Human cardiac gap junction coupling: effects of antiarrhythmic peptide AAP10

Anja Hagen*, Anna Dietze, Stefan Dhein

Clinic for Cardiac Surgery, University of Leipzig, Heart Centre, Strümpellstr. 39, 04289 Leipzig, Germany

*Current address and address for correspondence:
Anja Hagen
University of Leipzig
University Hospital for Children and Adolescents
Liebigstr. 20a
04103 Leipzig
Anja.Hagen@medizin.uni-leipzig.de
telephone: 0049-341-8651651
fax: 0049-341-8651452

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Abstract

Aims: Ventricular arrhythmia is one of the most important causes of death in industrial 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 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: We determined the influence of 50nM AAP10 (H$_2$N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH$_2$) on macroscopic gap junction conductance by dual whole-cell voltage clamping in human and rat cardiomyocytes. Cells were partially uncoupled by CO$_2$-mediated acidosis (pH 6.3) or kept at “normal” conditions (pH 7.4, T 36 °C). We furthermore investigated AAP10 effects in HeLa cells stably transfected with connexin 40, 43 or 45 and on metabolic coupling determined by dye transfer (Lucifer yellow).

Results: AAP10 (50 nM) enhanced gap-junctional intercellular coupling in human and rat cardiomyocytes, completely prevented CO$_2$-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.

Key Words: antiarrhythmic peptide, AAP10, gap junctions, human cardiomyocytes, dual whole cell voltage clamp
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 6 proteins called connexins. There are several connexin isoforms known, from which the most important ones in heart are Cx43 which is the predominant isoform in working myocardium, Cx40 which is mainly found in conduction system and atrium and Cx45 which plays a role during development and is found in adult hearts in conduction system and at the border between myocytes and fibroblasts (for review see 1).

Arrhythmia is one of the most important final causes of death in industrial 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 ischemia gap junction uncoupling occurs after about 15 minutes and has been linked to the initiation of ventricular fibrillation (type IB, typically after 20-40 minutes of ischemia).

In recent years a new antiarrhythmic principle has been proposed by modulation of gap junctional coupling by the use of 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 cardiomyocytes 6,8, and to prevent from ischemia-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:
1. Is the effect of the antiarrhythmic peptide AAP10 limited to certain cardiac connexin isoforms?

2. Is it effective on both electrical and metabolic coupling?

3. May the effect be enhanced in partially uncoupled cells?

4. 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 as well as the influence of AAP10 on electrical and metabolic coupling in cardiomyocytes as well as in Cx40, Cx43 or Cx45 transfected HeLa cells.
Methods:

(For a detailed description of methods see online data supplement.)

Cells

We determined the effect of AAP10 on intercellular coupling in freshly isolated human atrial cardiomyocytes, cultured neonatal rat cardiomyocytes and HeLa cells stable transfected with genes encoding for connexin 40, 43 or 45. Human right atrial biopsies (free atrial wall, undiseased atrium) were taken from patients connected to extracorporeal 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 to 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 before\textsuperscript{15,16} and in online data supplement).

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, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1, NaH\textsubscript{2}PO\textsubscript{4} 0.33, HEPES 10, glucose 10; pH 7.4, T 36°C). Two synchronized discontinuous patch clamp amplifier (SEC 05, npi electronic, Tamm, Germany)\textsuperscript{17}, were used. Electrodes were pulled from borosilicate glass capillaries with inner filament with resistances of 4-6MΩ. For perforated patch 240µmol amphotericin B\textsuperscript{18} were added to electrode solution (in mmol/l: NaCl 8, CaCl\textsubscript{2} 1, CsCl\textsubscript{2} 125, HEPES 10, EGTA 10, MgATP 3, Na\textsubscript{2}ATP 2, NaGTP 0.1, pH 7.2). Macroscopic gap junctional conductance was determined by applying transjunctional voltage differences from -50mV to +50mV for 200ms, thereby assessing the cord conductance. Junctional conductance (G\textsubscript{j}) was calculated as described previously\textsuperscript{6,8}. In addition, we applied transjunctional voltage differences of -120mV
to +120mV for 2000ms in order to assess the voltage-dependent inactivation. Data in the latter case were fitted to the two-state Boltzmann equation

To determine the time course of gap junctional conductance a transjunctional voltage difference of -10mV for 200ms was applied once a minute. Before wash in of AAP10 (50nmol/l for 30min) 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, BaCl₂ 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 studies we have chosen 50 nM AAP10 as a test concentration, since in all test systems the antiarrhythmic peptides and AAP10 were effective in 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.

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. There was microinjection of 0.1% LY in one cell and the number of coupled cells was determined after 1min dye transfer in cardiomyocytes and after 5min in HeLa cells, respectively. In HeLa Cx45 cells dye transfer was assessed by scrape load experiments using the gap junction permeable dye Lucifer yellow (LY, 0.05%) and the much larger gap junction impermeable dye rhodamine dextran (RD, MW 10000, 0.05%)²¹-²². The cells were incubated for 30min with AAP10 (50 nmol/l) before scrape load. For analysis we determined
the ratio \( \frac{N_{LY}}{N_{LY+RD}} \) as number of cells labelled by diffusion (only labelled by LY; \( N_{LY} \)) divided by number of all labelled cells (LY only + LY and RD; \( N_{LY+RD} \)) after 5min dye diffusion.

I(Na) measurement

Voltage clamp experiments were performed on isolated human cardiomyocytes following classical protocols \(^{23} \) 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].

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) and FITC-conjugated secondary antibodies: swine-anti-rabbit (F0205, DAKO, Hamburg, Germany) and rabbit-anti-mouse (F0261, DAKO). For further details see online data supplement.

Material:

AAP10 (\( \text{H}_2\text{N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH}_2 \)) was gained by our own synthesis, HPLC-purified, purity >99%, as described previously\(^{5,9} \).

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).
Results

AAP10 prevents acidosis-induced gap junctional electrical uncoupling in human and rat cardiomyocytes

Macroscopic gap junction conductance ($g_j$) was increased by AAP10 and uncoupling was prevented in human (fig 1A) and rat cardiomyocytes (fig 1C). Uncoupling was achieved by CO$_2$-mediated acidosis (pH 6.3) and conductance was reduced to about 1/3 of its initial value. Overall reduction ($\Delta j/\Delta t$) 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 50nmol/l AAP10 and furthermore, even at acidotic conditions, there was an increase in gap junctional conductance. Increase in electrical coupling ($\Delta j/\Delta t$) 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 ischemia). Exemplary current traces are given in fig. 2A (human) and 2C (rat).

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: $\Delta j/\Delta t$=0.34±0.08 nS/min (n=6) in human and $\Delta j/\Delta t$=0.36±0.10 nS/min (n=6) in rat cardiomyocytes. In addition of 50nmol/l AAP10 there was significant enhancement of electrical coupling. Increase in conductance was $\Delta j/\Delta t$=1.09±0.30 nS/min (n=4; p<0.05) in human cardiomyocytes (fig. 1B) and $\Delta j/\Delta t$=0.81±0.12 nS/min (n=6; p<0.05) in rat cardiomyocytes (fig. 1D). Exemplary time courses of calculated gap junctional conductance and corresponding current traces are shown in figure 1B and 1D and in figure 2B and D.
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 AAP10 effect was significant under both conditions (p<0.01), and was significantly enhanced in the uncoupled cells at pH 6.3 (p=0.011) (fig. 3A). The observed increase in $\Delta j/\Delta t$ was significantly higher in uncoupled cells (p=0.011). Regarding both species, AAP10 effects were comparable although somewhat (n.s., p=0.054) more pronounced in human cardiomyocytes.

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 $\Delta j/\Delta t$ 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 $\Delta j/\Delta t$ by +0.29±0.13 nS/min (n=4, p<0.05) and ending up in macroscopic gap junction conductance above the control level (fig. 3B).

AAP10 does not affect $I(\text{Na})$ in human cardiomyocytes

Under control conditions a maximum $I(\text{Na})$ of about 120 pA/pF was found at -45 mV in good correspondence with others. Voltage clamp revealed that neither the maximum $I(\text{Na})$ current nor the current-voltage relationship were altered by 50 nM AAP10 (fig. 3C; n=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 Lucifer Yellow from one cell labeled by microinjection to neighboring cells. The number of cells labeled after 1min dye transfer was determined. After 30min incubation with AAP10 there was a significant increase in the number of cells labeled by Lucifer Yellow via gap junctional diffusion: 10.31±1.72 cells in control group (n=16) vs. 17.41±2.10 cells
after 30min AAP10 treatment (n=17; p<0.05; fig. 7). Fig 4 (A, B) shows exemplary pictures and quantitative data of dye transfer experiments in controls and after AAP10 incubation.

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 50nmol/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) vs. Δj/Δt(AAP10)=0.196±0.051 nS/min (n=14)). Quantitative data are given in figure 5, for representative exemplary gap junction currents and time courses of calculated conductance see figure 1 of the online data supplement.

Permeability of gap junctions was determined using microinjection of the fluorescent dye Lucifer yellow (LY). In HeLa Cx43 controls there were 22±1.83 coupled cells (n=18) after 5min dye diffusion (fig. 4E). After 30min treatment with AAP10 there was an increase in metabolic coupling with 35.87±4.85 coupled cells (n=15, p<0.05; fig. 4F; fig. 5) after 5min dye diffusion. In accordance to 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; Fig. 4C-D; fig. 5).
It was not possible to determine gap junction permeability in HeLa Cx45 by the experimental design used for the other cell types due to 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 Lucifer Yellow (LY; 0.05%) and rhodamine dextran (RD; 0.05%). The amount of cells labeled with fluorescent dye by diffusion and not by membrane disruption through scraping (N_{LY}/N_{LY+RD}; see also methods section) was determined. In each group n=6 experiments were performed and about 2000 cells per group analysed in total. In contrast to other methods used there was dye diffusion in controls (N_{LY}/N_{LY+RD}=20.02±0.98%, n=6; fig. 4G). If the cells were incubated for 30min with AAP10 (50nmol/l) prior to scrape load there was an increase in dye diffusion via gap junctions (N_{LY}/N_{LY+RD}=29.40±2.39%, n=6, p<0.05, fig 4H).

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 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 (fig. 6) as well as expression of Cx40 and Cx45. In neonatal rat cardiomyocytes Cx40 and Cx45 were only clearly detectable when 70µg 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 (fig. 7). Western Blot analysis and immuncytochemical staining revealed expression of connexins Cx40, Cx43 and Cx45 and their location at the cell membrane.
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 transjunctional voltages ranging from -50mV to +50mV with short 200ms pulses revealed the typical ohmic behaviour with a linear current-voltage relationship and a cord conductance of 17.5±3.08 nS (n=29) (fig. 8). If long pulses (2000ms) ranging from -120mV to +120mV were applied, voltage-dependent inactivation was seen (fig. 8). A Boltzmann fit to the data of the voltage-dependent inactivation revealed $V_{50}$ values of -49.5±6.4 mV and +68.9±10.1 mV.

Discussion

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

Our present results show, 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 sensitivity25. Further investigations26 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 to the ball-and-chain-model for K⁺- channels. His-126 and His-142 were identified as additional histidine residues involved in pH-sensitivity28. 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 internalization29,30.
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 PKCα 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 ischemic 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 from Sprague-Dawley rats\(^\text{31}\) (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 ischemic zone but only minor effects in the non-ischemic zone, which could be recently shown in an isolated rabbit heart model of local ischemia\(^\text{32}\) (and see below). In continuation of our previous studies, our present data show that AAP10 also works in human cardiomyocytes.

Regarding the effects of AAP10 in comparison to the chemically closely related successor drug rotigaptide (formerly named ZP123) rotigaptide pre-treatment prevented from acidosis-induced conduction slowing in good accordance with our present data\(^\text{33}\). Moreover, prevention of ischemia-induced de-coupling by rotigaptide pre-treatment\(^\text{34}\) or AAP10 pre-treatment was described\(^\text{32}\). Interestingly, it was reported that rotigaptide suppressed atrial fibrillation only in the acute ischemic substrate but not in an atrial tachypacing model\(^\text{35}\), and not in a volume overload model\(^\text{36}\), although it improved conduction velocities in all these models. This was explained by the authors by differences in the contribution of gap junction dysfunction to the various models of atrial tachyarrhythmia\(^\text{35}\). An effect on I(Na) was not seen with rotigaptide\(^\text{33}\), 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\(^\text{37}\) as was pre-treatment\(^\text{31}\). On the molecular level this was explained by the findings that ischemia-induced de-phosphorylation was antagonized by both rotigaptide\(^\text{12}\) and AAP10 pre-treatment\(^\text{32}\).
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\textsuperscript{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 (see also below). Similarly, enhanced metabolic coupling was observed with rotigaptide in Cx43-coupled cells\textsuperscript{13}. Regarding the physiological meaning, metabolic coupling has been shown to allow the passage of second messengers such as cAMP from cell to cell\textsuperscript{39}, transfer of "death factors" \textsuperscript{40}, and, on the other hand, of "survival factors" passing via gap junction channels\textsuperscript{41}. In addition, metabolic coupling is necessary for growth and differentiation in many cells\textsuperscript{42} which might open other interesting applications for drugs like AAP10. However, our findings are related to the transfer of Lucifer Yellow (molecular weight 457) and must not be uncritically transferred to other molecules which may differ regarding 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 connexins 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 \textsuperscript{1,43}. 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 to 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 \(^\text{13}\), which also supports the view that these antiarrhythmic peptide 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 \(^\text{4,10-11,31-32}\). This might be beneficial if a sole action on working myocardium is desired.

From the 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 (fig. 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±3.08 nS (n=29). Human cardiac gap junction channels show voltage dependent inactivation (fig. 8E) with \(V_{50}\) values as determined by Boltzman-fit of -49.5±6.4 and +68.9±10.1 mV. Previously, we determined the \(V_{50}\) values for HeLaCx40, HeLaCx43, HeLa Cx45 with -46.9/+55.1; -78.3/+71.9 and -25.7/+18.4, respectively and for rat cardiomyocytes -47.8/+47.7 \(^\text{15}\), which is in good correspondence to other work \(^\text{44-46}\). Regarding \(g_{\text{min}}\) values in human cardiomyocytes these are higher than those found and published for HeLa cells. This might be due to much higher conductance levels between the human cardiomyocyte cell pairs. Although the measurement of voltage sensitivity can be contaminated by series resistance and is difficult to detect in well coupled cells, in our measurement system the series resistance is overcome by the electrical circuit provided by the switch clamp system \(^\text{17}\). From this data on voltage-dependent inactivation, the human gap junction currents probably may predominantly represent Cx43 currents, and to a lower extent
Cx40 and Cx45 currents. This view would also fit to our protein and immunohistology data regarding connexin expression in the human specimen, showing Cx43 as the predominant isoform.

Limitations
In freshly isolated human cardiomyocytes metabolic coupling cannot be investigated, since a subconfluent or confluent culture is necessary to measure dye transfer over several cells. However, it is impossible to study this effect in classical adult cardiomyocyte cultures since 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 expression, so that in adult cells there are no stable conditions. A second limitation due to technical and methodological 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 it is for ethical reasons not possible to get enough ventricular tissue for cell isolation from non-diseased human ventricles.

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Conflict of Interest: None declared.
References


Legends

Figure 1: Exemplary time courses of calculated gap junction conductance in rat and human cardiomyocytes at pH 7.4 and at uncoupling conditions (CO₂-acidosis). In addition of CO₂-acidosis there was gap junctional uncoupling in rat (A) and human (C) cardiomyocytes. Addition of 50nmol/l AAP10 prevented uncoupling and furthermore increased gap junction conductance (see also fig. 2 and 3). There was also increase in gap junction conductance by AAP10 at pH 7.4 (B, D).

Fig. 2: exemplary current traces of the same experiments as shown in fig. 1 at time points 10min and 40min. Gap junction currents were measured by application of transjunctional voltage difference of -10mV for 200ms. (A: CO₂-acidosis in rat CMC; B: pH 7.4 in rat CMC; C: CO₂-acidosis in human CMC; D: pH 7.4 in human CMC).

Fig. 3: Panel A: Alteration of gap junction conductance (Δj/Δt) in human and rat cardiomyocytes (CMC) at CO₂-acidosis (dark columns) and at pH 7.4 (light columns). 50nmol/l AAP10 increased gap junctional conductance under both conditions and prevented gap junctional uncoupling by CO₂ mediated acidosis in human and rat cardiomyocytes. (* p<0.05). Initial macroscopic gap junction conductance was 13.39±1.69 nS in human and 12.54±1.38 nS in rat cardiomyocytes.
Panel B: Effect of 50 nM AAP10 post-treatment on previously CO₂-uncoupled human cardiomyocytes. The upper part shows an original experiment, the lower panel gives the quantitative data for gap junction conductance (Δj/Δt; *= p<0.05).
Panel C: I(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 to +35 mV. Under control conditions a maximum I(Na) of about 120 pA/pF was found at -45 mV. Voltage clamp revealed that neither the maximum I(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].

Fig. 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 1min (rat CMC) and 5min 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). *(white) indicates cells that are coloured by dye diffusion (only LY, G: N\textsubscript{LY}/N\textsubscript{LY+RD}=6/28=21.4%; H: N\textsubscript{LY}/N\textsubscript{LY+RD}=5/17=29.4%), for further details see method section. AAP10 increased the percentage of cells coloured by dye diffusion. *(black) p<0.05

Fig. 5: 50nmol/l AAP10 increased $\Delta j/\Delta t$ (change in macroscopic gap junction conductance) in HeLa Cx43 and HeLa Cx45, but not in HeLa Cx40. (* p<0.05)

Fig. 6: Western blot analysis of HeLa Cx40, HeLa Cx43 and HeLa Cx45 as well as of rat and human cardiomyocytes (CMC). Total protein amounts of 20µg and 70µg for all cell types were applied. Analysis shows expression of all three investigated connexin isoforms in rat and human cardiomyocytes as well as expression of one connexin isoform in HeLa cells in accordance to their transfection.

Fig. 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 cardiomyocytes there is incorporation of all three
connexin isoforms into cell membrane. For inhibition of autofluorescence human cardiomyocytes were additionally stained by Chicago blue (scale bar 10µm).

Fig. 8: Characterization of human gap junctional currents: A: I-V-Curve (A) as initial measurements after achievement of whole cell configuration. Values of initial gap junctional conductance were 17.35±3.08 nS (n=29). B: Original voltage and current recordings. Potential in cell 1 was changed from -90mV to +10mV in 10mV steps as the other cell was still kept at the holding potential (-40mV), so that transjunctional voltage differences from -50mV to +50mV were achieved. C: Boltzman-fit of the voltage dependent inactivation data revealing V_{50} values of -49.5±6.4 mV and +68.9±10.1 mV. D: exemplary current traces for determination of voltage dependent inactivation of gap junction currents obtained by transjunctional voltage differences from -120mV to +120mV for 2000ms. E: isolated human cardiomyocyte. F: pair of human cardiomyocytes in dual whole cell voltage clamp.
Figure 1

CO₂-acidosis

Baseline

<table>
<thead>
<tr>
<th>CO₂-acidosis</th>
<th>AAP10</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>AAP10</td>
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</table>

pH = 7.4

Baseline

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<th>AAP10</th>
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</thead>
<tbody>
<tr>
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Baseline

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<tr>
<td>control</td>
<td>AAP10</td>
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</tbody>
</table>

<diagram with graphs A, B, C, D showing changes in conductance (g) over time (t) in min>
Figure 2

(A) Human cardiomyocytes, CO₂-acidosis
Baseline (10)
Acidosis (40)
200 pA
50 ms
+ AAP10

(B) Human cardiomyocytes, pH = 7.4
Control
pH
+ AAP10

(C) Rat cardiomyocytes, CO₂-acidosis
Baseline (10)
Acidosis (40)
100 pA
50 ms
+ AAP10

(D) Rat cardiomyocytes, pH = 7.4
Control
pH
+ AAP10
Fig. 3B
I(Na) (normalized current)

- normalized current
- [mV]

- control
- AAP10

1000 pA
2000 pA

control
AAP10

- [pA/pF]
Figure 4
Figure 5
Figure 6

- **anti-Cx45**
  - HeLa Cx40 (20 μg)
  - HeLa Cx40 (70 μg)
  - HeLa Cx43 (20 μg)
  - HeLa Cx43 (70 μg)
  - HeLa Cx45 (20 μg)
  - HeLa Cx45 (70 μg)

- **anti-Cx43**
  - rat CMC (20 μg)
  - rat CMC (70 μg)

- **anti-Cx40**
  - human CMC (20 μg)
  - human CMC (70 μg)
**Figure 7**

<table>
<thead>
<tr>
<th></th>
<th>anti-Cx40</th>
<th>anti-Cx43</th>
<th>anti-Cx45</th>
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<td><img src="he-la_cx45.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 8

A

B

C

D

E

F