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European Journal of Pharmacology 327 (1997) 65–72

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Increase in gap junction conductance by an antiarrhythmic peptide

Andreas Müller *, Michaela Gottwald, Tatjana Tudyka [1], Werner Linke, Wolfgang Klaus, Stefan Dhein

Institute of Pharmacology, University of Köln, Gleueler Straße 24, D-50931 Köln, Germany

Received 18 November 1996; revised 20 February 1997; accepted 14 March 1997

Abstract

Impaired cellular coupling is thought to be a very important factor for the genesis of cardiac arrhythmia. Cellular coupling is mediated by gap junctions. However, there are no therapeutic agents or experimental substances yet that increase cellular coupling. In addition, it has been shown that most antiarrhythmic drugs available now possess serious adverse effects. Thus, there is an urgent need for new antiarrhythmic agents. Previous studies using epicardial mapping in isolated rabbit hearts provided indirect evidence supporting the hypothesis that a newly synthesised antiarrhythmic peptide (Gly-Ala-Gly-4Hyp-Pro-Tyr-$CONH_2$ = AAP10) might act via an increase in cellular, i.e., gap junctional coupling. The aim of the present study was to test this hypothesis. Measurement of the stimulus–response interval in papillary muscle showed a decrease of about 10% after application of 1 μM AAP10. These results are compatible with the hypothesis of AAP10 acting on gap junctions. In order to prove this hypothesis, gap junction conductance was measured directly by performing double-cell voltage-clamp experiments in isolated pairs of guinea-pig myocytes. During a 10 min control period gap junction conductance slowly decreased with a rate of -2.5 ± 2.0 nS/min. After application of 10 nM AAP10 this behaviour reversed and gap junction conductance now increased with $+1.0 \pm 0.7$ nS/min. Upon washout of AAP10 gap junction conductance again decreased with a rate similar to that under control conditions. Another important finding was that we could not detect any other actions of AAP10 on cardiac myocytes. All parameters of the transmembrane action potential remained unchanged and, similarly, no changes in the IV relationship of single cardiac myocytes treated with 10 nM AAP10 could be observed. We conclude that AAP10 increases gap junction conductance, i.e., cellular coupling in the heart. This finding might be the first step towards the development of a new class of antiarrhythmic agents.

Keywords: Arrhythmia; Gap junction; Electrophysiology; Antiarrhythmic peptide

1. Introduction

In the heart, myocytes are electrically connected by gap junctions (Page, 1992; Spach, 1994). This is the basis for the intercellular spread of excitation in the normal heart. Uncoupling of gap junctions occurs, however, in a variety of pathological states including myocardial infarction (Peters et al., 1993; Smith et al., 1991; Campos de-Carvalho et al., 1992; Bastide et al., 1993). This is nowadays thought to be an important factor in the genesis of arrhythmia (Saffitz et al., 1993; Severs, 1994) which still is the main cause of death after myocardial infarction. Thus, improvement of cellular coupling would seem a good goal for antiarrhythmic therapy (Dhein and Tudyka, 1995).

In 1980 Aonuma et al. described a naturally occurring peptide which they called 'antiarrhythmic peptide' because it improved rhythmicity in cultured myocardial cell clusters (Aonuma et al., 1980). Starting from this hexapeptide we synthesised a new peptide (Gly-Ala-Gly-4Hyp-Pro-Tyr-$CONH_2$) which we called AAP10 (Dhein et al., 1994). This peptide decreased dispersion of refractoriness in isolated perfused rabbit hearts and exhibited antiarrhythmic effects during regional ischemia and reperfusion (Dhein et al., 1994) but had no other effects in our experimental setup. Electrophysiological studies on guinea-pig papillary muscle confirmed that AAP10 caused no changes in the parameters of the action potential. These results led to the hypothesis that AAP10 might improve gap junctional coupling. The aim of this study was to test this hypothesis and to determine whether AAP10 affects cellular coupling between cardiac myocytes.

* Corresponding author. Tel.: (49-221) 478-4196; Fax: (49-221) 478-5022; e-mail: andreas.mueller@uni-koeln.de

[1] Present address: Institute of Biochemistry, TU Darmstadt, Germany.

PII S0014-2999(97)00053-8

2. Materials and methods

2.1. Measurement of the stimulus–response interval

Male guinea-pigs (250–300 g) were killed by a single blow on the neck 30 min after intraperitoneal application of 1000 IU/kg body weight heparin. The heart was rapidly removed and put into a preparation chamber where the right ventricle was opened and an appropriate papillary muscle removed. The papillary muscle was then transferred into an organ bath, where it was connected to a pressure transducer (Hugo Sachs Elektronic, March-Hugstetten, Germany) and superfused with normal Tyrode's solution. The Tyrode's solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7.4. Temperature was maintained at 35°C. The preparation was stimulated by square pulses of double diastolic threshold strength at a frequency of 1 Hz.

Following an equilibration period of 45 min action potentials were recorded with glass microelectrodes filled with 3 M KCl having resistances of 10–20 MΩ using a custom-built microelectrode amplifier. Electrode capacity was compensated before each experiment using the capacity compensation circuit of the microelectrode amplifier. Data were recorded at a frequency of 20 kHz using the TIDA data acquisition system (version 5.72a, HEKA Electronic Lambrecht, Germany). Only recordings of propagated action potentials (time between end of the stimulus and start of the action potential > 2 ms) were accepted. Control recordings were done after 45, 55 and 60 min. Thereafter, perfusion with Tyrode's solution containing AAP10 (1 μM) was started. Recordings were carried out after 2, 15 and 30 min. Thereafter AAP10 was washed out. Experiments were accepted only if the recording remained stable over the whole period of the experiment. The concentration of AAP10 was chosen to give maximum effects which were observed at concentrations of 10-100 nM in isolated perfused hearts (Dhein et al., 1994). A ten times higher concentration was used in these experiments to account for the slower diffusion and distribution (as compared to perfused hearts) of the hydrophilic peptide in the superfused papillary muscle preparation.

The stimulus–response interval was measured as the time between the end of the stimulus and the crossing of the -60 mV line. At each measuring point ten action potentials were evaluated in each of six experiments. In addition several other action potential parameters were assessed: action potential duration, maximum upstroke velocity, resting membrane potential and overshoot potential.

2.2. Isolation of myocytes

Pairs of guinea-pig myocytes were isolated as described by Metzger and Weingart (1985). Briefly, guinea-pigs were anticoagulated and killed as described above. The heart was then cannulated and connected to a modified Langendorff apparatus for perfusion. The heart was perfused with modified Tyrode's solution for 10–15 min at room temperature. All following steps were performed at 37°C: (1) perfusion with solution A for 2 min; (2) perfusion with solution B for 2 min; (3) 12–17 min of perfusion with collagenase solution (recirculating). All solutions were gassed with 100% oxygen.

The ventricles were then minced and incubated in collagenase solution for about 5–10 min. After that the cells were filtered through nylon gauze (mesh width 250 μm) and centrifuged several times. The resulting cells were stored in solution A to which $CaCl_2$ was gradually added up to a final $CaCl_2$ concentration of 1.8 mM.

2.3. Double-cell voltage clamp

An aliquot of cells was transferred to a perfusion chamber mounted to the stage of an inverted microscope and the cells were allowed 5 min to settle to the bottom of the chamber. Cells were then superfused with normal Tyrode's solution to which 1 mM $BaCl_2$ was added during recordings.

The method for measuring gap junction conductance was essentially as described by Spray et al. (1981). Each cell of a pair was connected to a voltage-clamp amplifier (SEC 05, NPI-Electronic, Tamm, Germany) via suction pipettes filled with intracellular solution (resistances 2–3 MΩ). Giga-ohm seals were obtained as described by Hamill et al. (1981). Recordings were only started when the seal resistance exceeded 5 GΩ. Measurements were started 3–5 min after establishing the whole cell configuration. To avoid errors arising from series resistance (Wilders and Jongsma, 1992) we used two single-electrode voltage-clamp amplifiers which measure the membrane potential at a time when no current flows across the recording electrode. Great care was taken to correctly adjust the amplifiers to avoid errors arising from erroneous adjustments of voltage-clamp gain, capacity compensation or switching frequency (Halliwell et al., 1994). This approach made it possible to 'simultaneously' measure current and voltage in both cells and thus to accurately control the intercellular voltage. Data were sampled at 10 kHz per channel using the TIDA data acquisition and evaluation system (Wintida 2.6, HEKA-Electronic, Lambrecht, Germany). For analysis data were low-pass filtered at 1 kHz. The switching frequency of the synchronised voltage-clamp amplifiers was set to values between 25 kHz and 30 kHz. Voltage-clamp gain was set to give the fastest possible response while causing minimum overshoot in each experiment individually.

To measure gap junction conductance both cells were clamped to a common holding potential of -40 mV. Then the potential of one cell was changed for 200 ms to potentials between -90 and $+10$ mV in steps of 10 mV thereby establishing intercellular voltage differences between -50 mV and $+50$ mV. The current elicited in the

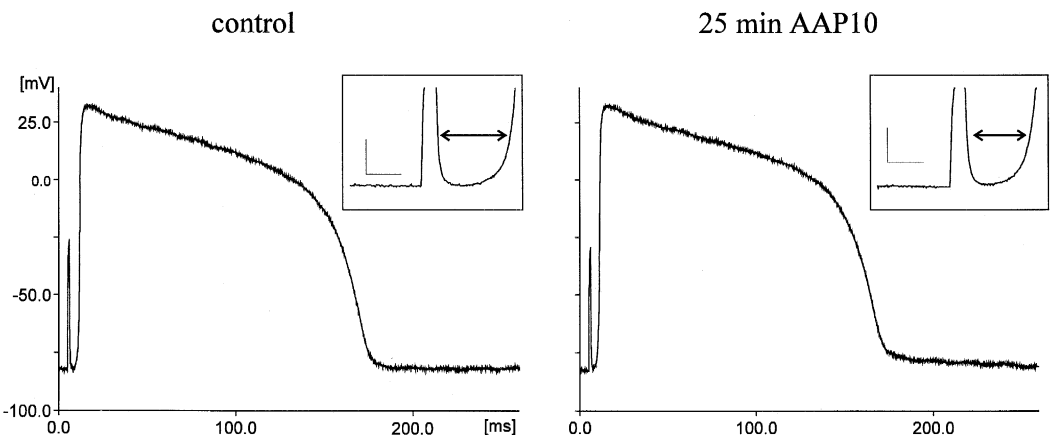

Fig. 1. Action potentials recorded before (left) and 25 min after application of 1 μM AAP10 (right). Note that there was no change in the shape of the action potential (cf., also Table 1). The insets show a magnification of the stimulus artifacts and the foot of the action potentials. Please note that the stimulus–response interval (indicated by the arrows) decreased from 5.0 ms (control) to 4.4 ms after application of AAP10. Scale bars in the insets are 15 mV and 2.5 ms, respectively.

non-pulsed cell was taken as the gap junctional current (Spray et al., 1981; Weingart, 1986). Current-voltage relationships were always linear and gap junction conductance was calculated as the slope of this straight line as obtained from linear regression analysis. Addition of the currents flowing in the resting and pulsed cell revealed the sarcolemmal current in the pulsed cell. From the current-voltage relationship obtained in this way, the input resistance of the cells was estimated from the chord conductance between −80 and −40 mV.

Measurements were done every 60 s in both cells alternatingly. Following a control period of 10 min AAP10 was applied for 10 min in a concentration of 10 nM. Thereafter, AAP10 was washed out for a further 10 min.

During the experiments it soon became clear that gap junction conductance did not remain constant but in most cases decreased during the experiment. It was, therefore, not in all cases possible to directly measure an absolute increase in gap junction conductance. Therefore, we decided to look for the rate of change of gap junction conductance during the control period, application of AAP10 and washout.

2.4. Composition of solutions

- Normal Tyrode's solution (mM): NaCl 136.80, KCl 5.36, NaH_2PO_4 0.42, $NaHCO_3$ 23.80, $MgCl_2$ 1.05, $CaCl_2$ 1.80, glucose 11.00, pH 7.4.
- Modified Tyrode's solution (mM): NaCl 135, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 10, glucose 10, pH 7.4.
- Solution A: modified Tyrode's solution without $CaCl_2$.
- Solution B (mM): NaCl 20, potassium aspartate 120, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4.

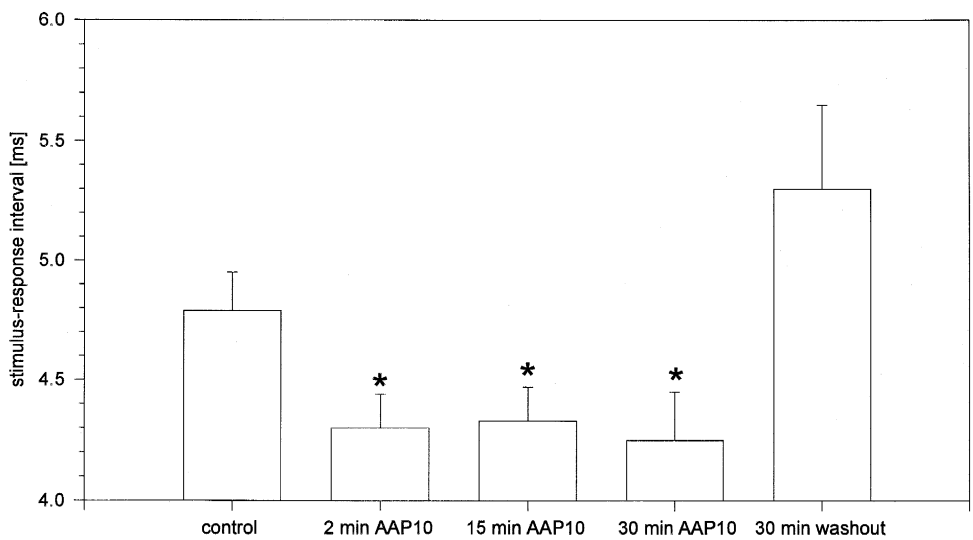

Fig. 2. Effect of AAP10 (1 μM) on the stimulus–response interval in guinea-pig papillary muscle. Data are mean ± S.E.M. of 6 experiments. Asterisks mark significant changes versus control ($P < 0.05$).

Table 1
Influence of AAP10 on electrophysiological parameters in guinea pig papillary muscle ($n = 6$)

Parameter	Control	AAP10 (1 μM)
Maximum upstroke velocity (V/s)	181 ± 10	190 ± 12
AD_{20} (ms)	80 ± 4	78 ± 8
AD_{90} (ms)	165 ± 5	164 ± 6
Overshoot potential (mV)	29 ± 2	30 ± 4
Resting membrane potential (mV)	−84 ± 1	−84 ± 3
Stimulus response interval (ms)	4.79 ± 0.16	4.30 ± 0.14

There were no significant effects of AAP10 on any of the measured parameters except for the stimulus–response interval (cf., Fig. 1A). AD_{20} and AD_{90} = action potential duration at 20% and 90% repolarisation, respectively.

- Collagenase solution: solution B + 1 mg/ml bovine serum albumin (fraction IV) + 25 μM $CaCl_2$.
- Intracellular solution (mM): CsCl 125, NaCl 8, $CaCl_2$ 1, EGTA 10, Na_2ATP 2, MgATP 3, Na_2GTP 0.1, HEPES 10, pH 7.2 with CsOH.

2.5. Chemicals

AAP10 was synthesised in our laboratory (purity: > 99%; HPLC grade) (Dhein et al., 1994). AAP10 was dissolved in normal Tyrode's solution and freshly prepared before each experiment. Bovine serum albumin was from Life Technologies (Eggenstein, Germany). Collagenase (Worthington, Type II) was purchased from Biochrom (Berlin, Germany). All other chemicals were from Sigma (Munich, Germany).

2.6. Statistical analysis

All data are mean ± S.E.M. Significance was tested using the *t*-test for paired observations (stimulus–response interval) or the non-parametric Wilcoxon test (double-cell voltage-clamp experiments).

3. Results

3.1. Effect of AAP10 on the stimulus–response interval

Before starting with technically difficult and time-consuming double-cell voltage-clamp experiments we wanted to have more indirect support for our hypothesis that AAP10 acts on gap junctions. A substance that increases the gap junction conductance should increase the conduction velocity in myocardial tissue provided that cellular coupling is not perfect. Thus, the time interval between the electrical stimulus and the time of the maximum upstroke velocity of a propagated action potential should be smaller after application of such a substance. We tested this prediction in electrically paced guinea-pig papillary muscle. Two minutes after application of 1 μM AAP10 the stimulus–response interval was significantly shortened from 4.79 ± 0.16 ms to 4.28 ± 0.14 ms ($P < 0.05$; Fig. 2). The effect remained stable over the whole period of time when AAP10 was present. After 30 min of washout of AAP10 the stimulus–response interval was prolonged to the control level again or even higher. It is important to note that this effect was not accompanied by changes in any of the

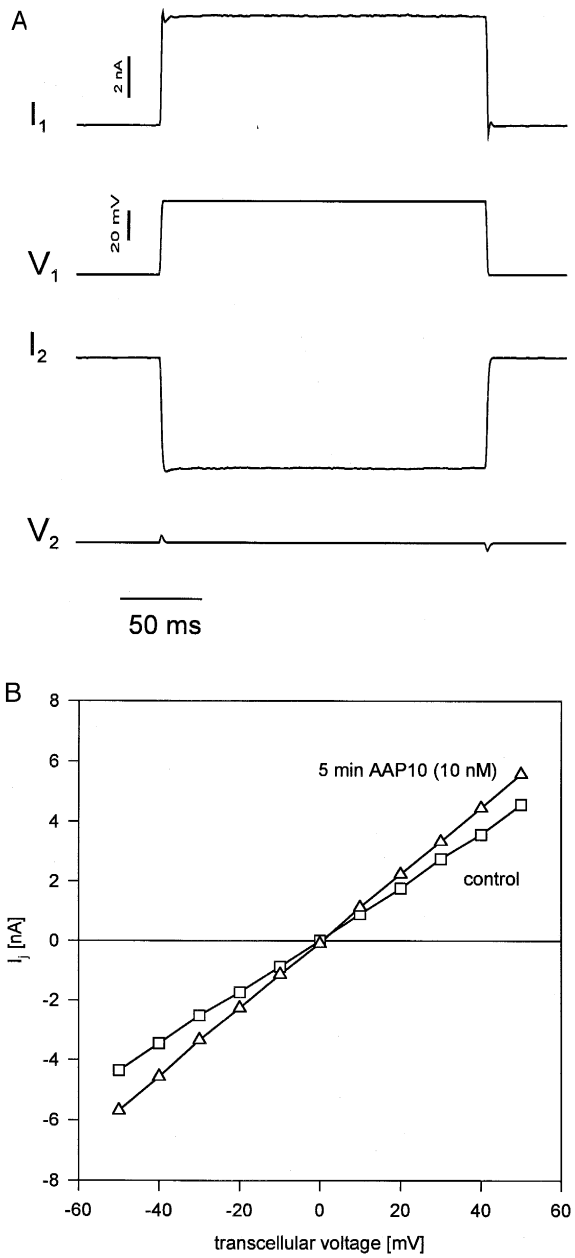

Fig. 3. (A) Original registrations showing the response to a 50 mV pulse applied to one cell of a pair of adult guinea-pig ventricular myocytes (holding potential −40 mV). V_1, V_2, I_1 and I_2 refer to voltage and currents in cell 1 and 2, respectively. Please note that the voltage traces represent actual measurements. (B) Current–voltage relationship obtained from the experiment shown in panel A. Current responses (I_j) elicited in the non-pulsed cell by changes in intercellular voltage (V_j) are plotted versus the intercellular voltage. Linear regression analysis yielded a gap junction conductance of 88.4 nS (= 11.3 MΩ; $r^2 = 0.99$; □) under control conditions. Five minutes after application of AAP10 gap junction conductance had increased to 112.5 nS (= 8.9 MΩ; $r^2 = 0.99$; △).

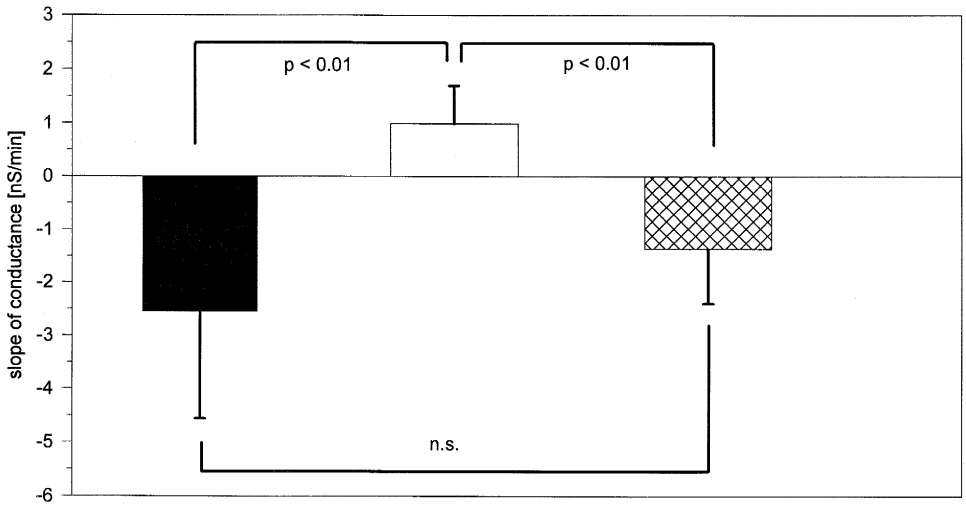

Fig. 4. Effect of AAP10 (10 nM) on the rate of changes of the gap junction conductance in pairs of guinea-pig myocytes measured over a period of 10 min. Gap junction conductance decreased during the control period (closed bar). Application of AAP10 led to a reversal of this behaviour (open bar). After washout of AAP10 gap junction conductance decreases again (cross-hatched bar). Data are means ± S.E.M. of 9 experiments. Details of the experimental protocol are given in Section 2.

other measured action potential parameters (see Fig. 1 and Table 1). Neither the resting membrane potential nor action potential duration nor overshoot potential changed and, most importantly, there was no change in the maximum upstroke velocity of the action potential. Time control experiments showed no change in the stimulus–response interval over a period of 10 min (5.11 ± 0.90 ms vs. 5.1 ± 0.91 ms; $n = 6$).

3.2. Influence of AAP10 on gap junction conductance

Fig. 3 shows representative original recordings of measurements of gap junction conductance in a pair of adult guinea pig ventricular myocytes, demonstrating that the membrane potential in both cells and, therefore, the intercellular voltage difference were adequately controlled. The current-voltage relationship of the gap junctional current was linear (Fig. 3B). Also, the gap junctional current did not decline during the voltage step in most experiments. Only in two experiments a small time-dependent decline of the transjunctional current was observed at the highest transjunctional voltages (i.e., ±50 mV). For these experiments only the measurements at lower transjunctional voltages were included in the analysis. The input resistance of the cells was very high and remained unchanged during the experiments (control: 1.5 ± 0.5 GΩ; AAP10: 1.5 ± 0.31 GΩ; washout: 1.9 ± 0.6 GΩ; differences not significant).

As already mentioned above (see Section 2) gap junction conductance did not remain constant during the experiments. In most cases there was a steady decrease in gap junction conductance. The starting value (3–5 min after establishing the whole cell mode) of gap junction conductance was 157 ± 23 nS. During the control period of 10 min the average rate of decrease was -2.5 ± 2.0 nS/min (Fig. 4). Application of 10 nM AAP10 led to a reversal of this behaviour: gap junction conductance now increased with a rate of $+1.0 \pm 0.7$ nS/min ($P < 0.01$ vs. control; $n = 9$). During washout of AAP10 gap junction conductance decreased again with a rate (-1.3 ± 1.0 nS/min) which was not significantly different from the control value (Fig. 4).

4. Discussion

In this study we show that the synthetic antiarrhythmic peptide AAP10 can increase gap junction conductance in guinea-pig cardiac ventricular muscle. Neither in this nor in previous studies could we detect any other effect of AAP10 on cardiac function and electrophysiology including left ventricular pressure, coronary flow, QRS morphology and AV conduction time (Dhein et al., 1994). Here we show that the ventricular action potential is not affected by AAP10 either (Fig. 1 and Table 1). We, thus, conclude that the antiarrhythmic effect of AAP10, which was demonstrated previously (Dhein et al., 1994; Dhein and Tudyka, 1995), is probably due to the increase in gap junction conductance (i.e., improvement in cellular coupling) mediated by AAP10.

4.1. Methodological considerations

Measurement of gap junction conductance is quite error-prone (see, e.g., Wilders and Jongsma, 1992) especially in adult cardiac myocytes because of their low input resistance and high gap junction conductance. However, experimental conditions were designed to maximise input

resistance by blocking the main membrane conductances in the voltage range used in the study. This was achieved by holding the cells at −40 mV which inactivates the sodium current and partly inactivates the L-type calcium current. In addition, Cs^+ was used as the major intracellular cation and Ba^{2+} was added to the extracellular solution to block potassium currents. These measures resulted in input resistances of more than 1 GΩ. Furthermore, series resistance problems which often affect the measurements were avoided by using discontinuous single-electrode voltage-clamp amplifiers, where series resistance errors do not occur. The independence of the measurement of the series resistance even in situations where series resistance changes with time was excellently demonstrated in a study by Jarolimek and Misgeld (1993).

Although other experimental models for assessing gap junction conductance as, e.g., neonatal rat myocytes or transfected cells exist, we decided to use adult cardiac myocytes because the effect of AAP10 was demonstrated in the adult heart. In addition, nothing is known about the molecular mechanism of action of AAP10 at the moment and it is not clear whether AAP10 is effective in neonatal myocytes which were shown to be different from adult myocytes (Page, 1992) or in transfected cells which only express one specific connexin and which may not express other factors that might be necessary for AAP10 to exert its effect.

4.2. Effects of AAP10 on the stimulus–response interval

Under control conditions application of AAP10 decreased the stimulus–response interval by about 10%. The fact that this effect was reversible upon withdrawal of AAP10 indicates that it is specific. A decrease in the stimulus–response interval corresponds to an increase in conduction velocity which could be a consequence of several different possible changes as, e.g., an increase in sodium current, an increase in membrane space constant or an increase in gap junction conductance. From the results obtained with this model we cannot unequivocally differentiate between these alternatives. However, because no significant changes in the action potential parameters (cf., Fig. 1 and Table 1) were observed (note that the change in V_{max} was not significant and that the resting membrane potential remained constant), the latter would appear to be the most likely explanation. It should be noted, however, that if AAP10 increases gap junction conductance the action potential will arise further away from the stimulus electrode thus shortening the way the action potential has to travel to reach the recording electrode. This will lead to an overestimation of the effect of AAP10 on the conduction velocity. Because the effects on conduction velocity and on the point of initiation of the action potential cannot be separated in the experimental setup used here, it is not possible to draw conclusions about the effects of AAP10 on conduction velocity.

4.3. Effects of AAP10 on gap junction conductance

AAP10 counteracted the time-dependent decrease in gap junction conductance measured in this study. As already seen in the experiments in papillary muscle, the effect of AAP10 was readily reversible upon washout, again indicating that the effect was due to the presence of AAP10.

With respect to the causes for the time-dependent decrease in gap junction conductance observed in the majority of the experiments we can only speculate. However, it is generally recognised that gap junction conductance decreases during long-term experiments (i.e., > 15–20 min; see also Weingart and Maurer, 1988). One explanation would be that frequent application of high intercellular voltage differences (> 30 mV) could lead to closure of gap junction channels (Veenstra, 1990; Wang et al., 1992). It is also possible that dialysis of the cells with the pipette solution leads to washout of cytoplasmic factors needed to keep gap junction channels in the open state (cf., Weingart and Maurer, 1988).

However, the decrease in gap junction conductance could be fully reversed by AAP10 and a net increase in gap junction conductance was induced. Thus, one might speculate that AAP10 may be beneficial in states characterised by reduced cellular coupling as, e.g., hypoxia or ischemia as has been suggested by Dhein and Tudyka (1995). In addition, an antiarrhythmic effect of AAP10 has been shown in hearts submitted to regional ischemia (Dhein et al., 1994).

4.4. Possible implications

Although there is no direct evidence yet, changes in gap junction distribution and density as well as changes in connexin expression in ischemic heart disease and cardiac hypertrophy have been implied as important factors contributing to arrhythmias associated with these diseases (for reviews see Spach, 1994 and Severs, 1994).

Cellular uncoupling also occurs in regional ischemia and myocardial infarction (Kléber et al., 1987; Steendijk et al., 1993) and leads to dispersion of action potential duration and refractoriness (Dhein et al., 1994). Dispersion of refractoriness is known to be an important factor in arrhythmogenesis (Kuo et al., 1983; Merx et al., 1977). It has been shown that pre-treatment with AAP10 can counteract arrhythmogenesis and enhanced dispersion of refractoriness in ischemic hearts (Dhein et al., 1994). The results obtained in this study indicate a possible mechanism of action: the increase in gap junction conductance, which was the only effect of AAP10 on all physiological and electrophysiological parameters investigated so far.

It is generally assumed that improvement of cellular coupling can act antiarrhythmically (Dhein and Tudyka, 1995). However, due to the lack of substances increasing cellular coupling these assumptions and also conclusions

from theoretical studies (Cole et al., 1988; Lesh et al., 1989; Cai et al., 1994; Balke et al., 1988; Müller and Dhein, 1993) could not be investigated in existing experimental models. As a consequence of the results presented in this study, those studies may now become possible. One should, however, keep in mind that in some situations, as, e.g., pre-excitation syndromes or possibly catecholamine overload, an increase in cellular coupling (i.e., an increase in conduction velocity) might also lead to pro-arrhythmic effects.

In addition, our experiments showed that an increase in gap junction conductance can be reflected by a decrease in the stimulus–response interval in guinea-pig papillary muscle. Thus, this model which is rather simple as compared to the double-cell voltage-clamp approach might be used when looking for substances that are supposed to increase gap junction conductance. However, great care should be taken to assure that all other parameters remain constant during the course of such experiments.

4.5. Conclusions

AAP10 increases gap junction conductance in guinea-pig ventricular muscle. No other effects of AAP10 were observed. This study might open new pathways for the search for new antiarrhythmic drugs with a novel mechanism of action. In addition, it may be possible to test several hypotheses and theoretical predictions concerning the effects of improving cellular coupling in the heart.

Acknowledgements

This work was supported by the DFG (Grant Dh 3-1/4 to S.D.).

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