

Time-dependent changes in spontaneous respiratory activity in turtle brainstems in vitro

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Abstract

Our goal was to determine whether time-dependent changes in respiratory motor output in vitro could be minimized by altering bath solution composition. Adult turtle brainstems were bathed in standard solution, nutrient-rich Dulbecco's Eagle media (100 or 25% concentration), or standard solution with phenylbiguanide (PBG, 5-HT₃ agonist which increases respiratory drive). Except for a 63% frequency increase in PBG solution, hypoglossal bursts were unaltered within 100 min of observation. Respiratory activity was abolished within 7 h in 100% Dulbecco's compared with a mean of 24–31 h in other test solutions. At 12 h, burst frequency decreased faster in standard solution and 25% Dulbecco's (-0.28 ± 0.07 and -0.13 ± 0.05 bursts/h, respectively) compared with PBG solution (-0.09 ± 0.04 bursts/h); amplitude declined at $\sim 2\%/h$ in all solutions. The tendency for episodic discharge decreased gradually in standard solution, but was eliminated in 25% Dulbecco's and PBG solution. Certain bath solutions may minimize time-dependent frequency reductions but may also cause breathing pattern changes.

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1. Introduction

The neural network that underlies breathing expresses long-lasting (hours to days) changes in respiratory motor output in response to physiological stimuli, disease, injury, etc. (i.e. plasticity; reviewed in Mitchell and Johnson, 2003). The

cellular mechanisms underlying such plasticity are not currently known. One strategy for studying cellular mechanisms is to conduct experiments on reduced in vitro preparations that contain the respiratory network, where cellular investigations are more feasible. For example, several unique in vitro preparations have revealed critical insights into the cellular/synaptic bases of respiratory rhythm generation (Richter and Spyer, 2001; Feldman et al., 2003). To study respiratory plasticity under in vitro conditions (e.g. Johnson et al., 2001), it is necessary to have a preparation

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that is viable and produces stable respiratory motor output for many hours. Despite the abundant use of in vitro preparations to study fictive respiration, no studies have documented time-dependent changes in spontaneous respiratory motor output in different bath solutions.

To address this question, long-term (up to 40 h) changes in respiratory activity were measured in adult in vitro turtle brainstems bathed in different solutions. In vitro brainstem preparations from mature turtles are hypoxia-resistant and generate spontaneous respiratory neural discharge similar to breathing in intact turtles (Douse and Mitchell, 1990; Johnson et al., 1998b; Johnson and Mitchell, 1998). The solutions chosen for this study include a standard solution (HEPES-based), Dulbecco's Eagle media (100 and 25% concentration), and PBG solution (standard solution containing phenylbiguanide [PBG; 5-HT₃ agonist]). Dulbecco's may preserve respiratory activity for longer periods because the media is enriched with amino acids and vitamins, and has been shown to maintain stable evoked EMG potentials for 3–5 days in an in vitro turtle spinal cord–nerve–muscle preparation (Lennard et al., 1989). PBG solution, which increases hypoglossal burst frequency (Johnson et al., 2001), allows us to test whether increased respiratory drive prolongs the production of spontaneous respiratory motor output.

2. Materials and methods

The University of Wisconsin Institutional Animal Care and Use Committee approved all surgical procedures and experimental protocols. Adult red-eared slider turtles (*Trachemys scripta*) were obtained from commercial suppliers and kept in a large open tank with access to water, heat lamps, and rocks for basking. Room temperature was kept between 27 and 28 °C with a 14-h light:10-h dark cycle. Turtles were fed daily (ReptoMin[®], Tetra, Blacksburg, VA, USA).

2.1. Brainstem isolation

Turtles ($n = 37$, 721 ± 15 g) were intubated and ventilated (10–15 breaths/min) with 5% isoflurane in O₂. Head withdrawal, corneal reflexes and the response to noxious front limb pinch were eliminated within 5–10 min. Turtles were decapitated and tissue and bone encasing the brain were removed. The preparation was immersed in standard solution (22 °C) bubbled with 5% CO₂–95% O₂ (pH 7.4 ± 0.1 ; calomel pH electrode; Cole-Parmer Instruments, Vernon Hills, IL, USA). Standard solution was composed of (in mM): 100 NaCl, 23 NaHCO₃, 10 D-glucose, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)–sodium salt, 5 HEPES-free acid, 2.5 CaCl₂, 2.5 MgCl₂, 1 KCl, and 1 KH₂PO₃ (Sigma/Aldrich, St. Louis, MO, USA). HEPES was included to increase buffering capacity as discussed in Richerson and Getting (1987). Brain tissue rostral to the optic nerves and caudal to the first spinal nerve was discarded, the cerebellum and meninges removed, and the brainstem pinned ventral side up in a recording chamber (13 ml volume; Fig. 1A). Preparations were bathed with standard solution (flow rate = 5–6 ml/min) and allowed to equilibrate 3–5 h to establish stable baseline neural discharge. Four brainstem preparations were placed simultaneously in the recording chamber.

2.2. Nerve recording

Hypoglossal nerve rootlets were fitted with glass suction electrodes to measure bursts of respiratory motor output (Fig. 1B–E; Douse and Mitchell, 1990; Johnson et al., 2001). Signals were amplified ($\times 10000$), band-pass filtered (10 Hz–10 kHz) using a differential amplifier (model 1700, A-M Systems, Everett, WA, USA), and rectified and integrated (time constant = 200 msec) with a moving averager (MA-821/RSP, CWE, Inc., Ardmore, PA, USA). All signals were digitized using AXOSCOPE 7.0 software (Axon Instruments, Foster City, CA, USA) and stored on computer for analysis.

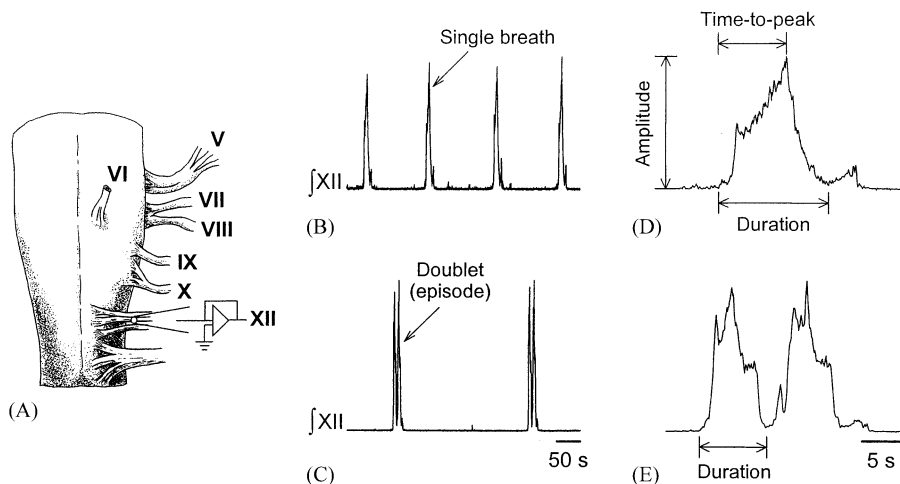


Fig. 1. (A) In vitro turtle brainstem drawing (ventral view) indicating cranial nerves and suction electrode placement. Compressed and extended integrated hypoglossal discharge traces for single (B, D) and episodic bursts (e.g. doublets) (C, E). Respiratory variables were measured as shown.

2.3. Experimental protocols

Hypoglossal bursts were recorded for 30 min in standard solution (baseline) before switching to one of the following test solutions: (1) standard solution, (2) 100% Dulbecco's solution, (3) 25% Dulbecco's (one part 100% Dulbecco's to three parts standard solution), or (4) PBG solution (standard solution with 20 μ M PBG). 100% Dulbecco's solution consisted of HEPES-free Dulbecco's Modified Eagle media (GibcoBRL, Life Technologies, Grand Island, NY, USA) supplemented with NaHCO_3 , D-glucose, CaCl_2 and MgCl_2 to achieve a final ionic composition similar to standard solution (i.e. 23, 10, 2.9 and 2.5 mM, respectively). Test solutions contained antibiotic/antimycotic solution (10 ml/L; 5000 units/ml penicillin; 5000 μ g/ml streptomycin; 12.5 μ g/ml amphotericin B; GibcoBRL, Life Technologies), except for some experiments ($n = 4$ each in the 25% Dulbecco's and PBG solution groups). Preparations were continuously bathed in a test solution until all activity ceased or up to 40 h. At the end of some experiments, glutamate (10 mM, $n = 19$) was added to the bath reservoir to determine if hypoglossal motoneurons could still be activated. Solutions were not recycled during the experiment.

2.4. Data analysis

After recording baseline data, test solutions were applied and preparations allowed to equilibrate for 40 min. The next 60 min was designated as the 0-h time point. Every 4 h thereafter, a 60-min data bin was analyzed (Fig. 2A). Burst and episode frequencies were measured within the entire 60-min bin. Episodes were defined as two or more bursts separated by less than the average duration of a single burst (Fig. 1C, E). Burst amplitude, duration and time-to-peak (reported as percent of burst duration) were measured during the 20–40 min period of each 60-min bin (Fig. 1D). Amplitude was measured in arbitrary units and normalized to either baseline data (acute effects) or 0-h data (time-dependent effects). For episodes, only the amplitude of the first burst was measured (see Johnson et al., 1998b). All values are given as mean \pm S.E.M. Baseline and 0-h data were compared with a Student's t-test. Time-dependent data (0–12-h) were compared using a two-way repeated measures ANOVA (data after 12 h could not be compared due to loss of active preparations). Post-hoc pair-wise comparisons were made using Student–Neuman's–Keul's test. Data were considered significant when $P \leq 0.05$.

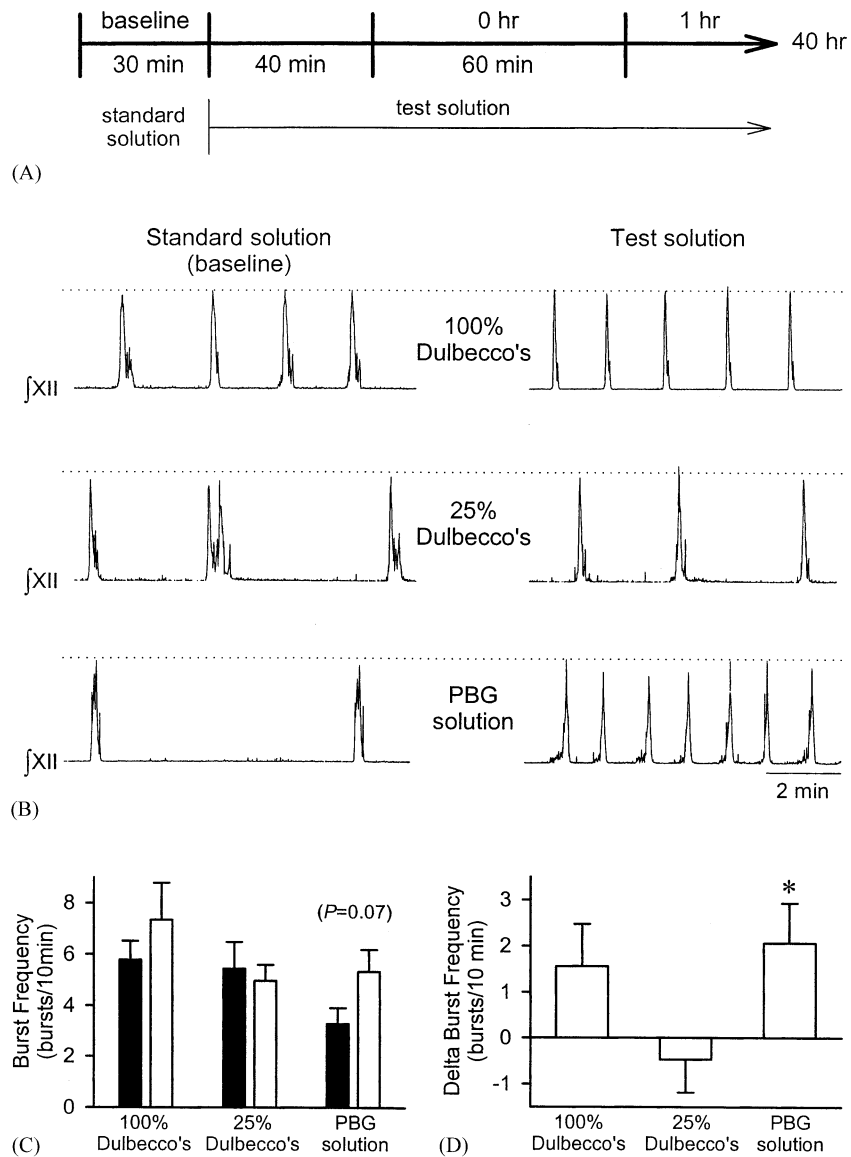


Fig. 2. (A) Time line for experimental protocol. (B) Integrated hypoglossal traces comparing standard solution and 100% Dulbecco's (top), 25% Dulbecco's (middle), or PBG solution (bottom). The horizontal dotted line indicates the average burst amplitude during baseline. Burst frequency (C) and delta burst frequency (D) are altered in 100% Dulbecco's and PBG solution (open bars) relative to standard solution (closed bars). Delta burst frequency is the difference between baseline and 0-h burst frequency values. Values are given as mean \pm S.E.M. Asterisk indicates $P \leq 0.05$ relative to baseline.

3. Results

3.1. Acute effects of test solutions on hypoglossal motor output

Application of 100% Dulbecco's and PBG solution tended to increase burst frequency while 25% Dulbecco's produced a small decrease (Fig. 2B, C). When analyzed as the change in frequency from baseline, only PBG solution elicited a significant increase in frequency (Fig. 2D; $P \leq 0.05$). Burst amplitude was not altered by 100% Dulbecco's, 25% Dulbecco's or PBG solution (1.04 ± 0.03 , 1.15 ± 0.10 , 1.03 ± 0.08 relative to baseline, respectively; $P > 0.05$). Burst duration and time-to-peak were not altered by any test solution (Table 1; comparison of baseline and 0-h data).

3.2. Time-dependent effects of test solutions on hypoglossal motor output

In standard solution ($n = 11$), respiratory motor output was produced for 24.1 ± 3.5 h (range = 6.9 to > 40 h [two brainstems active at > 40 h], Fig. 3A, Fig. 4A). 100% Dulbecco's ($n = 3$) caused burst frequency to decrease more rapidly; bursts continued for only 5.6 ± 1.3 h (range = 4.5–7.0 h; Fig. 3B). Brainstems bathed in 25% Dulbecco's ($n = 12$) were active for 25.4 ± 1.0 h (range = 20.1–30.5 h; Fig. 3C, Fig. 4B), while PBG solution enabled brainstems ($n = 11$) to remain active for 31.2 ± 2.9 h (range = 14.5 to > 40 h [four brain-

stems active at > 40 h], Fig. 3D, Fig. 4C). In brainstems that had stopped bursting ($n = 8$ in standard solution; $n = 3$ in PBG solution), glutamate application (10 mM) produced tonic discharge in hypoglossal nerve roots, indicating that hypoglossal motoneurons were still responsive, but the respiratory rhythm was not reestablished. However, glutamate application did not cause motoneuron discharge in brainstems bathed in 25% Dulbecco's ($n = 8$), suggesting that hypoglossal motoneurons were no longer responsive.

In standard solution, burst frequency decreased from 4.9 ± 0.8 to 2.1 ± 0.6 bursts/10 min within 12 h, stayed at ~ 2 bursts/10 min between 12 and 24 h, then declined from 2.1 ± 0.5 to 0.3 ± 0.1 bursts/10 min between 24 and 40 h (Fig. 5A). In 25% Dulbecco's solution, burst frequency decreased from 5.0 ± 0.6 to 1.5 ± 0.3 bursts/10 min over 24 h, whereas preparations in PBG solution decreased from 5.3 ± 0.9 to 2.4 ± 0.5 bursts/10 min over 40 h (Fig. 5A). Between 0 and 12 h, two-way ANOVA revealed no differences between test solutions ($P = 0.09$). When analyzed as the change in burst frequency from baseline, significant decreases were found at 4, 8 and 12 h in standard solution, and at 12 h in 25% Dulbecco's (Fig. 5B). In PBG solution there were no significant decreases in burst frequency between 0 and 12 h, but there was a significant time-dependent difference between standard and PBG solution treatments (Fig. 5B).

Burst amplitude decreased at a similar rate ($\sim 2\%/h$) for each test solution (Fig. 5C). Within the first 12 h, amplitude decreased significantly at

Table 1
Acute and time-dependent changes in burst duration and time to peak

	Standard	100% Dulbecco's	25% Dulbecco's	20 μ M PBG
<i>Duration (s)</i>				
Baseline	14.7 ± 1.8 (11)	14.7 ± 3.2 (3)	17.4 ± 1.6 (12)	18.0 ± 3.1 (11)
0 h	14.8 ± 1.7 (11)	9.5 ± 2.0 (3)	17.0 ± 1.1 (12)	16.6 ± 3.0 (11)
12 h	14.2 ± 2.5 (8)	–	$13.4 \pm 0.9^*$ (12)	14.2 ± 1.9 (10)
<i>Time to peak (% of duration)</i>				
Baseline	33 ± 4	39 ± 3	30 ± 3	40 ± 4
0 h	43 ± 5	43 ± 2	36 ± 3	43 ± 5
12 h	32 ± 5	–	$46 \pm 4^*$	44 ± 4

Values are given as mean \pm S.E.M.; numbers in parentheses indicate the number of preparations; $*P \leq 0.05$ relative to 0 h data within the test solution group.

the 12-h point for standard and PBG solutions (Fig. 5D). There were no significant differences in amplitude for 25% Dulbecco's, and no significant time-dependent differences between test solutions. When suction electrodes were repositioned (> 16 h after test solution application), amplitude increased in only 2/11 experiments, suggesting that the time-dependent decrease was not due to detachment of the suction electrode from the nerve roots.

Burst duration and time-to-peak were not altered after 12 h for standard and PBG solutions ($P > 0.05$; Table 1). In 25% Dulbecco's, mean burst duration decreased from 17.0 ± 1.1 to 13.4 ± 0.9 sec ($P \leq 0.05$) and mean time-to-peak in-

creased from 36 ± 3 to $46 \pm 4\%$ ($P \leq 0.05$; Table 1). There were no significant time-dependent differences between test solutions on burst duration and time-to-peak.

3.3. Episodic discharge decreases in 25% Dulbecco's and PBG solution

Episodic discharge (i.e. bursts/episode ratio > 1.0) during baseline conditions was observed in 21 brainstem preparations and consisted of doublets (Fig. 1D, E, Fig. 6A) with occasional triplets and quadruplets (data not shown). To determine whether test solutions altered episodic discharge, brainstems ($n = 15$) with baseline bursts/episode

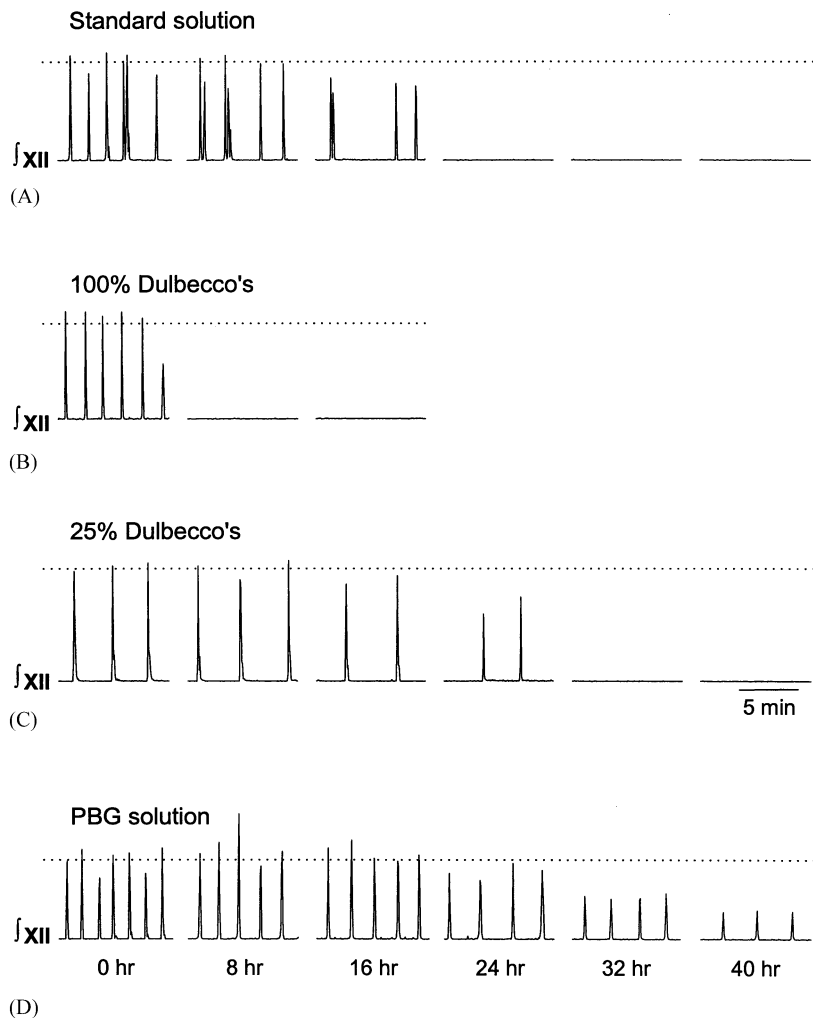


Fig. 3. Integrated hypoglossal discharge traces illustrating time-dependent effects in (A) standard solution, (B) 100% Dulbecco's (no data > 16 h), (C) 25% Dulbecco's, and (D) PBG solution. The horizontal dotted line indicates the average amplitude at 0-h.

ratios ≥ 1.5 were analyzed. Acute and long-term bursts/episode ratios in standard solution ($n = 6$) were unchanged with a decreasing trend over 12 h ($P > 0.05$; Fig. 6). 25% Dulbecco's ($n = 6$) or PBG solution ($n = 3$) acutely decreased the bursts/episode ratio from 1.83 ± 0.06 to 1.04 ± 0.03 and 2.17 ± 0.37 to 1.00 ± 0.00 , respectively ($P \leq 0.05$; Fig. 6A, B). The reduction in bursts/episode ratios with 25% Dulbecco's and PBG solution was maintained after 12 h (Fig. 6C). Significant time-dependent treatment differences were found between standard solution and 25% Dulbecco's or PBG solution ($P \leq 0.05$).

4. Discussion

This study is the first to characterize time-dependent changes in spontaneous respiratory motor output in several different test solutions in an adult in vitro brainstem preparation. We found that turtle brainstems produced respiratory motor output for up to 40 h, and that test solutions had different effects on burst frequency and pattern. Thus, turtle brainstems are valuable for studying long lasting forms of respiratory motor plasticity (e.g. Johnson et al., 2001), but solution-dependent and time-dependent changes must be considered carefully.

4.1. Caveats and limitations

The general advantages and limitations of measuring respiratory motor output in turtle brainstems in vitro were previously discussed (see Johnson et al., 1998a,b). Since these experiments were performed under non-sterile conditions, bacterial or fungal growth may have damaged tissues, consumed nutrients, or produced harmful waste products. To minimize these potential adverse effect, antibiotics and antimycotics were added to the test solutions. Although the effects of antimicrobial drugs on respiratory motor output in vitro are not known, there were no obvious changes in respiratory variables immediately following bath application of standard solution with antimicrobial drugs (Table 1).

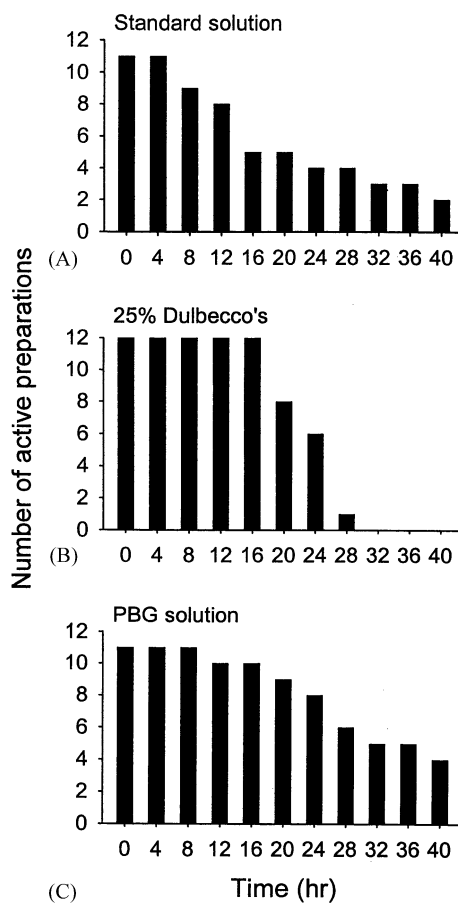


Fig. 4. Number of active preparations over time in (A) standard solution, (B) 25% Dulbecco's, and (C) PBG solution.

Another caveat is that brainstem preparations were superfused with test solutions rather than perfused arterially, and thus, nutrient, O_2 , CO_2 and pH gradients may have been established. However, poikilothermic vertebrate brain preparations have low metabolic rates, thereby minimizing O_2 , CO_2 and pH gradients relative to similar mammalian preparations (Hounsgaard and Nicholson, 1990; Torgerson et al., 1997). Furthermore, the turtle central nervous system is highly resistant to anoxia (Jackson, 1968; Brooks and Storey, 1994; Doll et al., 1994; Buck et al., 1998). For example, respiratory motor output is unaltered for ≥ 2 h by severe hypoxia in in vitro turtle brainstem preparations (Johnson et al., 1998b).

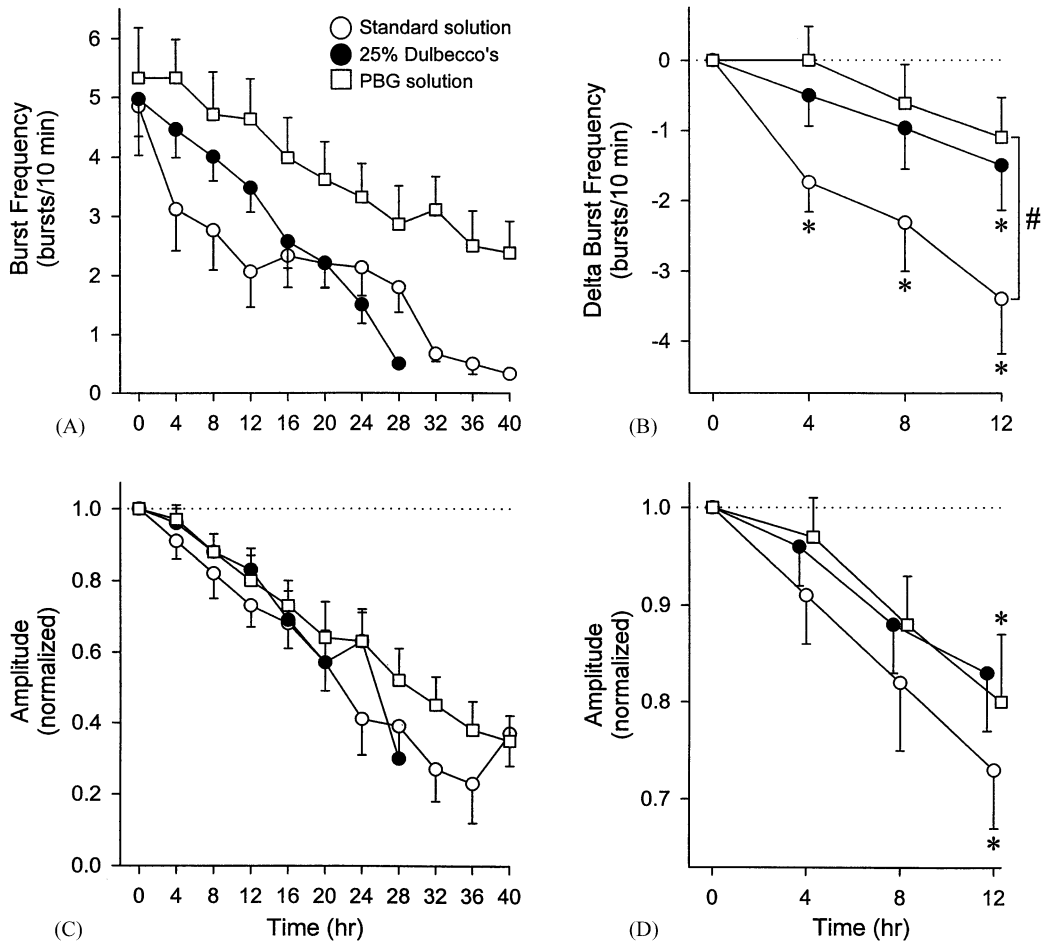


Fig. 5. Time-dependent changes in burst frequency (A, B) and amplitude (C, D) in standard solution (○), 25% Dulbecco's solution (●), and PBG solution (□). Delta burst frequency was calculated with respect to 0-h; amplitude values were normalized to 0-h data. Asterisk indicates $P \leq 0.05$ relative to 0-h data within a treatment group; “#” indicates $P \leq 0.05$ between standard and PBG solutions. Amplitude data in (D) were transformed (square root) for statistical analysis.

4.2. Poikilothermic versus mammalian *in vitro* preparations

To our knowledge, no studies have systematically examined time-dependent changes in spontaneous respiratory motor output from *in vitro* or *in situ* preparations; but instead list only the duration of preparation viability. For example, neonatal rat brainstem–spinal cord preparations produce rhythmic motor discharge for 5.9 ± 0.8 h (Smith et al., 1990). Neonatal rodent medullary slices produce rhythmic activity for 8–48 h, but only at reduced temperatures (27 °C) and with

increased extracellular potassium ion concentrations (e.g. 7–10 mM; Johnson et al., 1996; Koshiya and Smith, 1999). *In vitro* semi-intact preparations from mature mammals, such as the adult mouse working heart–brainstem preparation, produce stable motor output for 5–6 h at 31 °C (Paton, 1996). Similarly, perfused adult guinea pig brainstem preparations produce respiratory motor output for 4.3 ± 0.4 h at 27 °C (Morin-Surun et al., 1992). Lastly, an *in situ* arterially perfused adult rat brainstem–spinal cord preparation generates phrenic nerve output for up to 15 h (mean = 8.5 h; Hayashi and Lipski, 1992). Each of these mam-

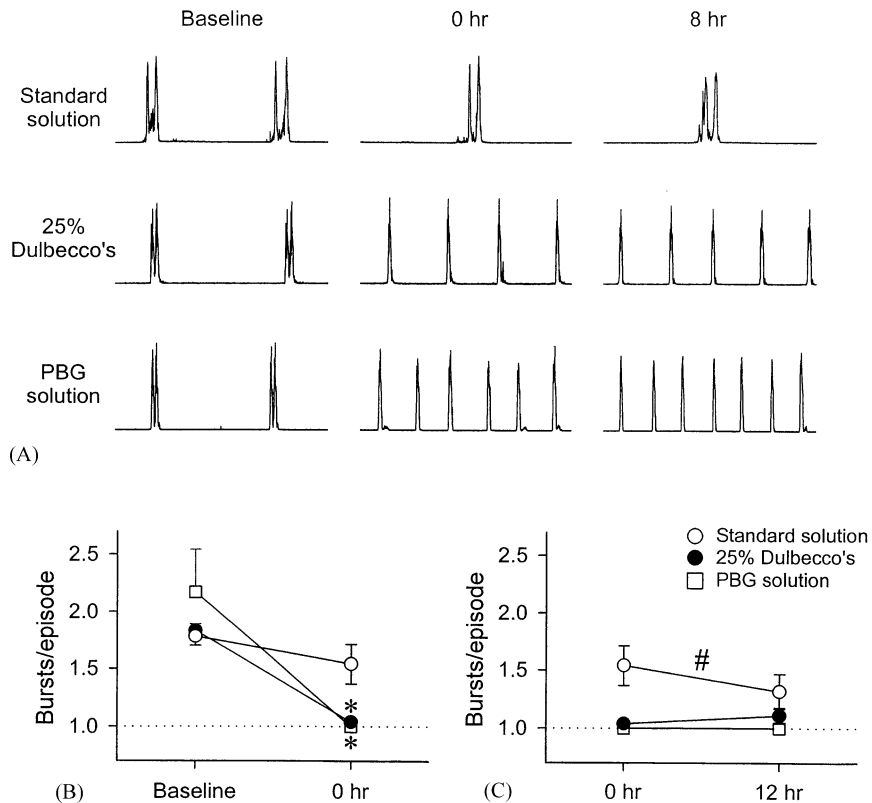


Fig. 6. (A) Time-dependent changes in episodic hypoglossal discharge from brainstems bathed in standard solution, 25% Dulbecco's, or PBG solution. (B) 25% Dulbecco's and PBG solution acutely alter the number of bursts/episode. Asterisk indicates $P \leq 0.05$ relative to baseline. (C) Time-dependent changes between 0- and 12-h data were not found, but a significant treatment difference was found between standard and 25% Dulbecco's and PBG solution (# indicates $P \leq 0.05$). The dotted line represents singlet breaths (i.e. 1.0 bursts/episode).

malian preparations requires decreased temperatures and/or an increased extracellular potassium concentration, which may affect synaptic physiology (Mitchell, 1993).

In contrast, *in vitro* brainstem and brainstem–spinal cord preparations from poikilothermic vertebrates produce spontaneous rhythmic neural discharge for long time periods at physiologically relevant temperatures without increased extracellular potassium ion concentrations. For example, spontaneous respiratory motor output continues for > 40 h in turtle brainstem or brainstem–spinal cord preparations (this study; Johnson et al., 1998a,b; Johnson and Mitchell, 1998) and 12 h in adult frog brainstems (Perry et al., 1995). When kept at 4 °C and periodically warmed to room

temperature for neural recordings, turtle brainstems produce fictive respiratory activity after 2–21 days (S.M. Johnson and G.S. Mitchell, unpublished observations; Douse and Mitchell, 1990).

Although poikilothermic vertebrates are advantageous for long-term studies *in vitro* on respiratory control, the choice of bath solution (and its specific time-dependent effects) needs to be considered carefully. For example, long-term studies *in vitro* may best be accomplished using 25% Dulbecco's or PBG solution because all brainstems continued to burst with relatively minor decreases in burst frequency and amplitude within the first 4–8 h. However, both solutions significantly altered respiratory motor output by abolishing episodic breathing. Standard solution did

not dramatically alter breathing pattern, but burst frequency and amplitude decreased more rapidly compared with other solutions. Thus, choice of bath solution depends upon the nature of the experiment to be performed.

The cause of respiratory “run-down” in turtle brainstems is not known. High nutrient levels (e.g. amino acids) in Dulbecco’s solution may cause depolarization block, excitotoxicity, or glutamate receptor downregulation. Consistent with this hypothesis, brainstems bathed in 25% Dulbecco’s solution did not respond to glutamate application at the end of some experiments. Alternatively, run-down may have been due to washout or decreased endogenous production of neurotrophins, thereby decreasing (moto)neuronal excitability. For example, most spinal motoneurons in cultured turtle spinal cord slices are unable to fire repetitively in response to depolarization unless brain-derived neurotrophic factor, neurotrophin-3, glial cell line-derived neurotrophic factor, and ciliary neurotrophic factor are added to the culture media (Perrier et al., 2000). Run-down may also be due to the build-up of cellular waste products. Turtle brainstems, however, are U-shaped with a thickness of ~ 1.0 mm, and most neurons are within 500–600 μm of the tissue surface (Cruce and Nieuwenhuys, 1974). Thus, it seems unlikely that waste product accumulation is a major cause of run-down.

4.3. Time- and solution-dependent changes in episodic breathing

Intact reptiles and amphibians exhibit breathing patterns that can be classified as single breath, episodic or continuous (single breaths one after another; reviewed in Shelton et al., 1986; Milsom, 1991; Kinkead, 1997; Milsom et al., 1999). Under in vitro conditions, isolated brainstems from these animals still produce similar patterns (e.g. Douse and Mitchell, 1990; Milsom et al., 1997; Johnson et al., 1998a; Reid and Milsom, 1998; Reid et al., 2000). To our knowledge, this is the first study to quantitatively report time-dependent changes in episodic breathing. In standard solution, episodic breathing patterns in vitro gradually transformed into a singlet pattern, suggesting that tissue “run-

down” may be associated with decreased respiratory drive, lower burst frequency and less fictive breath clustering. In contrast, PBG solution reduces the frequency of episodic bursts, despite a trend towards increased respiratory drive.

5. Summary

In vitro preparations are valuable models for examining mechanisms underlying respiratory rhythm generation, modulation, and plasticity. In this paper, the pattern of respiratory motor output was shown to be sensitive to the composition of the bath solution in an acute and time-dependent manner. Thus, for in vitro studies on respiratory motor control, the bathing medium must be chosen to minimize preparation run-down and alterations in breathing pattern.

Acknowledgements

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