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MODULATION OF THE RESPIRATORY RHYTHM BY GAP-JUNCTION BLOCKERS 18-α-GLYCYRRHETINIC ACID AND 18-β-GLYCYRRHETINIC ACID

BENJAMIN JUNTERMANNS[1], KIRSTEN GÖPELT[1] AND DIETRICH BÜSSELBERG[2]*

[1]*Universitätsklinikum Essen, Institut für Physiologie, Hufelandstrasse 55, 45122 Essen, Germany*

[2]*Weill Cornell Medical College in Qatar, Qatar Foundation – Education City, POB 24144, Doha, Qatar*

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Abstract : Gap junction proteins are expressed in the pre-Bötzinger complex of the respiratory network but it remains under discussion how they modulate the respiratory rhythm generation. In the present study we tested whether the gap junction blockers 18-β-glycyrrhetinic acid (18-β-GA) and 18-α-glycyrrhetinic acid (18-α-GA) change either the phrenic nerve (PN) discharge frequency or amplitude. The PN discharge was recorded using the working heart-brainstem preparation of adult wistar rats (P22-P28) exposed to increasing concentrations of the gap junction blockers (0.1; 1; 10; 20 µM). With the two lower concentrations (0.1; 1 µM) of 18-β-GA, PN discharge frequency decreased to 46±15% (p=0.007) of the control value while it increased to 173±57% (p=0.16) with the two higher concentrations (10; 20 µM). Surprisingly, with 18-α-GA the PN discharge frequency was not significantly changed with any of the concentrations used. Enhancing the respiratory drive with 12% CO_2, the PN discharge frequency increased tendencially with rising concentrations of 18-β-GA, but again no significant change was observed with 18-α-GA. PN-amplitudes were slightly reduced over the course of the experiments, while the frequency of the heartbeat was not significantly changed at any time with any concentration.

Key words : gap junctions gap junction blockers respiratory rhythm generation working heart brainstem preparation 18-α-glycyrrhetinic acid 18-α-glycyrrhetinic acid

INTRODUCTION

Breathing in mammals is controlled by a neuronal network of medullary neurons, which generates rhythmic activity and leads to periodic contractions of abdominal, diaphragm and thoracic muscles. The neurons involved are connected through

*Corresponding author : Tel.: +974 492 8334; Mobile : +974 348 0728; Fax : +974 492 8333
E-mail: dib2015@qatar-med.cornell.edu

a respiratory network, which is located in the pre-Bötzinger complex (PBC), a morphologically defined region within the ventral brainstem (1). Respiration is divided into three distinct phases: inspiration, post-inspiration and expiration. Depending on their discharge pattern, the neurons of this network can be distinguished by their activity in relation to the respiratory cycle. Accordingly, they are classified as early-inspiratory (early-I), augmenting-inspiratory (aug-I), late-inspiratory (late-I), post-inspiratory (post-I), augmenting-expiratory (aug-E) and pre-inspiratory (pre-I) neurons (2). Each subtype of the inspiratory neurons has its specific function for rhythm generation and pattern formation (3, 4).

Gap junction channel-proteins consist of connexins that establish a direct connection between neighbouring cells, and therefore provide a direct exchange path for small molecules and ions. They also couple neurons electrically (5, 6). Electric communication via gap junctions is compulsory for pacemaker neuron synchronization in mammals, as it was demonstrated for the heart and neocortical pattern generator (7, 8). Gap junctions have also been detected in the brainstem, and recently, it has been demonstrated that gap junctions are also important for rhythmogenic inspiratory neurons in the pre-Bötzinger complex of both neonatal and adult rats (9, 10). Using immunohistochemical methods Solomon and co-workers (9, 11) detected connexin 26 (Cx26) as well as connexin 32 (Cx32), although their relative amounts and distribution varied with age. Connexin (Cx26) was present in high amounts in neonatal rats (<7d) but declined with age, while connexin 32 (Cx32) was sparse in the juvenile tissue but increased with age.

The influence of gap junction coupling within the respiratory network was demonstrated by Bou-Flores and co-workers (12) by pharmacological blockade, which resulted in a reduced inspiratory burst frequency. These experiments were done using the brain stem-spinal cord (en bloc) or medullary slice preparation of neonatal (P1-P5) Swiss Webster mice (12). On the other hand, Solomon and co-workers (13) found an increase of the respiratory frequency when blocking gap junctions. They performed their experiments with adult rats (5-6 weeks old) utilizing an *in situ* "working heart-brainstem preparation" (WHBP) (14). It is unknown whether differences in these results are due to the way the tissue was prepared, or are simply a reflection of the different ages or species of the animals used.

Here we test if the gap junction blockers 18-β-glycyrrhetinic acid (18-β-GA) and 18-α-glycyrrhetinic acid (18-α-GA) change PN discharges using the *in situ* WHBP with adult wistar rats (P22-P28). In light of the results presented above, our objective was to study a concentration response relationship for the gap-junctions blocker 18-β-GA and 18-α-GA. Our study provides data that might help to illustrate the functional role of gap junctional communication in the respiratory network.

METHODS

Preparation

In order to test the effects of gap junction blockers 18-α-GA and 18-β-GA (both purchased from Sigma-Aldrich Chemie GmbH, Germany), we used the *in situ* perfused brainstem preparation (14) with adult

Wistar rats P22-P28 (*Rattus norvegicus familiaris*). The animals were bred in the animal facility of the University-Clinic of Essen. Our procedures have been extensively described (15, 16) and ensured a maximum reduction of discomfort and pain to the animals. The following are the main steps of the preparation: animals were deeply anaesthetized with isoflurane, and the absence of the tail flick reflex was taken as an indicator that the animals were pain free. The skull was opened with a scalpel and the cortex was removed at the pre-collicular level. The animals were then transected right above the diaphragm and the lower part of the body was removed. These steps were performed as quickly as possible to avoid ischemia time. The preparation was transferred into ice-chilled artificial cerebrospinal fluid (ACSF) of the following composition: 125 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$,1.25 mM $MgSO_4$, 1.25 mM KH_2PO_4, 1.1 mM glucose. The ACSF had an osmolarity of 290 mosmol and was gassed with carbogen (95% O_2/5% CO_2) for at least 15 minutes. The pH was adjusted with HCl or NaOH and maintained at 7.3 to assure *in vivo* like conditions. In addition, 3.5 g Ficoll (Sigma-Aldrich Chemie GmbH, Germany) was applied, and the total osmolarity was increased to 300-330 mosmol. The animals were transferred to the recording chamber and were retrograde perfused with ACSF via the abdominal aorta. To assure the respiratory drive, we gassed the perfusion with oxygen which contained either 5% CO_2 (ACSF pH 7.35) or 12% CO_2 (ACSF pH 7.2) to achieve a higher chemostimulation. The temperature of the perfusate was maintained at 31°C. We used a peristaltic pump (Watson Marlow 205 U) to circulate the ACSF through the preparation. Bubble traps and filters in the flow system minimized the risk of embolism by air bubbles in the animal. The pump was running at 60-80 rotations per minute, which provided a pressure between 50-80 mmHg at the level of the abdominal aorta. To assure muscular paralysis we applied Vecuronium bromide 0.04 µg/ml (Norcuron, Inresa Arzneimittel GmbH, Germany). The PN was isolated, cut and the end was taken into the tip of a glass electrode to record the nerve discharges.

Drug solutions

The gap junction blockers 18-α-GA and 18-β-GA were dissolved in ethanol (stock solution 10 mM). Final concentrations (0.1-20 µM) were made immediately before the experiments, diluting the stock solution with ACSF.

Electrophysical recordings

PN discharges were recorded by suction electrodes in order to monitor central respiratory activity. The electrodes were made from soda glass without filament, with an outer diameter (Ø) of 1.4 mm (Hilgenberg, Malsfeld/Germany). The glass electrodes were produced with a micropipette puller Model Flaming/Brown P-87 (Sutter Instrument Co. USA). The electrode was filled with the ACSF, which was connected by a silver wire to an amplifier (custom-made at the electronics workshop of the University of Mainz/Germany). PN activities were amplified (2,000–10,000x), band pass filtered (100-5000 Hz) and monitored on a strip chart recorder. This set-up allowed recording of both PN electrical discharge and heartbeat frequency simultaneously.

Experimental protocol

The major focus of this study was to observe PN discharges before and during pharmacological blockade (i.e., uncoupling) of gap junctional proteins. To monitor the PN discharges under control conditions (CTR), they were recorded for 15-20 minutes during perfusion with normal ACSF gassed with either 5% CO_2 or 12% CO_2 The preparation was then perfused with the gap junction uncoupling agents. During the experiment the concentrations increased in steps from 0.1, to 1 and 10 µM and finally to 20 µM. The concentrations were changed after 30 minutes of application. PN discharges were recorded continuously throughout the entire experiment.

Data analysis

The data were recorded on hard disc for off-line analysis. PN discharges were also recorded on paper charts throughout the entire length of the experiment. To standardize our experiments, we analyzed the PN discharges after 30 minutes exposure of gap junction uncoupling agents. Mean values (±S.D.) were calculated with Microsoft Excel. Frequencies of both the PN discharges and the recorded heartbeats were standardized by expressing all values as a percentage of the control value, which was set as 100%. To evaluate the statistical significance of the experiments we used the student's t-test and presented them as P-values. The statistical significance was set as (*) $P<0.05$, (**) $P<0.01$ and (***) $P<0.001$.

RESULTS

Results were obtained from a total of 37 experiments (22 experiments using 5% CO_2 and 15 experiments with 12% CO_2). 18-α-GA and 18-β-GA were applied in 19 experiments each.

Phrenic nerve discharge characteristics under control conditions

Eupnoenic discharges are characterized by a ramp like discharge pattern (Figs. 1A, B, C, D). PN discharge frequency under control conditions (5% of CO_2) was 10±2.43 discharges/min and with 12% of CO_2 the frequency was 11.6±3.58 discharges/min. The effects of the uncoupling agents on PN discharge were mostly reversible in experiments using the ACSF without gap junction uncoupling agents after 45-60 min (data not shown; n=3).

Concentration dependence of phrenic nerve discharge frequency after blockade of gap junctions

When the solution was gassed with 5% CO_2, the PN discharge frequency varied depending on the different 18-β-GA concentrations used (Fig. 2A). At a concentration of 0.1 µM, PN discharge frequency was decreased to 46±15.4% ($p=0.008$, n=5) compared to control conditions. The PN frequency decreased slightly further when 1 µM was applied (49.3±26.9%; $p=0.007$, n=5). But, when the concentration of 18-β-GA was increased to 10 µM, PN discharge frequency was no longer reduced, but rather increased to 134.6±66.1% (not significantly different from the control value; $p=0.4$, n=4). Further increasing the 18-β-GA concentration to 20 µM made this observation more pronounced (173.4±57.2%; $p=0.2$, n=3).

18-α-GA did not change the PN discharge

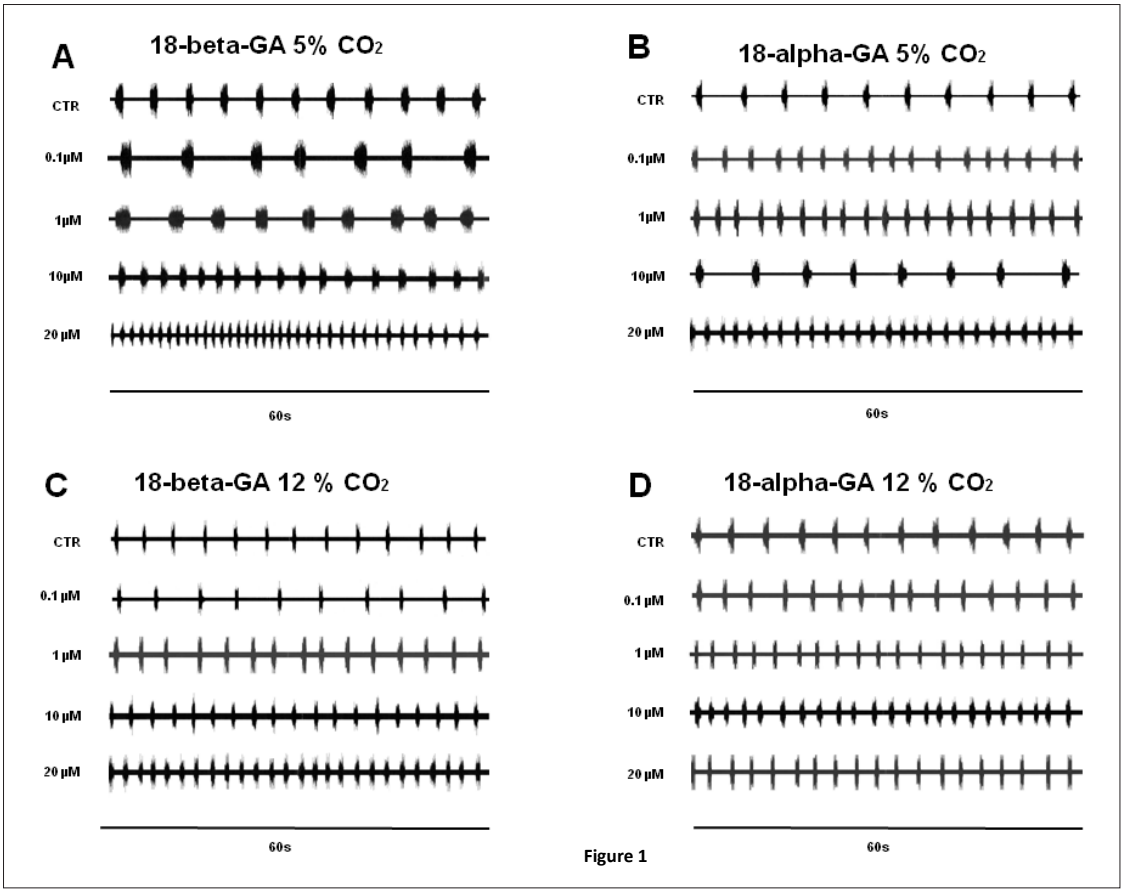

Fig. 1 : Characteristic changes of burst discharges in the PN of adult wistar rats (P22–P28) under control conditions (CTR) and after blockade of gap junction proteins with 18-α-glycerrhetinic acid and 18-β-glycerrhetinic acid with rising concentrations: A) Changes of PN frequency after application of 18-β-GA (0.1-20 µM; 5% of CO_2). B) Changes in PN discharge frequency after different applications of 18-α-GA (0.1-20µ M; 5% CO_2). C) Changes of PN frequency after application of 18-β-GA (0.1-20 µM; 12% of CO_2. D) Changes in PN discharge frequency after increasing applications of 18-α-GA (0.1-20 µM; 12% CO_2).

frequency significantly at any concentration using 5% CO_2 (Fig. 2B; 0.1 µM: 107.4±50.6%, p=0.9, n=5; 1 µM: 132.8±36.8%, p=0.2, n=3; 10 µM: 107.4±58.1%, p=0.7, n=3; 20 µM: 145.6±25%, p=0.09, n=3).

When the respiratory drive was boosted by the use of 12% CO_2 in the ASCF, neither of the two gap junction blockers altered the frequency of the PN discharges significantly at any concentration used. With 18-β-GA (0.1-20 µM) the observed PN discharge frequency was: 0.1 µM: 97±43%, p=0.7, n=2; 1 µM: 116.8±34%, p=0.09, n=8; 10 µM: 142±64%, p=0.18, n=7; 20 µM: 146±75%, p=0.54, n=2). Applying 18-α-GA, the frequencies were as follows: 0.1 µM: 91±31%, p=0.5 n=4; 1 µM: 131±15%, p=0.05, n=3; 10 µM: 123±40%, p=0.3, n=4; 20 µM: 96±19%, p=0.7, n=4.

Variations in the amplitudes of the PN discharges were measured as an indicator to

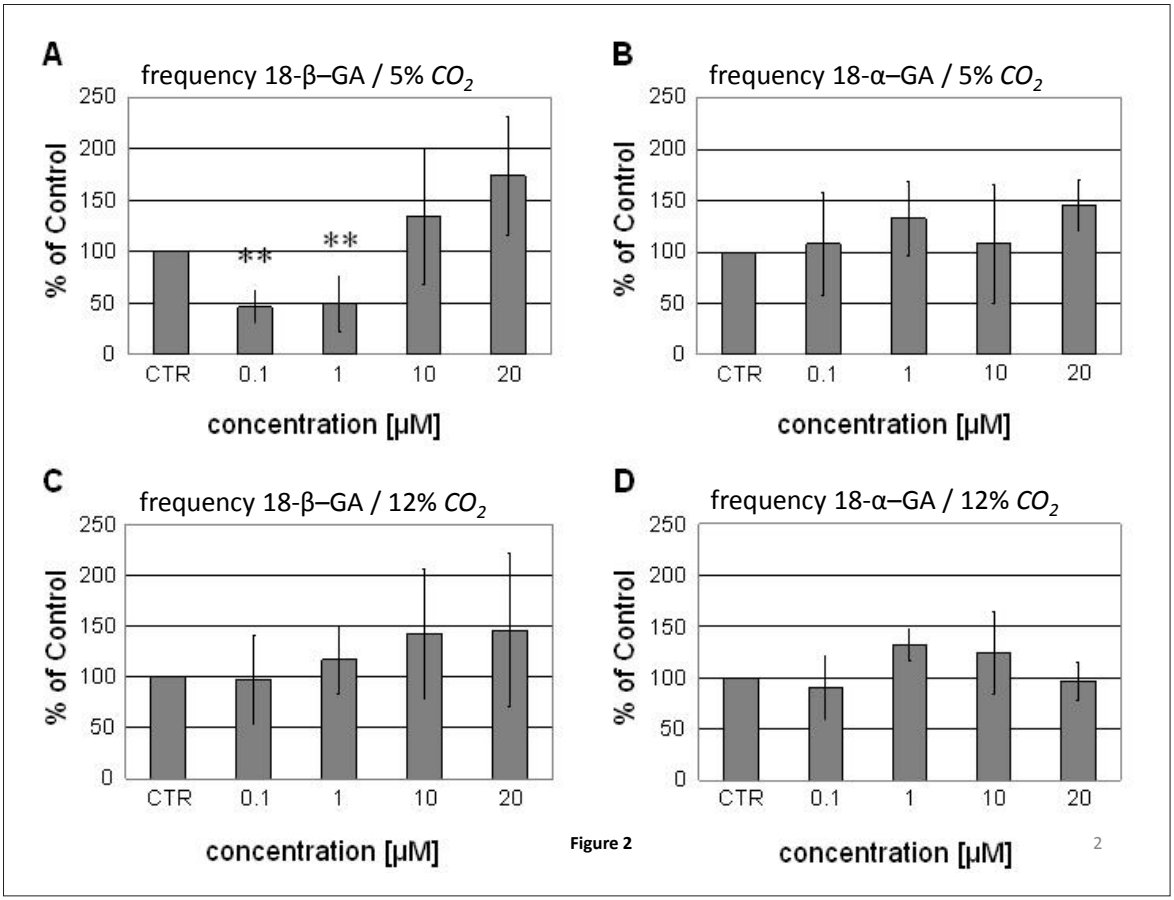

Fig. 2 : PN discharge frequency under control conditions (CTR) and after pharmacological blockade of gap junction proteins with 18-α-glycerrhetinic acid or 18-β-glycerrhetinic acid. A) Changes in the phrenic nerve discharge frequency with increasing concentrations of 18-β-GA (0.1-20 µM; 5% of CO_2). B) Phrenic nerve burst frequency changes after application of 18-α-GA (0.1-20 µM) into the perfusate (5% CO_2). C) Changes in discharge frequency using 18-β-GA (0.1-20 µM; 12% CO_2). D) Changes in the phrenic nerve discharge frequency applying 18-α-GA (0.1-20 µM; 12% CO_2).

see whether the gap-junction blockers change the respiratory drive. In 5% CO_2, 18-β-GA and 18-α-GA changed the amplitude of the PN output inconsistently and insignificantly at the different concentrations (Figs. 3A, 3B). Using 18-β-GA (5% CO_2), changes in amplitude were: 0.1 µM: 72±20%, p=0.9 n=5; 1 µM: 67.7±14%, p=0.8, n=4; 10 µM: 71±12%, p=0.3, n=; 20 µM: 64±10%, p=0.03, n=3. For 18-α-GA (5% CO_2), the results were: 0.1 µM: 53±23%, p=0.003 n=6; 1 µM: 65±15%, p=0.03, n=4; 10 µM: 77±11%, p=0.05, n=4; 20 µM: 58±11%, p=0.05, n=3.

With 12% CO_2,the amplitude continuously decreased with increasing concentrations of the blockers using 18-β-GA: 0.1 µM: 94±23%, p=0.7 n=8; 1 µM: 80±19%, p=0.03, n=2; 10 µM: 62±21%, p=0.02, n=7; 20 µM: 76±4%, p=0.07, n=2. In the presence of 18-α-GA

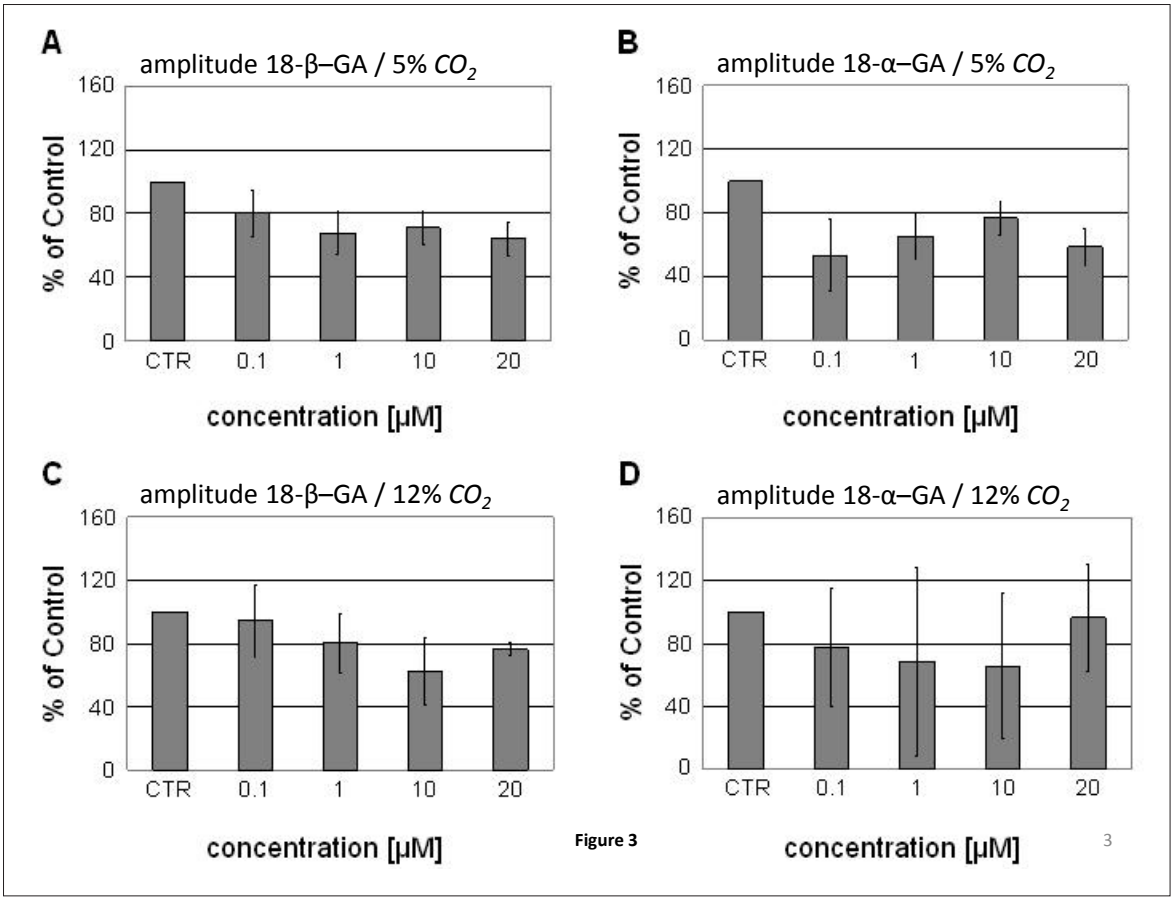

Fig. 3 : PN discharge peak amplitude under control conditions (CTR) and after pharmacological blockade of gap junction proteins with 18-α-glycerrhetinic-acid or 18-β-glycerrhetinic-acid. A) Changes of the phrenic nerve discharge peak amplitude with increasing concentrations of 18-β-GA (0.1-20 µM; 5% CO_2). B) Phrenic nerve discharge peak amplitude changes after application of 18-α-GA (0.1-20 µM; 5% CO_2). C) Changes in discharge peak amplitude using 18-β-GA (0.1-20 µM; 12% CO_2). D) Changes in the phrenic nerve discharge peak amplitude applying 18-α-GA (0.1-20 µM; 12% CO_2).

phrenic nerve amplitude changed as follows: 0.1 µM: 77±37%, p=0.38 n=4; 1 µM: 68±59.8%, p=0.8, n=3; 10 µM: 65±46.7%, p=0.4, n=4; 20 µM: 96±34%, p=0.83, n=4 (Figs. 2C, D, and 3C, D).

Since the myocardial cells are also connected via gap junctions (17), we expected a change in the heartbeat frequency; however the frequency did not change significantly from control conditions during the application of gap junction uncoupling agents, regardless of whether the perfusion was gassed with either 5% CO_2 or 12% CO_2 (Figs. 4A, B, C, D).

DISCUSSION

Recent studies have shown the expression of connexin 26 (Cx26) and connexin 32 (Cx32)

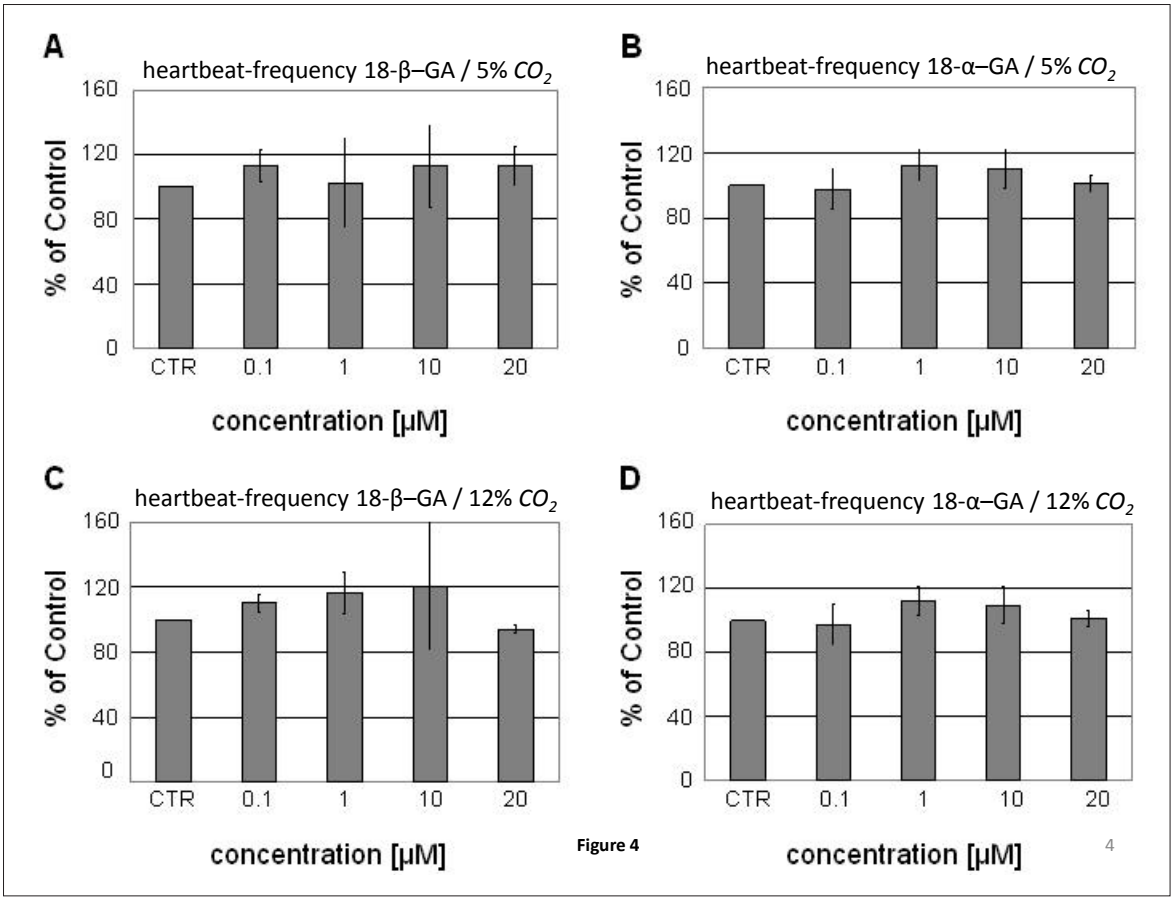

Fig. 4 : Changes in the heartbeat-frequency with varying concentrations of gap junction blockers 18-β-glycerrhetinic acid (A, C) 18-α-glycerrhetinic acid (B, D) gassed with either 5% CO_2 (A, B) or 12% CO_2 (C, D).

in the pre-Bötzinger complex (11), a CO_2-chemosensitive region of the brain stem (9), as well as in motor neurons (18).

While several gap junction blockers have been used to induce a change in the frequency of the PN discharge, we examined the effects of the most common gap junction blockers 18-α-GA and its isomer 18-β-GA to induce a pharmacological blockade of gap junction proteins. These two substances were tested in a variety of experiments, which have demonstrated a functional gap junction blockade (19-27). Clearly, when using the heart-brainstem preparation (14) all gap junction proteins in the brainstem and spinal cord will be blocked simultaneously, independent of whether they are involved in respiratory activity or not.

With rising concentrations of these gap junction blockers we observed a biphasic modification of the frequency of PN discharges while their amplitude decreased with rising concentrations; applying 18-β-GA in a concentration range from 0.1 µM to

1 µM reduced the discharge frequency, while concentrations from above 1 µM increased the PN discharge frequency.

Our results are comparable with the effects described by Solomon and co-workers (13) as well as with those presented by Bou Flores and Berger (12). Both also underline the importance of gap junctions for the maintenance of the respiratory rhythm. The findings presented here support both reported effects (decrease and increase of PN discharge) in a concentration-dependent manner. Like Solomon and co-workers (13), who used concentrations of the gap junction blockers in the range of 25-100 µM (5% CO_2), we observed an increase of PN discharge frequency using a concentration of 20 µM (18-β-GA; 18-α-GA). In contrast, Bou-Flores and Berger (12) described a reduction of PN discharge frequency over a concentration range between 50 and 100 µM. While there remain discrepancies in the results, the following differences in experimental design could be a possible explanation:

I. Type of preparation

Like Solomon and co-workers (13), we used the *in-situ* perfused heart-brainstem preparation and systemically applied gap junction blockers into the perfusion system; therefore, all gap junctional proteins were uncoupled simultaneously (as well as those in the entire preparation).

Gap junction proteins have been detected in the putative CO_2-chemosensitive brainstem areas (9) and in presumptive phrenic and hypoglossal moto-neurons (18). These areas could have contributed to the increase or decrease of PN discharge frequency due to the blockade of gap junction uncoupling agents, which block connexins throughout the whole brain and spinal cord. Nevertheless, it remains unanswered which cells are responsible for the effects. On the other hand, Bou-Flores and Berger (12) performed their experiments using the medullary slice preparation, which isolates the respiratory centre from inhibitory (or excitatory) input of other areas of the brain or the spinal cord, which might have influenced the results described in their presentation.

II. Age of animals

As in this study, Solomon and co-workers (13) used adult animals, whereas the animals used by Bou-Flores and Berger (12) were 1-5 days old. A different expression of gap junctions throughout the brainstem in relation to the age of the rats have been reported by Solomon and co-workers (9), which provides another possible explanation of the different respiratory effects which were reported.

III. Concentration of uncoupling agents

The biphasic effect we observed for 18-β-GA might be due to specific binding affinities at different receptors. Lower concentrations of gap junction blockers could specifically block connexins that contribute to the respiratory generation or synchronization of the signal within the respiratory network. Of the two gap junction blockers used in this study, 18-β-GA showed a bigger magnitude in its potency of modification than 18-α-GA. Using the same concentrations of both uncoupling agents, 18-β-GA had a more

pronounced effect on the frequency of phrenic nerve discharges. The gap junction blockers that we used in our experiments were not composed for specific Cx isoforms. Unfortunately, more specific agents are currently not available (25).

IV. Respiratory drive

In order to test the effect of the respiratory drive (28), we gassed the preparation with either 5% or 12% CO_2. With 12% of CO_2, any effects of gap junction blockade were not obvious because chemosensitive receptors and their modulation of the respiratory neurons might have masked the effects of the gap junction blockers.

The changes to the respiratory rhythm that we observed were definitely mediated by blockade of gap junction proteins, and not by nonspecific effects, because both glycyrrhetinic derivates performed similar modification to the respiratory rhythm. Furthermore, in our experiments, the effects of both gap junction uncoupling agents were reversible even after an exposure of more than 30 minutes. Additional evidence is that glycyrrhetinic derivates have been shown to functionally block ≤ 80% of electrical coupling between cells (21, 22, 25, 29, 30, 31). Nevertheless, it remains unclear where the precise site of gap junctional blockade is located on the connexin (25).

To determine the actual site of action of the gap junction blockade and to analyse whether a gap junction blockade at other parts of the nervous system might have influence the results were beyond the scope of this study but will be addressed in future studies. For example, we expected to see changes in heartbeat frequency after gap junction blockers were applied, but it did not change significantly in the concentration range we have used. Therefore, we assume that higher concentration will be needed to change the frequency of the heartbeat.

Conclusion

Electrical coupling between neurons in the respiratory network is compulsory for physiological respiratory rhythm generation. 18-β-GA as well as 18-α-GA changed PN discharge frequency (i.e., respiratory rhythm) due to the blockade of gap junctional proteins. Respiratory rhythm is modulated but not stopped; therefore, we assume that under this condition synaptic transmission is driving the respiratory network.

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