiomyocytes, we treated uncoupling with AAP10 using the following protocol (AAP10 post-treatment): cell pairs were patched as described above and submitted to acidosis-induced uncoupling. After establishing uncoupling (10 min uncoupling), acidosis was continued and the cell pairs were additionally treated for 30 min with 50 nM AAP10.

2.3 Dye coupling
In cultured neonatal rat cardiomyocytes and transfected HeLa cells, permeability was determined by diffusion of the fluorescent dye Lucifer yellow (LY, MW 457.2) as described.20 There was microinjection of 0.1% LY in one cell and the number of coupled cells was determined after 1 min dye transfer in cardiomyocytes and after 5 min in HeLa cells, respectively. In HeLa Cx45 cells dye transfer was assessed by scrape load experiments using the gap junction-permeable dye LY (0.05%) and the much larger gap junction-impermeable dye rhodamine dextran (RD, MW 10 000, 0.05%).21,22 The cells were incubated for 30 min with AAP10 (50 nmol/L) before scrape load. For analysis we determined the ratio (NLY/NLY + RD) as number of cells labelled by diffusion (only labelled by LY; NLY) divided by number of all labelled cells (LY only + LY and RD; NLY + RD) after 5 min dye diffusion.

2.4 I(Na) measurement
Voltage clamp experiments were performed on isolated human cardiomyocytes following classical protocols23 at a holding potential of −80 mV, which were clamped to −80 to +35 mV (pulse duration: 100 ms) (solutions: see above). The measured current was related to the cell capacity and expressed as pA/pF.

2.5 Biochemistry
For biochemical analysis we used well-established procedures for immunocytochemistry and western blot analysis using the following antibodies: rabbit-anti-Cx40 (AB1726, Chemicon), rabbit-anti-Cx43 (C6219, Sigma–Aldrich, Munich, Germany), mouse-anti-Cx45 (MAB3100, Chemicon, Millipore, Billerica, MA, USA), and FITC-conjugated secondary antibodies: swine-anti-rabbit (F0205, DAKO, Hamburg, Germany) and rabbit-anti-mouse (F0261, DAKO). For further details see Supplementary material online.

2.6 Material
AAP10 (H2N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH2) was gained by our own synthesis, high-performance liquid chromatography-purified, purity >99%, as described previously.4,5

2.7 Statistics
We performed a multivariate analysis of variance using species; pH and treatment as factors, followed by an ad hoc statistic test (t-test, with Bonferroni correction, if necessary).

3. Results
3.1 AAP10 prevents acidosis-induced gap-junctional electrical uncoupling in human and rat cardiomyocytes
Macroscopic gap-junctional conductance (gj) was increased by AAP10 and uncoupling was prevented in human (Figure 1A) and rat cardiomyocytes (Figure 1C). Uncoupling was achieved by CO2-mediated acidosis (pH 6.3) and conductance was reduced to about one-third of its initial value. Overall reduction (Δj/Δt) was −0.76 ± 0.30 nS/min (n = 6) in human and −1.36 ± 0.59 nS/min (n = 6) in rat cardiomyocytes. Gap-junctional uncoupling was completely prevented by addition of 50 nmol/L AAP10 and furthermore, even at acidic conditions, there was an increase in gap-junctional conductance. Increase in electrical coupling (Δj/Δt) at
pH 6.3 was 0.96 ± 0.17 nS/min (n = 5) in human and 0.24 ± 0.06 nS/min (n = 6) in rat cardiomyocytes, during acidosis-related uncoupling (simulating ischaemia). Exemplary current traces are given in Figure 2A (human) and C (rat).

3.2 AAP10 enhances gap-junctional conductance in human and rat cardiomyocytes under normal conditions (pH = 7.4)

We also investigated the effect of AAP10 on cardiomyocytes without acidosis, i.e. under normal, nearly physiological conditions (pH 7.4, T 36 °C). There was a slight increase in gap-junctional conductance in control groups: ΔDj/Δt = 0.34 ± 0.08 nS/min (n = 6) in human and ΔDj/Δt = 0.36 ± 0.10 nS/min (n = 6) in rat cardiomyocytes. In addition of 50 nmol/L AAP10 there was significant enhancement of electrical coupling. Increase in conductance was ΔDj/Δt = 1.09 ± 0.30 nS/min (n = 4; P < 0.05) in human cardiomyocytes (Figure 1B) and ΔDj/Δt = 0.81 ± 0.12 nS/min (n = 6; P < 0.05) in rat cardiomyocytes (Figure 1D). Exemplary time courses of calculated gap-junctional conductance and corresponding current traces are shown in Figures 1B and D and in Figure 2B and D.

3.3 The effect of AAP10 on gap-junctional electrical coupling is enhanced in partially uncoupled cells

Comparing the quantitative effects of AAP10 in partially uncoupled cells (pH 6.3) and in normal cells (pH 7.4) revealed that the effect of AAP10 was significant under both conditions (P < 0.01), and was significantly enhanced in the uncoupled cells at pH 6.3 (P = 0.011) (Figure 3A). The observed increase in ΔDj/Δt was significantly higher in uncoupled cells (P = 0.051). Regarding both species, AAP10 effects were comparable although somewhat (n.s., P = 0.054) more pronounced in human cardiomyocytes.

3.4 Post-treatment with AAP10 reverses acidosis-induced uncoupling

If human cardiomyocytes were uncoupled and thereafter treated with AAP10, AAP10 also was effective in reversing acidosis-induced uncoupling: acidosis (pH 6.3) led to a decrease in ΔDj/Δt by −0.54 ± 0.11 nS/min (n = 4, P < 0.05). After uncoupling, cells were treated with 50 nM AAP10, which reversed uncoupling leading to increase in ΔDj/Δt by +0.29 ± 0.13 nS/min (n = 4, P < 0.05) and ending up in macroscopic gap-junction conductance above the control level (Figure 3B).

3.5 AAP10 does not affect I(Na) in human cardiomyocytes

Under control conditions, a maximum I(Na) of about 120 pA/pF was found at −45 mV in good correspondence with others. Voltage clamp revealed that neither the maximum I(Na) current nor the current–voltage relationship were altered by 50 nM AAP10 (Figure 3C; n = 6).
3.6 AAP10 enhances gap-junctional permeability (metabolic coupling) in rat cardiomyocytes

Gap-junctional metabolic coupling (pore permeability) was measured by transfer of the fluorescent dye LY from one cell to another by microinjection to neighboring cells. The number of cells labeled after 1 min dye transfer was determined. After 30 min incubation with AAP10, there was a significant increase in the number of labeled cells by LY via gap junctional diffusion: 10.31 ± 1.72 cells in control group (n = 16) vs. 22.41 ± 2.10 cells after 30 min AAP10 treatment (n = 17; P < 0.05; Figure 4). Figure 4A and B shows exemplary pictures and quantitative data of dye-transfer experiments in controls and after AAP10 incubation.

3.7 AAP10 enhances conductivity and permeability in HeLa Cx43 and HeLa Cx45, but not in HeLa Cx40

Regarding the question, which of the connexin isoforms relevant in heart (Cx43, Cx40, Cx45) may be affected by AAP10, we investigated electrical and metabolic coupling under the influence of AAP10 in transfected HeLa cells. Thus, in pairs of HeLa cells transfected with genes encoding for Cx40, Cx43, or Cx45 macroscopic gap-junctional conductance (gj) was determined. For analysis, the change of gj during the experiment was determined as Δj/Δt. Without treatment there was a slight increase in gj with time (Δj/Δt(control) = 0.134 ± 0.030 nS/min (n = 12)) in HeLa Cx43 controls. This increase was significantly enhanced by 50 nmol/L AAP10 (Δj/Δt(AAP10) = 0.267 ± 0.047 nS/min (n = 11; P < 0.05). Similar results were obtained from HeLa Cx45. In controls, there was a slow increase in gj with time [Δj/Δt(control) = 0.065 ± 0.026 nS/min (n = 4)]. This was also significantly enhanced if AAP10 was added (Δj/Δt(AAP10) = 0.245 ± 0.032 nS/min (n = 4, P < 0.05). In contrast, AAP10 had no effect on macroscopic gap-junctional conductance in HeLa Cx40 [Δj/Δt(control) = 0.205 ± 0.039 nS/min (n = 14)]. Quantitative data are given in Figure 5 for representative exemplary gap-junction currents and time courses of calculated conductance see Supplementary material online, Figure S1.

Permeability of gap junctions was determined using microinjection of the fluorescent dye LY. In HeLa Cx43 controls, there were 22 ± 1.83 coupled cells (n = 18) after 5 min dye diffusion (Figure 4E). After 30 min treatment with AAP10 there was an increase in metabolic coupling with 35.87 ± 4.85 coupled cells (n = 15, P < 0.05; Figures 4F and 5) after 5 min dye diffusion. In accordance with dual whole-cell voltage clamp measurements there was no effect of AAP10 on dye diffusion in HeLa Cx40 [control: 6.625 ± 0.586 coupled cells (n = 24) vs. AAP10: 6.957 ± 0.771 (n = 23) coupled cells; Figures 4C and D and 5].

It was not possible to determine gap-junction permeability in HeLa Cx45 by the experimental design used for the other cell types because of the fact that HeLa Cx45 are weakly coupled and only a few cells in a cluster communicate via gap junctions. With microinjection of LY in one cell there was only infrequent diffusion to other cells (data not shown). Therefore, additional scrape load experiments were performed using the fluorescent dyes LY (0.05%) and RD (0.05%). The amount of cells labelled with fluorescent dye by diffusion and not by membrane disruption through scraping (NLY/NLY+RD; see also methods section) was determined. In each group n = 6 experiments were performed and about 2000 cells per group were analyzed in total. In contrast to other methods used there was dye diffusion in controls (NLY/NLY+RD = 20.02 ± 0.98%, n = 6; Figure 4G). If the cells were incubated for 30 min with AAP10 (50 nmol/L) prior to scrape load there was an

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/83/2/405/320508/fig2)

Figure 2: Exemplary current traces of the same experiments as shown in Figure 1 at time points 10 min and 40 min. Gap-junction currents were measured by application of transjunctional voltage difference of -10 mV for 200 ms. (A) CO₂-acidosis in rat cardiomyocytes; (B) pH 7.4 in rat cardiomyocytes; (C) CO₂-acidosis in human cardiomyocytes; (D) pH 7.4 in human cardiomyocytes.
increase in dye diffusion via gap junctions ($N_{LY}/N_{LY+RD}=29.40 \pm 2.39\%$, $n=6$, $P<0.05$, Figure 4H).

3.8 Immuncytochemistry and western blot analysis show expression of Cx40, Cx43, and Cx45 and their location at cell membrane in human and rat cardiomyocytes as well as in transfected HeLa cells

For control we investigated the expression of connexins in the used cells as well as their incorporation into cell membrane. Western blot analysis clearly shows expression of Cx43 in human and rat cardiomyocytes (Figure 6) as well as expression of Cx40 and Cx45. In neonatal rat cardiomyocytes Cx40 and Cx45 were only clearly detectable when 70 mg of total protein amount was applied. In each of the HeLa cell lines there was only one connexin isoform expressed in accordance to their transfection.

Immuncytochemical staining showed membrane staining for connexins Cx40, Cx43, and Cx45 into cell membrane in cardiomyocytes and transfected HeLa cells (Figure 7). Western blot analysis and immuncytochemical staining revealed expression of Cx40, Cx43, and Cx45 and their location at the cell membrane.

3.9 Characteristics of human cardiac gap-junction currents

Since this is the first report on human cardiac gap-junction currents, we tried to characterize the currents in human cardiomyocytes a little bit more. Applying transjunctio

Figure 3 (A) Alteration of gap-junction conductance ($\Delta G/\Delta t$) in human and rat cardiomyocytes at CO$_2$-acidosis (dark columns) and at pH 7.4 (light columns). About 50 nmol/L of AAP10 increased gap-junctional conductance under both conditions and prevented gap-junctional uncoupling by CO$_2$-mediated acidosis in human and rat cardiomyocytes. (*$P<0.05$). Initial macroscopic gap-junction conductance was $13.39 \pm 1.69$ nS in human and $12.54 \pm 1.38$ nS in rat cardiomyocytes. (B) Effect of 50 nM AAP10 post-treatment on previously CO$_2$-uncoupled human cardiomyocytes. The upper part shows an original experiment, the lower panel gives the quantitative data for gap-junction conductance ($\Delta G/\Delta t$; *$P<0.05$). (C) $I_{\text{Na}}$ was measured on isolated human cardiomyocytes by voltage clamp experiments with a holding potential of -80 mV and a 100 ms pulse from -80 mV to +35 mV. Under control conditions, a maximum ($I_{\text{Na}}$) of about 120 pA/pF was found at -45 mV. Voltage clamp revealed that neither the maximum ($I_{\text{Na}}$) current nor the current–voltage relationship were altered by 50 nM AAP10 ($n=6$). The measured current was related to the cell capacity and expressed as pA/pF.
Rat cardiomyocytes, HeLa Cx40, and HeLa Cx43 were loaded with Lucifer yellow (LY) by microinjection and pictures were made 1 min (rat CMC) and 5 min later (HeLa cells), respectively. AAP10 increased metabolic gap-junctional coupling in rat cardiomyocytes (rat CMC), HeLa Cx43, and HeLa Cx45, but not in HeLa Cx40. HeLa Cx45 were scrape-loaded with LY and rhodamine dextran (RD). Asterisk (* white) indicates cells that are coloured by dye diffusion (only LY, G: $N_{LY}/N_{LY+RD} = 6/28 = 21.4\%$; H: $N_{LY}/N_{LY+RD} = 5/17 = 29.4\%$); for further details see Section 2. AAP10 increased the percentage of cells coloured by dye diffusion. Asterisk (* black) indicates $P < 0.05$. 

Figure 4  Dye diffusion in rat cardiomyocytes, HeLa Cx40, HeLa Cx43, and HeLa Cx45 in controls (A, C, E, G) and after 30 min treatment with AAP10 (B, D, F, H). Rat cardiomyocytes, HeLa Cx40, and HeLa Cx43 were loaded with Lucifer yellow (LY) by microinjection and pictures were made 1 min (rat CMC) and 5 min later (HeLa cells), respectively. AAP10 increased metabolic gap-junctional coupling in rat cardiomyocytes (rat CMC), HeLa Cx43, and HeLa Cx45, but not in HeLa Cx40. HeLa Cx45 were scrape-loaded with LY and rhodamine dextran (RD). Asterisk (* white) indicates cells that are coloured by dye diffusion (only LY, G: $N_{LY}/N_{LY+RD} = 6/28 = 21.4\%$; H: $N_{LY}/N_{LY+RD} = 5/17 = 29.4\%$); for further details see Section 2. AAP10 increased the percentage of cells coloured by dye diffusion. Asterisk (* black) indicates $P < 0.05$. 

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The present data indicate (i) that AAP10 enhances gap-junctional electrical coupling in both human and rat cardiomyocytes under normal conditions, (ii) that this effect is significantly enhanced, if cells are previously uncoupled by acidosis, (iii) that pre-treatment and post-treatment with AAP10 were effective against acidosis-induced de-coupling, (iv) that in the test systems investigated here AAP10 affects Cx43 and Cx45, but not Cx40, and (v) that AAP10 not only can affect electrical coupling but also metabolic coupling. Furthermore (vi), we present a characterization of human cardiac gap-junction currents.

Our present result shows that the AAP10 effect is enhanced in partially uncoupled cells. The mechanism of acidosis-induced decoupling, which was described to occur at pH < 6.5, 24 is still a matter of debate. A common hypothesis is that the connexin carboxy tail is responsible for the pH sensitivity. 25 Further investigations 26 revealed that the carboxy terminal serves as an independent domain, which can bind to another separate domain of the connexin protein, e.g. a region including His-95, 27 and close the channel, comparable with the ball-and-chain-model for K+ channels. His-126 and His-142 were identified as additional histidine residues involved in pH-sensitivity. 28 Moreover, decrease in pH to 6.5 results in association of c-Src kinase to Cx43 and activation of c-Src kinase, thereby suppressing Cx43/ZO-1 association, leading to Cx43 internalization. 29, 30

The fact that the AAP10 effect is enhanced in acidosis-uncoupled cells may involve many factors like receptor sensitivity, receptor coupling, and unknown interactions between PKCθ and those factor involved in pH-dependent channel closing. However, this enhancement of the AAP10-effect in uncoupled cells could mean some preference of AAP10 for uncoupled tissue, such as ischaemic tissue. In accordance with this hypothesis, that Zp123 (now called rotigaptide, a peptide chemically closely related to AAP10) prevents atrial conduction slowing during metabolic stress in isolated atria of Sprague-Dawley rats 31 (and see below). From our present finding that AAP10 effects are more pronounced in uncoupled cells, one could suggest that AAP10 may exert local effects in e.g. an ischaemic zone but only minor effects in the non-ischaemic zone, which could be recently shown in an isolated rabbit heart model of local ischaemia 32 (and see below). In continuation with our previous studies, our present data show that AAP10 also works in human cardiomyocytes.

Regarding the effects of AAP10 in comparison with the chemically closely related successor drug rotigaptide (formerly named ZP123), rotigaptide pre-treatment prevented acidosis-induced conduction slowing in good accordance with our present data. 33 Moreover, prevention of ischaemia-induced de-coupling by rotigaptide pre-treatment 34 or AAP10 pre-treatment was described. 32 Interestingly, it was reported that rotigaptide suppressed atrial fibrillation only in the acute ischaemic substrate but not in an atrial tachypacing model, 35 and not in a volume overload model, 36 although it improved conduction velocities in all these models. This was explained by the authors with differences in the contribution of gap-junction dysfunction to the various models of atrial tachyarrhythmia. 35 An effect on I(Na) was not seen with rotigaptide, 33 as we also could not find such an effect in the human cardiomyocytes with AAP10. In atria submitted to metabolic stress, rotigaptide treatment was also effective to revert conduction slowing 37 as was pre-treatment. 31 On the molecular level this was explained by the findings that ischaemia-induced de-phosphorylation was antagonized by both rotigaptide 12 and AAP10 pre-treatment. 32

Electrical and metabolic coupling may react in a similar manner to a given stimulus, but may also respond differentially or in some cases even in opposite manner. 20, 38 Since it was unknown whether AAP10 may affect metabolic coupling, it was necessary to investigate both electrical and metabolic coupling. Our present data show that AAP10 exerted a similar effect on metabolic coupling as on electrical coupling with effects on Cx43 and Cx45, but not on Cx40
Similarly, enhanced metabolic coupling was observed with rotigaptide in Cx43-coupled cells. Regarding the physiological meaning, metabolic coupling has been shown to allow the passage of second messengers such as cAMP from cell to cell, transfer of 'death factors', and, on the other hand, of 'survival factors' passing via gap-junction channels. In addition, metabolic coupling is necessary for growth and differentiation in many cells, which might open other interesting applications for drugs like AAP10. However, our findings are related to the transfer of LY (molecular weight 457) and must not be uncritically transferred to other molecules which may differ based on size or electrical charges.

Since cardiomyocytes may express different isoforms of connexins, a further, still open question was which of the cardiac connexin isoforms are affected. Western blot analysis and immunohistochemistry data showed expression of Cx40, Cx43, and Cx45 as well as their incorporation into cell membrane in the used biopsies of human myocardium and in the rat cardiomyocytes. The human biopsies were taken from the atrial-free wall, where it is known that Cx40 and Cx43, as well as low amounts of Cx45 can be found. Thus, we decided to investigate which of these three isoforms, solely expressed in HeLa cells, may respond to the application of AAP10. The data show that only Cx43 and Cx45, but not Cx40 are affected by AAP10 regarding both electrical and metabolic coupling, both reacting in the same direction. In accordance with prior studies we also found a coupling effect on Cx43, but in addition to that also Cx45 can be affected by AAP10. Thus, in contrast to previous assumptions, the AAP10 effect is not limited to Cx43. However, the present data indicate that not all connexins are affected by AAP10, since there was no effect on Cx40 as far as investigated here. Regarding the AAP10-successor rotigaptide, it was shown that rotigaptide acts on Cx43, but not on Cx26 or Cx32, which also supports the view that these antiarrhythmic peptides do not affect all connexins.

A first conclusion is that AAP10 probably will exert only minor effects on the cardiac-specific conduction system, since in these structures (including sinus node, AV-node, bundle of His, bundle branches, Purkinje fibres) cells are mostly coupled by Cx40. In good accordance with this consideration, no effect on atrioventricular conduction or on the specific conduction system has been seen with AAP10, ZP123, or rotigaptide so far. This might be beneficial if a sole action on working myocardium is desired.

From our present data and the considerations above it can be concluded that AAP10 indeed works in human cardiomyocytes. Furthermore, the gap-junction currents in human cardiomyocytes show a typical ohmic linear current–voltage relationship for short pulses and low transjunctional voltages (Figure 8A) as previously shown for gap junctions in other tissues and species (see below). Initial gap-junction conductance in pairs of isolated human cardiomyocytes was $17.5 \pm 3.08$ nS ($n = 29$). Human cardiac gap-junction

Figure 7 Immuncytochemical staining of human and rat cardiomyocytes (CMC) and HeLa cells. Left column is stained anti-Cx40, the middle one anti-Cx43, and the right pictures are made from anti-Cx45-stained cells. All three types of HeLa cells incorporate their connexin isoform into cell membrane. In rat and human CMCs there is incorporation of all three connexin isoforms into cell membrane. For inhibition of autofluorescence human CMCs were additionally stained by Chicago blue (scale bar 10 μm).
Characterization of human gap-junctional currents. (A) $I-V$ curve as initial measurements after achievement of whole cell configuration. Values of initial gap-junctional conductance were $17.35 \pm 3.08 \text{nS} (n=29)$. (B) Original voltage and current recordings. Potential in cell 1 was changed from $-90 \text{mV}$ to $+10 \text{mV}$ in $10 \text{mV}$ steps as the other cell was still kept at the holding potential ($-40 \text{mV}$), so that transjunctional voltage differences from $-50 \text{mV}$ to $+50 \text{mV}$ were achieved. (C) Boltzman-fit of the voltage-dependent inactivation data revealing $V_{50}$ values of $-49.5 \pm 6.4 \text{mV}$ and $+68.9 \pm 10.1 \text{mV}$. (D) Exemplary current traces for determination of voltage-dependent inactivation of gap-junction currents obtained by transjunctional voltage differences from $-120 \text{mV}$ to $+120 \text{mV}$ for 2000 ms. (E) Isolated human cardiomyocyte. (F) Pair of human cardiomyocytes in dual whole-cell voltage clamp.
6.4 and

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Supplementary material

ditions.47 A second limitation owing to technical and meth-
expression, so that in adult cells there are no stable con-
days after isolation in culture they change their morphology
in classical adult cardiomyocyte cultures as these cells

4.1 Limitations

In freshly isolated human cardiomyocytes metabolic coupl-
ng cannot be investigated, since a subconfluent or conflu-
ent culture is necessary to measure dye transfer over
several cells. However, it is impossible to study this effect
in classical adult cardiomyocyte cultures as these cells
after isolation decrease their Cx43 expression and several
days after isolation in culture they change their morphology
to a more embryonic type and then again increase Cx43 ex-
pression, so that in adult cells there are no stable condi-
tions.47 A second limitation owing to technical and meth-
odological facts is, that our human current data do not give
information, which of the connexins is expressed in the
very myocyte-pair which was patched. However, the
HeLa cell data show that only Cx43 and Cx45 are affected,
and the protein and immunohistology data (in congruence
with the literature) show that expression of Cx43 is probably
more pronounced than Cx45.

Human data refer to atrial human myocytes. It would be
interesting to investigate human ventricle, but for ethical
reasons it is not possible to get enough ventricular tissue
for cell isolation from non-diseased human ventricles.

Supplementary material

Supplementary material is available at Cardiovascular
Research online.

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