Are pacemaker properties required for respiratory rhythm generation in adult turtle brain stems in vitro?

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Johnson SM, Wiegel LM, Majewski DJ. Are pacemaker properties required for respiratory rhythm generation in adult turtle brain stems in vitro? Am J Physiol Regul Integr Comp Physiol 293: R901–R910, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00912.2006.—The role of pacemaker properties in vertebrate respiratory rhythm generation is not well understood. To address this question from a comparative perspective, brain stems from adult turtles were isolated in vitro, and respiratory motor bursts were recorded on hypoglossal (XII) nerve rootlets. The goal was to test whether burst frequency could be altered by conditions known to alter respiratory pacemaker neuron activity in mammals (e.g., increased bath KCl or blockade of specific inward currents). While bathed in artificial cerebrospinal fluid (aCSF), respiratory burst frequency was not correlated with changes in bath KCl (0.5–10.0 mM). Riluzole (50 μM; persistent Na+ channel blocker) increased burst frequency by 31 ± 5% (P < 0.05) and decreased burst amplitude by 42 ± 4% (P < 0.05). In contrast, flufenamic acid (FFA, 20–500 μM; Ca2+-activated cation channel blocker) reduced and abolished burst frequency in a dose- and time-dependent manner (P < 0.05). During synaptic inhibition blockade with bicuculline (50 μM; GABA_A channel blocker) and strychnine (50 μM; glycine receptor blocker), respiratory motor activity persisted, and burst frequency was directly correlated with extracellular KCl (0.5–10.0 mM; P = 0.005). During synaptic inhibition blockade, riluzole (50 μM) did not alter burst frequency, whereas FFA (100 μM) abolished burst frequency (P < 0.05). These data are most consistent with the hypothesis that turtle respiratory rhythm generation requires Ca2+-activated cation channels but not pacemaker neurons, which thereby favors the group-pacemaker model. During synaptic inhibition blockade, however, the rhythm generator appears to be transformed into a pacemaker-driven network that requires Ca2+-activated cation channels.

Most data supporting the hybrid pacemaker-network and group-pacemaker models are derived from perinatal and juvenile mammals, whereas network models are supported primarily by data from adult mammals. The maturation network-burst hypothesis (43) attempts to harmonize these findings by stating that the respiratory control system is pacemaker-driven in young animals, but becomes network-driven in adults due to increasing synaptic inhibition and activation of specific ionic conductances (17, 18, 31, 32, 33). However, in some nonmammalian adult vertebrate preparations, there is evidence suggesting that pacemaker properties may be required for respiratory rhythm generation. For example, in brain stems isolated from adult lampreys (44) and adult turtles (23), rhythmic motor activity persists during synaptic inhibition blockade. This persistent rhythmic activity in isolated adult turtle brain stem (and brain stem-spinal cord) preparations is hypothesized to be respiratory related, because motor bursts are produced on a spinal expiratory nerve (23) and abolished by characteristic respiratory depressants such as μ-opiate receptor activation (23) and high-pH/low-CO2 conditions (unpublished observations). These data suggest that expiratory pacemaker neurons or clusters of expiratory neurons with pacemaker properties drive the rhythm, rather than nonrespiratory neurons taking over control of the respiratory control system and producing a seizure-like pattern (4, 43).

To test the hypothesis that pacemaker properties are involved in turtle respiratory rhythm generation, brain stems from adult turtles were isolated and tested under in vitro conditions. Specifically, we tested whether depolarization via increased bath KCl increases the frequency of spontaneous respiratory bursts of motor activity, since the frequency of endogenous bursting in respiratory pacemaker neurons is voltage dependent (5, 53). We also tested whether riluzole or flufenamic acid (FFA) altered respiratory burst frequency produced by turtle brain stems. Riluzole blocks persistent Na+ currents, and FFA blocks Ca2+-activated cation currents, in respiratory neurons in the mammalian medulla (11, 33, 59). Both currents are hypothesized to be required for pacemaker activity in mammalian inspiratory neurons (33, 59), although their obligatory role for respiratory rhythm generation has been challenged (11). In separate experiments under conditions of synaptic inhibition blockade, the effects of altered bath KCl, riluzole, and FFA were examined to test whether synaptic inhibition blockade transforms the respiratory rhythm generator into a pacemaker-driven network (45, 47, 48, 49, 50). A preliminary report of this work was published in abstract form (20).

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MATERIALS AND METHODS

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine. Adult red-eared slider turtles (Trachemys scripta, n = 161, weight = 696 ± 10 g) were obtained from commercial suppliers and kept in a large open tank where they had access to water for swimming and heat lamps and dry areas for basking. Room temperature was set to 27–28°C with light 14 h/day. Turtles were fed ReptoMin floating food sticks (Tetra, Blacksburg, VA) 3–4 times per week.

Brain stem preparations. Turtles were intubated and anesthetized with 5% isoflurane (balance O2) until limb withdrawal to noxious foot pinch was eliminated. Turtles were rapidly decapitated and decerebrated. Brain stems were removed and pinned down in a recording chamber (13-ml vol) with the ventral surface facing upward (Fig. 1A). Brain stems were superfused (4–6 ml/min) with artificial cerebrospinal fluid (aCSF) at 23°C containing HEPES buffer as follows (in mM): 100 NaCl, 23 NaHCO3, 10 glucose, 5 HEPES (sodium salt), 5 HEPES (free acid), 2.5 CaCl2, 2.5 MgCl2, 1.0 K2PO4, and 1.0 KCl (bubbled with 5% CO2–95% O2). The pH of solution in the reservoir was measured periodically with a calomel glass pH electrode (Digi-Sense; Cole-Parmer Inst., Vernon Hills, IL) and averaged 7.30 ± 0.01 during data collection. All brain stems were allowed to equilibrate for 2–5 h prior to initiating an experimental protocol. To record respiratory motor bursts, glass suction electrodes were attached to hypoglossal (XII) nerve rootlets (Fig. 1A). Signals were amplified (×10,000) and band-pass filtered (1–500 Hz) using a differential alternating current amplifier (model 1700; A-M Systems, Everett, WA) before being rectified and integrated (time constant = 200 ms) using a moving averager (model MA-821/RSP; CWE, Ardmore, PA). Analysis was performed using Axoscope (Axon Instruments, Foster City, CA) and MiniAnalysis software (Synaptosoft, Decatur, GA). All drugs used in this study were obtained from Sigma/RBI Aldrich (St. Louis, MO) and include: (+)-bicuculline (GABA_A receptor antagonist), FFA (blocks Ca2+-activated cation currents), riluzole (blocks persistent Na+ channels), and strychnine (glycine receptor antagonist). Riluzole and FFA were dissolved in 100 µl of dimethyl sulfoxide (DMSO) and then dissolved slowly in 0.5–1.0 liters of HEPES-buffered solution before being applied to turtle brain stems. Control experiments with equivalent amounts of DMSO in HEPES-buffered solution showed that DMSO had no effect on respiratory burst frequency and amplitude (data not shown).

Experimental protocols. To determine the effects of bath KCl on burst frequency, baseline data were recorded for 20 min in aCSF (2 mM KCl) before switching to aCSF with altered KCl for 60 min and then washing out with aCSF (2 mM KCl) for 60 min. To determine the effects of riluzole and FFA, baseline data were recorded for 20 min before switching to aCSF with riluzole (50 µM) or FFA (20–500 µM) for 2 h before washing out with aCSF. To determine the effects of KCl, riluzole, and FFA during synaptic inhibition blockade, bicuculline (50 µM) and strychnine (50 µM) were added to the aCSF to block synaptic inhibition. After allowing for complete transformation of the motor output within 100 min (22), bath KCl was altered for 1 h, or riluzole (50 µM) or FFA (100 µM) were added along with bicuculline and strychnine for 2 h.

Data analysis. XII burst amplitude was measured at the highest point of integrated discharge trajectory in arbitrary units and normalized to the average amplitude recorded during the baseline period. Burst frequency was calculated as the number of bursts per minute. All measurements were averaged into 20-min bins and reported as means ± SE. For statistical inferences, linear regression, one-way ANOVA, or two-way ANOVA with repeated measures design were used (Sigma Stat; Jandel Scientific Software, San Rafael, CA). Post-hoc comparisons were made using the Dunnett or Student-Newman-Keul test. P values <0.05 were considered significant.

RESULTS

Increased bath KCl did not alter burst frequency in aCSF. While bathed in aCSF, increasing bath KCl from 2.0 mM to 4.0–9.0 mM often transiently increased burst frequency within the first 10–20 min, but burst frequency was highly variable during the next 40 min (Figs. 2, A, B, and D). There were no changes in frequency when bath KCl was increased from 2.0 mM KCl to 5.0 mM (n = 4) or 7.0 mM KCl (n = 6; Fig. 2B). There was, however, a sustained 80–110% frequency increase when bath KCl was increased from 2.0 to 9.0 mM KCl (n = 3; P < 0.05 for KCl- and time-dependent effects; Fig. 2B). At other increased bath KCl levels, burst frequency decreased below baseline (Fig. 2D). Increasing bath KCl to levels greater than 10.0 mM resulted in tonic activity and disruption of the respiratory rhythm (n = 3; data not shown). Decreasing bath KCl from 2.0 to 0.5 mM (n = 3) did not alter burst frequency. A graph of burst frequency after 20–40 min of increased KCl exposure (i.e., data at the 60-min time point in Fig. 2B) vs. bath KCl revealed no correlation (r = 0.066, r² = 0.129; Fig. 2D). This time period was chosen for analysis because KCl-induced frequency changes often reached steady-state without the rhythm being disrupted by prolonged KCl exposures. Although
there was an occasional initial burst frequency increase during the KCl application, there was no correlation for burst frequency vs. bath KCl during the 0–20 min period ($P = 0.313$, $r^2 = 0.041$). Burst amplitude was highly variable with time-dependent decreases (5.0 and 7.0 mM KCl; $n = 4, 6$, respectively) and increases (9 mM KCl; $n = 3$) after 60 min (Fig. 2C). Overall, there was no correlation between bath KCl and burst amplitude after 60 min of increased KCl exposure ($P = 0.296$, $r^2 = 0.129$; Fig. 2E).

Effects of riluzole and FFA in aCSF. Bath-applied riluzole (50 μM; $n = 18$) produced a time-dependent increase in burst frequency (Figs. 3 and 4, A and B) and decrease in burst amplitude (Figs. 3 and 4D). In the presence of riluzole, frequency increased from $0.71$ ± $0.06$ bursts/min (baseline) to a maximum of $0.92$ ± $0.07$ bursts/min ($P < 0.05$) at 80 min before reaching a steady-state frequency of $0.83$–$0.87$ bursts/min for the next 40 min ($P < 0.05$; Fig. 4A). In contrast, burst frequency in time control experiments ($n = 12$) did not change; burst frequency was $0.52$ ± $0.07$ bursts/min at baseline and $0.52$ ± $0.07$ bursts/min at the 140-min time point ($P < 0.05$; Fig. 4A). When graphed as percent change in frequency, riluzole steadily increased frequency by $31$ ± $5\%$ within
80-min before reaching a steady-state increase of 23–26% ($P < 0.05$ for drug and time-dependent effects; Fig. 4B). With respect to the number of bursts/episode, riluzole had no effect and was constant at 1.4–1.6 bursts/episode throughout the riluzole exposure ($P > 0.05$; Figs. 3B and 4C). For burst amplitude, riluzole produced an immediate, time-dependent decrease such that amplitude was $58 \pm 4\%$ of baseline after 120 min ($P < 0.05$) compared with an $8 \pm 3\%$ decline ($P > 0.05$) in time control experiments (Fig. 4D). To rule out the caveat that riluzole was not penetrating the brain stems and altering rhythm-generating neurons deep within the tissue, riluzole (50 μM) was applied to turtle hemi-brain stems (i.e., brain stems completely transected along the midline). In these reduced preparations ($n = 2$), riluzole produced a similar time-dependent frequency increase and amplitude decrease (data not shown).

In contrast to riluzole, FFA produced a time- and dose-dependent decrease in burst frequency with minor decreases in amplitude (Fig. 5A). Although bath application of 20 μM FFA ($n = 6$) had little effect on amplitude, FFA at higher concentrations (50, 100, and 500 μM; $n = 5, 7,$ and 8, respectively) decreased burst frequency to new steady-state levels within 40–100 min ($P < 0.05$; Fig. 5B). The percent decrease in frequency at 140 min post-drug application was significantly decreased compared with time controls at FFA concentrations between 50–500 μM (Fig. 5C). Similar to riluzole, FFA had no effect on the number of bursts per episode compared with time controls (Fig. 5D). With respect to amplitude, biphasic, dose-dependent effects were observed. There was no change when brain stems were exposed to 20 μM FFA (Fig. 5E). During the 50 μM exposure, however, there was a transient increase of $25 \pm 30\%$ ($P < 0.05$) after 40 min, but amplitude was decreased by $38–47\%$ during the next 40 min ($P < 0.05$). For the 100 μM exposure, amplitude was decreased by $17–33\%$ ($P < 0.05$) during 40–80 min of the FFA application before returning to baseline. The amplitude data after 100–120 min of 50–100 μM FFA application are highly variable because only 1–3 brain stems produced respiratory bursts at a low frequency.

Increased extracellular KCl increased burst frequency during synaptic inhibition blockade. As demonstrated previously (23), the motor output of the turtle respiratory neural control

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Fig. 3. Riluzole does not abolish respiratory motor output. Traces of integrated XII motor discharge are shown for two different brain stems bathed in aCSF and exposed to riluzole (50 μM, 2 h). Riluzole increased frequency and decreased amplitude in brain stems producing either singlet (A) or episodic (B) discharge.

Fig. 4. Effects of riluzole on respiratory variables for brain stems bathed in aCSF. A: bath-applied riluzole (50 μM, 2 h) significantly increased burst frequency (open circles) while frequency remained constant in time control experiments (closed circles). B: when frequency data were graphed as percent change from baseline, riluzole increased frequency during the first 80 min of application until a plateau was reached. C: riluzole did not alter the number of bursts/episode. D: riluzole caused a steady time-dependent decrease in burst amplitude. *$P < 0.05$ compared with time control data. †$P < 0.05$ compared with baseline. ‡$P < 0.05$ for drug effect. §$P < 0.05$ for time-dependent effect.

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system was transformed by blocking fast inhibitory synaptic transmission via bath application of bicuculline (50 μM) and strychnine (50 μM), resulting in rhythmic bursts that had an increased frequency and amplitude and a rapid onset/slow decrementing shape (Fig. 6A). Time control experiments (n = 14) showed that after 100 min of exposure to bicuculline and strychnine, rhythmic bursts reached a steady-state frequency of 0.86 ± 0.10 bursts/min. After another 80 min of bicuculline and strychnine, burst frequency was 0.85 ± 0.09 bursts/min and amplitude decreased by only 6 ± 4% (P > 0.05; data not shown). Thus, increased bath KCl was applied after establishing a 20-min baseline during the 100–120 min period of bicuculline and strychnine application (Fig. 6A).

During synaptic inhibition blockade, burst frequency was not altered when bath KCl was increased from 2.0 to 5.0 mM KCl (n = 8; Fig. 6B). However, within 60 min of increasing bath KCl to 7 mM (n = 7) or 8 mM KCl (n = 7), burst frequency increased to 320 ± 70% and 380 ± 110% of baseline, respectively (P < 0.05; Fig. 6B). Decreasing bath KCl to 0.5 mM (n = 8) produced a slight 12 ± 7% decrease in burst frequency. Burst frequency was correlated (P = 0.005, r² = 0.167) with bath KCl after 60 min of KCl exposure (i.e., 80-min time point in Fig. 6B) (Fig. 6D). In contrast, burst amplitude generally decreased with time (Fig. 6A) in a dose-dependent manner with amplitude falling by 18 ± 12% and 27 ± 8% when bath KCl was 7 mM or 8 mM KCl, respectively (P < 0.05 for a time effect after 60 min of increased KCl; Fig. 6C). After 60 min of KCl exposure, burst amplitude was inversely correlated with bath KCl (P = 0.012, r² = 0.139; Fig. 6E).

Effects of riluzole and FFA during synaptic inhibition blockade. To test whether the rhythmic bursts produced during synaptic inhibition blockade were altered by riluzole or FFA, turtle brain stems were exposed to bicuculline (50 μM) and strychnine (50 μM) for 100 min, and then a 20-min baseline was established. Against a background of bicuculline and strychnine, either riluzole (50 μM; n = 12) or FFA (100 μM; n = 7) was applied. Under these conditions, riluzole had no
Effect on burst frequency (Figs. 7A and 8A). In contrast, FFA decreased burst frequency in a time-dependent manner from 0.96 ± 0.10 bursts/min (baseline) to 0.36 ± 0.08 bursts/min after 60 min ($P < 0.05$) and 0.10 ± 0.05 bursts/min after 120 min ($P < 0.05$; Figs. 7B and 8A). Burst amplitude was not altered by either riluzole or FFA, although there were significant time effects for all the data during the last 80 min of drug exposure (Fig. 8B).

**DISCUSSION**

This is the first study to show that FFA, but not riluzole, decreases respiratory burst frequency in an adult ectothermic vertebrate using in vitro isolated brain stems. This suggests that riluzole-sensitive persistent Na$^+$ currents in pacemaker neurons are not required for rhythm generation in aCSF or during synaptic inhibition blockade. In contrast, the finding that FFA abolished burst frequency in aCSF as well as during synaptic inhibition blockade suggests that Ca$^{2+}$-activated cation currents are required for rhythm generation. Burst frequency was not correlated with bath KCl in aCSF, it appears unlikely that pacemaker neurons with FFA-sensitive currents are required for rhythm generation. Following network transformation via synaptic inhibition blockade, the frequency of the FFA-sensitive rhythmic activity was correlated with bath KCl, suggesting that synaptic inhibition “releases” pacemaker neurons or properties which then produce rhythmic motor activity. Taken together, we hypothesize that recurrent excitatory synaptic transmission involving postsynaptic Ca$^{2+}$-activated cation currents are responsible for rhythm generation in turtles (i.e., group-pacemaker model).

Pacemaker properties in vertebrate respiratory rhythm generation. The maturation network-burster hypothesis (43) proposes that the hybrid-pacemaker network model applies during the early postnatal period in mammals because glycine-mediated synaptic inhibition is weak, respiratory neurons are relatively depolarized, and persistent Na$^+$ currents in pacemaker neurons are active. During maturation, however, neuronal membrane potentials are hypothesized to become more negative and thereby permit a large repertoire of ionic conductances to contribute to rhythm generation. Furthermore, synaptic inhibition is hypothesized to become necessary for rhythm generation, and the obligatory role of pacemaker neurons is di-
minished in adult animals. In support of this model, respiratory-related pacemaker neurons are found in primarily in vitro preparations from perinatal rodents in key putative rhythm-generating sites, such as the pre-Bötzinger complex (pre-BoTC; 22, 53, 57) and the parafacial respiratory group (pFRG; 1, 26, 27). Consistent with the maturation network-burster hypothesis, there is little evidence for respiratory-related pacemaker neurons in adult mammals (although the precise age at which the pacemaker-driven respiratory rhythm presumably transitions to a network-driven rhythm is not well defined). Interestingly, pre-I neurons in a perfused in situ preparation from 21- to 42-day-old mice burst rhythmically in low-Ca$^{2+}$/high-Mg$^{2+}$ solution, but it was not clear whether synaptic transmission was completely blocked (28). In amphibians, the maturation network-burster hypothesis also appears to apply since the lung rhythm persists during synaptic inhibition blockade in isolated brain stems from tadpoles but not from adult frogs (3, 17).

In contrast, evidence suggests that the maturation network-burster hypothesis may not apply in all vertebrates. For example, respiratory-related motor bursts are produced by isolated adult lamprey brain stems during bath application of picrotoxin (GABA$\alpha$ antagonist) and strychnine (44). Also, as shown here (Fig. 6A) and in Johnson et al. (23), rhythmic motor activity persists in isolated adult turtle brain stems, and this rhythm is abolished by well-known respiratory depressants, such as $\mu$-opioid receptor agonists (23) and high-pH/low-CO$_2$ conditions (unpublished observations). This suggests that pacemaker properties, whether they are expressed in individual neurons or as an emergent network property, may be involved in respiratory rhythm generation in some adult vertebrates.

Since respiratory-related pacemaker neurons (in mammals) have intrinsic, voltage-dependent bursting properties, action potential burst frequency is directly related to membrane potential (5, 53). Thus, under in vitro conditions, increasing bath KCl would be expected to depolarize pacemaker neurons, increase action potential burst frequency in pacemaker neurons, and thereby increase respiratory burst frequency within the network. There are several caveats associated with this approach. For example, networks without pacemaker neurons have voltage-dependent conductances whose properties will be altered by depolarization. Also, increased bath KCl may increase neurotransmitter release, alter intrinsic membrane properties, alter the driving force through K$^+$-permeable conductances, or alter the function of membrane ion pumps. Although correlating bath KCl with rhythm frequency is not specific, it is useful when combined with other experimental approaches, such as synaptic inhibition blockade. For example, the tadpole lung rhythm frequency was directly related to bath KCl levels (61) and persisted during synaptic inhibition blockade (3, 17), which is consistent with the network being driven by pacemaker neurons. In contrast, the adult frog lung rhythm was not correlated with bath KCl (61) and was abolished during synaptic inhibition blockade (3, 17), which is consistent with a nonobligatory role for pacemaker neurons. In this study, the finding that burst frequency in isolated turtle brain stems was
not correlated with increased bath KCl is consistent with the hypothesis that pacemaker neurons with voltage-dependent properties are not required for rhythm generation.

Can the turtle respiratory rhythm generator be transformed into a pacemaker-driven network? In mammalian in vitro brain stem preparations, the “switching” hypothesis states that the respiratory network can undergo a nonphysiological switch from a network-driven to a pacemaker-driven system when there is decreased synaptic inhibition and increased extracellular K⁺ ions (45, 47, 48, 49, 50, 55). Under these conditions, pacemaker neurons, which are not required for normal breathing, are proposed to become rhythmically active and force respiratory neurons downstream to oscillate rhythmically (4, 45, 47, 48, 49, 50). Accordingly, the precise nature of the motor output produced by reduced perinatal mammalian preparations is part of an ongoing debate as to what constitutes normal breathing (7, 12, 29, 34, 40, 54).

In contrast to mammalian preparations, turtle brain stems in vitro are highly resistant to hypoxia and have a low metabolic rate at physiologically relevant temperatures (e.g., room temperature). Thus, turtle brain stems are unlikely to have hypoxia-induced increases in extracellular H⁺ and K⁺ levels (58, 62) that are found in some mammalian in vitro brain stem-splinal cord preparations. It should be noted that turtle brain stems used in this study were at pH 7.30, which is acidic compared with the normal blood pH (~7.60) in red-eared sliders. Whether low pH biases the turtle respiratory rhythm generator toward pacemaker- vs. network-based function is not known. In addition, isolated turtle brain stems produce appropriate phasic expiratory and inspiratory activity similar to intact turtles, thereby negating the mammalian “eunea vs. gasping” controversy (21). Nevertheless, blockade of synaptic transmission in turtle brain stems produces a rhythm that was directly correlated with extracellular KCl levels, which is consistent with the hypothesis that turtle brain stems, in a manner similar to mammalian preparations, can undergo a switch to a pacemaker-driven network. It should be noted that this correlation was statistically significant but only 17% of the variation was due to bath KCl. One difference is that only synaptic inhibition blockade appears to be necessary to make the switch in turtles, whereas increased extracellular K⁺ levels may also be necessary in mammals. Thus, this capacity for switching to a pacemaker-driven network may be a conserved mechanism within the respiratory systems of mature vertebrates.

Role of riluzole- and FFA-sensitive conductances in respiratory rhythm generation. In mammals, the ionic conductances hypothesized to be responsible for pacemaker properties of respiratory neurons include persistent Na⁺ currents (5, 9, 10, 25, 47, 48), Ca²⁺-activated cation currents (33, 57), and intracellular Ca²⁺ oscillations (2). For example, persistent Na⁺ currents are required for voltage-dependent bursting in a subpopulation of respiratory pacemaker neurons in the pre-Bötz (10, 33). Riluzole blocks respiratory activity in rhythmically active rodent slices (37, 48) and in postpartum d21 (and older) perfused in situ murine preparations (37), suggesting that persistent Na⁺ currents are required for respiratory rhythm generation. In contrast, riluzole did not change the frequency of respiratory motor output produced by rhythmically active rodent slices (10) or in 6-wk-old perfused in situ rat preparations (30, 56), although positive controls showing the penetration of riluzole into the tissue were not shown. These data were interpreted as consistent with the hypothesis that persistent Na⁺ currents are required for gasping and not eupnea (30, 56). Another interesting observation is that rhythmic motor activity (presumed to be respiratory related) persists during simultaneous blockade of both persistent Na⁺ currents (with riluzole) and Ca²⁺-activated cation currents (with FFA), which suggests that pacemaker neurons are not required for mammalian respiratory rhythm generation (11). Although some of the contradictory results may be due to different experimental conditions, protocols, preparations, and animal strains and species, clearly more work is required to resolve these questions.

Since both riluzole and FFA block currents other than persistent Na⁺ and Ca²⁺-activated cation currents, respectively, the strongest finding in this paper was that riluzole did not abolish respiratory burst frequency in aCSF or during synaptic inhibition blockade. For example, riluzole blocks N-methyl-D-aspartic acid (NMDA)-dependent responses in frog oocytes with an IC₅₀ = 18.2 μM (8). Riluzole appeared to penetrate the tissue rapidly, because the same effects were observed in aCSF for intact and hemi-brain stems. If persistent Na⁺ currents are present in turtle respiratory-related neurons, then these currents do not appear to be essential for rhythm generation. On the other hand, it’s possible that persistent Na⁺ currents are not present in turtle respiratory neurons. Since persistent Na⁺ currents are hypothesized to be necessary for mammalian gasping (30, 56) and turtles do not appear to gasp during 6 h of anoxia (unpublished observations), we hypothesize that the turtle respiratory neurons express low levels, if any, of persistent Na⁺ channels.

In contrast, FFA blocked turtle respiratory rhythm generation in a time- and dose-dependent manner with 50 μM FFA producing significant decreases in burst frequency within 40 min of exposure. One interpretation is that FFA blocked Ca²⁺-activated cation currents in pacemaker neurons that are required for respiratory rhythm generation and that increased bath KCl elicits offsetting complex effects on frequency so that increased bath KCl does not increase burst frequency in aCSF. If so, intracellular recordings in the absence of synaptic transmission will be required to identify and characterize candidate pacemaker neurons. On the other hand, the lack of correlation between burst frequency and bath KCl in aCSF argues against the role of pacemaker neurons. Instead, Ca²⁺-activated cation currents may be expressed in nonpacemaker neurons, and the positive feedback interaction of this current with excitatory glutamatergic synaptic currents may underlie respiratory rhythm generation (i.e., group pacemaker model; Refs. 10, 11, 14, 15, 39). In this case, Ca²⁺-activated cation currents would play a critical role in initiating and maintaining inspiratory bursts. However, other interpretations are plausible, because FFA blocks other membrane currents, such as transient receptor potential currents (19) and L-type Ca²⁺ channels (51), which may be necessary for rhythm generation. Also, FFA may inactivate other neurons that project to the turtle rhythm generator and provide critical neurotransmitters that maintain respiratory neurons at appropriate levels of excitability.

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