Blockade of Brain Stem Gap Junctions Increases Phrenic Burst Frequency and Reduces Phrenic Burst Synchronization in Adult Rat

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Solomon, Irene C., Ki H. Chon, and Melissa N. Rodriguez. Blockade of brain stem gap junctions increases phrenic burst frequency and reduces phrenic burst synchronization in adult rat. J Neurophysiol 89: 135–149, 2003; 10.1152/jn.00697.2002. Recent investigations have examined the influence of gap junctional communication on generation and modulation of respiratory rhythm and inspiratory motoneuron synchronization in vitro using transverse medullary slice and en bloc brain stem-spinal cord preparations obtained from neonatal (1–5 days postnatal) mice. Gap junction proteins, however, have been identified in both neurons and glia in brain stem regions implicated in respiratory control in both neonatal and adult rodents. Here, we used an in vitro arterially perfused rat preparation to examine the role of gap junctional communication on generation and modulation of respiratory rhythm and inspiratory motoneuron synchronization in adult rodents. We recorded rhythmic inspiratory motor activity from one or both phrenic nerves before and during pharmacological blockade (i.e., uncoupling) of brain stem gap junctions using carbenoxolone (100 μM), 18α-glycyrrhetinic acid (25–100 μM), 18β-glycyrrhetinic acid (25–100 μM), octanol (200–300 μM), or heptanol (200 μM). During perfusion with a gap junction uncoupling agent, we observed an increase in the frequency of phrenic bursts (∼95% above baseline frequency; P < 0.001) and a decrease in peak amplitude of integrated phrenic nerve discharge (P < 0.001). The increase in frequency of phrenic bursts resulted from a decrease in both T₁ (P < 0.01) and T₂ (P < 0.01). In addition, the pattern of phrenic nerve discharge shifted from an augmenting discharge pattern to a “bell-shaped” or square-wave discharge pattern in most experiments. Spectral analyses using a fast Fourier transform (FFT) algorithm revealed a reduction in the peak power of both the 40- to 50-Hz peak (corresponding to the MFO) and 90- to 110-Hz peak (corresponding to the HFO) although spurious higher frequency activity (≥130 Hz) was observed, suggesting an overall loss or reduction in inspiratory-phase synchronization. Although additional experiments are required to identify the specific brain stem regions and cell types (i.e., neurons, glia) mediating the observed modulations in phrenic motor output, these findings suggest that gap junction communication modulates generation of respiratory rhythm and inspiratory motoneuron synchronization in adult rodents in vitro.

INTRODUCTION

Accumulating evidence from both neonatal and adult rodents indicates that gap junctions may participate in multiple aspects of respiratory control (for a recent review, see Solomon and Dean 2002). Gap junctions are intercellular channels that form a transmembrane pathway for the direct exchange of small molecules and ions and provide an avenue for both metabolic and electrical coupling between neighboring cells (Bennett and Goodenough 1978; Bruzzone and Ressot 1997). Recent investigations have demonstrated electrical coupling between presumptive rhythmonic type-1 inspiratory neurons located in the preBötzinger complex (preBötC; the hypothesized site for respiratory rhythm generation) of neonatal mice (Rekling et al. 2000) and pharmacological blockade of gap junctions in the in vitro transverse medullary slice and en bloc brain stem-spinal cord preparations obtained from neonatal (1–5 days postnatal) mice has been shown to reduce inspiratory burst frequency, suggesting that gap junctions may participate in respiratory rhythm generation (Bou-Flores and Berger 2001; Rekling et al. 2000). Additionally, inspiratory motoneuron synchronization appears to be enhanced in these preparations during blockade of gap junctions, suggesting that gap junction coupling may play a role in reducing the magnitude of short time-scale synchrony in an electrically coupled network (Bou-Flores and Berger 2001).

Although evidence supporting a role for gap junction communication in generation of respiratory rhythm and inspiratory motoneuron synchronization has been demonstrated in young neonatal rodents, gap junction proteins have been identified in brain stem regions implicated in respiratory control, including presumptive rhythmonic type-1 inspiratory preBötC neurons in both neonatal and adult rats (Solomon et al. 2001a,b) and adult mice (Parenti et al. 2000). These studies, however, provide no insight into the functional role of gap junction communication (i.e., metabolic and/or electrical coupling) in the generation and modulation of respiratory rhythm and synchroniziation of inspiratory motor output in adult mammals. Thus the purpose of the present investigation was to examine phrenic nerve discharge during pharmacological blockade (i.e., uncoupling) of brain stem gap junctions in a more mature rodent preparation. We used an in vitro arterially perfused rat preparation (Paton 1996) to examine the role of gap junctional coupling on generation and modulation of respiratory rhythm and pattern and inspiratory motoneuron synchronization in adult rats.

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adult (≥35 days) rat. This preparation was selected so that we could simultaneously uncouple gap junctions in all brain stem regions implicated in the generation and modulation of respiratory rhythm and pattern. We hypothesized that if gap junctions play a role in generation and modulation of respiratory rhythm and inspiratory motoneuron synchronization, uncoupling brain stem gap junctions would reduce synchronization of inspiratory activity, which would result in modulation of inspiratory burst pattern and frequency of rhythmic inspiratory motor bursts.

METHODS

General methods

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals. Experiments were conducted using an in vitro arterially perfused rat (81–125 g, ∼5–6 wk old; n = 48) preparation (Paton 1996). The rats were deeply anesthetized using isoflurane (2–5%) via inhalation. Prior to beginning any surgical procedures, the level of anesthesia was assessed by testing for the absence of a withdrawal reflex or increase in ventilation in response to a noxious paw pinch. The rat was then rapidly transected sub-diaphragmatically and immediately submerged in ice-cold artificial cerebrospinal fluid (ACSF) gassed with 95% O₂-5% CO₂ containing (in mM) 125 NaCl, 24 NaHCO₃, 5.0 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄, and 10 dextrose. The skull was removed, and a decerebration was performed at the precollicular level using aspiration. It should be noted that with decerebration at this level, all of the brain centers involved in the sensation and perception of pain are removed; thus following decerebration, no supplemental anesthesia is necessary. The skin and lungs were then removed, the thoracic aorta was separated from the vertebral column, the preparation was transferred to the recording chamber, and the descending thoracic aorta was cannulated (double-lumen catheter; French 3.5). One lumen of the catheter was used to perfuse the preparation with a modified ACSF (containing 2.5% Ficoll 70, an oncotic agent; Sigma Chemical, St. Louis, MO) using a roller pump (PeriStar, World Precision Instrument, Sarasota, FL). The other lumen of the catheter was used to continuously measure perfusion pressure, which was gradually increased until phrenic nerve activity returned with a eupneic-like (“ramp-like”) discharge pattern; the pressure was then maintained at this level (∼50 mmHg) throughout the experiment. The perfusate was gassed continuously with 95% O₂-5% CO₂, warmed to 31°C, filtered using a nylon mesh (pore size: 40 μm; Millipore, Bedford, MA), and passed through a bubble trap to remove gas bubbles and to dampen pulsations from the roller pump. Flow was assessed by testing for the absence of a withdrawal reflex or increase in ventilation in response to a noxious paw pinch. Flow was measured using a third-order Paynter filter with a 20- or 50-ms time constant. In some experiments, inspiratory burst pattern and frequency of rhythmic inspiratory motor bursts were carefully dissected from the surrounding connective tissue and sectioned at their insertion point on the diaphragm. Phrenic nerve activity was recorded using bipolar platinum rod hook electrodes. Phrenic nerve discharge was amplified and filtered (100 Hz to 5 kHz), and a moving average was obtained using a third-order Paynter filter with a 20- or 50-ms time constant. In some experiments, phrenic nerve discharge was filtered at 10 Hz to 5 kHz for analysis of spectral composition (see Data analysis). Both raw and averaged phrenic nerve activity were recorded on a chart recorder, computer (sampling rate of 1 to 10 kHz; Chart 4.0, PowerLab, ADInstruments, Mountain View, CA), and digital tape (DAT, Cygnus, Delaware Water Gap, PA) for off-line analyses. Prior to the recording protocol, vucuronium bromide (2 mg) was added to the perfusate to eliminate motor movements associated with respiratory efforts.

Experimental protocol

The primary focus of these experiments was to examine phrenic nerve discharge before and during pharmacological blockade (i.e., uncoupling) of brain stem gap junctions. Baseline levels of phrenic nerve discharge were recorded for 5–10 min during perfusion with normal ACSF bubbled with 95% O₂-5% CO₂. The preparation was then perfused with ACSF containing an uncoupling agent. All uncoupling agents were dissolved in normal ACSF. The following uncoupling agents were used in these experiments: CBX (100 μM final concentration; n = 19), 18k-glycyrrhetinic acid (18k-GA; 25–100 μM final concentration; n = 4), 18β-glycyrrhetinic acid (18β-GA; 25–100 μM final concentration; n = 3), octanol (200–300 μM final concentration; n = 5), and heptanol (200 μM final concentration; n = 6). The first three uncoupling agents listed are glycyrrhetinic acid derivatives and the last two uncoupling agents are higher-order (i.e., long chain) alcohol gap junction inhibitors. At the applied concentrations, all of these uncoupling agents have been demonstrated to selectively block functional gap junction coupling in a reversible manner (Christ et al. 1999; Dean et al. 2001; Ishimatsu and Williams 1996; Travagili et al. 1995; Yamane et al. 2002; also see Rozental et al. 2001). At least 10–15 min was allowed for the uncoupling agent to exert an effect; the ACSF was then returned to normal ACSF (without an uncoupling agent) for 10–20 min, during which time phrenic nerve activity was continuously recorded. In seven experiments, the preparation was continuously perfused for 30–45 min with ACSF containing CBX before returning to normal ACSF; these experiments were conducted to examine the effects of a longer duration of uncoupling of brain stem gap junctions on phrenic nerve discharge.

Control experiments

Control experiments were conducted using glycyrrhizic acid (GZA; 25–100 μM final concentration; n = 7) and hexanol (200 μM final concentration; n = 4), the inactive analogs of glycyrrhetic acid derivatives and higher-order alcohol gap junction inhibitors (i.e., heptanol and octanol), respectively. The same protocol described in the preceding text was employed; however, instead of perfusion with an uncoupling agent, GZA or hexanol was used.

Data analyses

Peak amplitude of integrated phrenic nerve discharge, frequency of phrenic bursts, inspiratory duration (T₁), expiratory duration (Tₑ), duration to peak amplitude (Tₚₑₚₑ), and area of integrated phrenic nerve activity were determined during control conditions, during pharmacological blockade of brain stem gap junctions, and following washout of the uncoupling agent (recovery). Average values were calculated from five consecutive breathing cycles under each condition. Data for the control period were taken just prior to application of the uncoupling agent. Uncoupling data were taken during the 14- to 15-min time point of perfusion with a gap junction blocker and during the 29- to 30-min time point and/or last minute of perfusion with CBX in experiments examining the effects of a longer duration of uncoupling. Recovery data were taken at the end of the recovery period. Amplitude of integrated phrenic nerve discharge, frequency of phrenic bursts, Tₚₑₚₑ (normalized to T₁), and area of integrated phrenic nerve activity are reported as a percentage of the control levels of discharge, which were set at 100% in each preparation.

Spectral composition of phrenic nerve discharge was determined on data obtained from seven preparations before and during perfusion with CBX. Similar analyses were also performed on data obtained from nine control (i.e., GZA and hexanol) experiments. The data used for these analyses were resampled with a sampling rate of 500 Hz,
after digital low-pass filtering (250 Hz, using a 100th-order Hamming window) to avoid aliasing. Each data burst was demeaned to eliminate the power at the zero frequency and normalized by subtracting out the mean and dividing by the SD of the data record to allow for direct comparisons of spectral composition between preparations (and conditions). Thus all analyzed data sets had zero mean and unit variance.

Spectral analyses of phrenic nerve discharge were performed using Blackman and Tukey’s correlograms method (Marple 1987), defined as

\[ S_\omega(\omega) = \sum_{m=-M}^{M} \phi_\omega(m)w(m)e^{-j\omega m} \]

where \( S_\omega \) denotes the spectrum and

\[ w(m) = 0.54 + 0.46 \cos(\pi m/M) \]

is the Hamming window of maximum lag \( M = 128 \). The corresponding correlation function estimate \( \phi_\omega \) was computed over lags \( m = -128 \) to 128, resulting in spectral resolution of 1.953125 Hz. Note that one lag corresponds to 0.002 s. Power spectral density was calculated as an ensemble average derived from analyses of five inspiratory bursts under each condition in each these experiments. Both relative power and area of the spectral peaks were computed; relative power is reported as a percentage of the control. In one preparation, the spectral composition of the power spectral density was calculated as an ensemble average derived from analyses of five inspiratory bursts under each condition in each these experiments. Both relative power and area of the spectral peaks were computed; relative power is reported as a percentage of the control. In one preparation, the spectral composition of the right and left phrenic nerve discharges were compared by computing the percentage of the control. In one preparation, the spectral composition of the right and left phrenic nerve discharges were compared by computing the percentage of the control.

All data are reported as means ± SE. Statistical significance was evaluated using one-way ANOVA with repeated measures and the nonparametric Friedman’s test, followed by Scheffé post hoc analyses for multiple comparisons, as appropriate. Data obtained from control experiments (i.e., GZA, hexanol) were analyzed using a Student’s paired t-test or the paired nonparametric Wilcoxon signed-rank test, as appropriate. The criterion level for determination of statistical significance was set at \( P < 0.05 \) for all experiments.

R E S U L T S

General characteristics of phrenic nerve activity before blockade of gap junctions

Under control conditions (normal ACSF), phrenic nerve discharge was recorded in 48 arterially perfused rat preparations. In all experiments (under these conditions), phrenic nerve discharge exhibited an augmenting or ramp-like discharge pattern, characteristic of the eupneic pattern of discharge observed in vivo. Examples of phrenic nerve discharge under control conditions can be seen in figures throughout the results section (e.g., Figs. 1–3, 5, 7, 8, and 10). In general, these phrenic bursts exhibited little variability in patterning (i.e., peak amplitude, area of integrated phrenic nerve discharge, and rate of rise) and timing (i.e., \( T_I, T_E, \) and \( T_{peak} \)) within a single preparation although some differences in patterning and timing were seen among the preparations (see Fig. 3, A–D, “Control” for an example). Overall, the mean control values for frequency of phrenic bursts, \( T_I, \) and \( T_E \) were \( 13.3 ± 0.6 \) bursts/min, \( 0.96 ± 0.10 \) s, and \( 4.12 ± 0.24 \) s, respectively. These values are similar to those previously reported for this preparation (St. John and Paton 2000; St. John and Rybak 2002; St. John et al. 2002; Wilson et al. 2001).

General effects of blockade of gap junctions on phrenic nerve activity

Phrenic nerve activity was recorded in response to pharmacological blockade of gap junctions in 37 arterially perfused rat preparations. In these experiments, five different uncoupling agents were used. Regardless of the uncoupling agent employed, in 36 of the preparations tested, pharmacological blockade of gap junctions elicited a decrease in the peak amplitude of integrated phrenic nerve discharge and an increase in the frequency of phrenic bursts. In addition, in 27 of the preparations tested, the pattern of phrenic nerve discharge (i.e., shape of the burst) was also modified. Examples of the effects of pharmacological blockade of gap junctions on phrenic nerve discharge can be seen in Fig. 1 for each of the uncoupling agents employed. It should be noted that the effects of pharmacological blockade of gap junctions were bilaterally symmetrical in preparations in which bilateral recordings were made (\( n = 28 \)). Further, in most experiments, these effects were reversible following washout of the uncoupling agent, and both the amplitude and frequency of phrenic bursts returned to control levels within 10–20 min of return to perfusion with normal ACSF (see Fig. 5 for example).

Although five different uncoupling agents were employed in the current study, the magnitude of the effects on peak amplitude of integrated phrenic nerve discharge, frequency of phrenic bursts, and pattern of phrenic nerve discharge differed among the uncoupling agents. In general, blockade of gap junctions by perfusion with ACSF containing CBX (ACSF/CBX) appeared to be the most consistent; therefore most of the experiments were conducted using CBX. In addition, CBX has been used previously to study the effects of gap junction uncoupling on respiratory rhythm generation in transverse medullary slice and isolated brain stem-spinal cord preparations of neonatal rodents (Bou-Flores and Berger 2001; Rekling et al. 2000). A more detailed description of the effects of CBX on phrenic nerve discharge is provided in the following text (see Effects of CBX on phrenic nerve discharge). A brief description of the effects of 18α-GA, 18β-GA, octanol, and heptanol is provided for general comparisons to the effects of CBX.

During blockade of gap junctions by perfusion with ACSF containing either 18α-GA (ACSF/α-GA; \( n = 4 \)) or 18β-GA (ACSF/β-GA; \( n = 3 \)), a high degree of variability in the magnitude of the decrease in peak amplitude of integrated phrenic nerve discharge and in the increase in the frequency of phrenic bursts was observed. To address this variability, three different concentrations (i.e., 25, 50, and 100 \( \mu M \)) of these uncoupling agents were employed; however, the effects on peak amplitude of integrated phrenic nerve discharge and frequency of phrenic bursts appeared to be independent of the concentrations tested. When higher concentrations (i.e., 50–100 \( \mu M \)) were used, however, spurious action potential activity was also observed during and/or following perfusion with ACSF/α-GA or ACSF/β-GA. Overall, perfusion with ACSF/α-GA and ACSF/β-GA decreased the peak amplitude of integrated phrenic nerve discharge to ~40% of control (range = 29–71%) and increased the frequency of phrenic bursts by ~97% above control frequency (range = 42–163%). The effects on frequency resulted from reductions in both \( T_I \) and \( T_E \) which were observed in each of these experiments. Further, changes in the patterning of phrenic nerve discharge were observed in each of these experiments (see Fig. 1, B and C, for examples), and were similar to those observed during perfusion with ACSF/CBX (see Effects of CBX on phrenic nerve discharge).
During blockade of gap junctions by perfusion with ACSF containing octanol (ACSF/OCT; \( n = 5 \)), a marked reduction in the peak amplitude of integrated phrenic nerve discharge and a moderate increase in the frequency of phrenic bursts were observed. The peak amplitude of integrated phrenic nerve discharge decreased to \( \sim 39\% \) of control (range \( = 19–57\% \)) and the frequency of phrenic bursts increased by \( \sim 53\% \) above control frequency (range \( = 31–67\% \)). The effects on frequency resulted predominantly from a reduction in \( T_i \) although there was a small decrease in \( T_i \) in three experiments. Further, changes in the patterning of phrenic nerve discharge were observed in three of these experiments (see Fig. 1D for example) and were similar to those observed during perfusion with ACSF/CBX (see Effects of CBX on phrenic nerve discharge).

The effects of blockade of gap junctions by perfusion with ACSF containing heptanol (ACSF/HEP; \( n = 6 \)) appeared to be less robust than those of the other uncoupling agents. Perfusion with ACSF/HEP produced a decrease in the peak amplitude of integrated phrenic nerve discharge and an increase in the frequency of phrenic bursts in five of the six experiments conducted. In these experiments, the peak amplitude of integrated phrenic nerve discharge decreased to \( \sim 60\% \) of control (range \( = 40–87\% \)) and the frequency of phrenic bursts increased by \( \sim 44\% \) above control frequency (range \( = 41–55\% \)). The effects on frequency resulted from a reduction in \( T_i \), as there was little or no change in \( T_i \) in any of these experiments. Further, changes in the patterning of phrenic nerve discharge were only observed in two of these experiments (see Fig. 1E for example).

Effects of CBX on phrenic nerve discharge

In 19 experiments, blockade of gap junctions was accomplished by perfusion with ACSF/CBX. In each of these experiments, during perfusion with ACSF/CBX, there was a decrease in the peak amplitude of integrated phrenic nerve discharge and an increase in the frequency of phrenic bursts; however, some variability in the magnitude of the amplitude and/or frequency responses among the preparations was observed. Two examples depicting this variability are provided in Fig. 2. In most preparations, during application of CBX, the amplitude was reduced by \( \sim 40–60\% \) of control and the frequency of phrenic bursts increased at least threefold over control levels (Fig. 2B). There was a marked decrease in \( T_i \) in each of the experiments conducted; however, the effects on frequency appeared to be mediated by reductions in both \( T_i \) (\( P < 0.01 \)) and \( T_E \) (\( P < 0.01 \); Fig. 4). It should be noted, that in some cases, little or no change in \( T_i \) was observed. Further, in two experiments, a prolongation of \( T_i \) (i.e., apneusis) was seen (an example is provided in Fig. 3D); in these two experiments, the increase in frequency observed (63 and 83% over baseline) was due solely to a reduction in \( T_E \).

The pattern of phrenic nerve discharge (i.e., shape of the burst) was also modified in 15 of the 19 experiments during perfusion with ACSF/CBX. Under these conditions, the augmenting or ramp-like discharge pattern (seen under control
conditions) was replaced by “bell-shaped” and/or square-wave phrenic bursts. Examples depicting these patterning changes during blockade of gap junctions are provided in Fig. 3 (also see Fig. 1). During perfusion with ACSF/CBX, phrenic nerve discharge reached peak activity earlier in the phrenic burst, resulting in a reduction of \( T_{\text{peak}}/T_1 \) \((P < 0.01; \text{Fig. 4})\). Further, the changes in patterning in conjunction with the reduction in amplitude of integrated phrenic nerve discharge resulted in a decrease in the area of integrated phrenic nerve activity \((P < 0.01; \text{Fig. 4})\). In most cases, the modified phrenic bursts also exhibited a shift in the rate of rise (as demonstrated by the change in \( T_{\text{peak}}/T_1 \)). It should be noted that the abrupt onset of phrenic nerve activity, which preceded the augmenting or ramp-like discharge pattern under control conditions, was unaltered during perfusion with ACSF/CBX. In addition, postinspiratory activity, when encountered, remained unchanged or increased slightly (Fig. 3C). Summary data illustrating the effects of CBX-induced blockade of gap junctions on the timing, amplitude, area, and frequency of phrenic bursts are shown in Fig. 4 for all 19 of these experiments. These summary data also show that the CBX-induced effects of were reversed with washout of CBX (i.e., return to normal ACSF).

In seven of these experiments, perfusion with ACSF/CBX was maintained for 30–45 min before returning to normal ACSF to assess the effects of a longer duration of uncoupling of brain stem gap junctions on phrenic nerve discharge. An example of the data obtained from one of these experiments for 30 min of perfusion with ACSF/CBX is provided in Fig. 5, and summary data illustrating the effects of 30 min of CBX-induced blockade of gap junctions on the timing, amplitude, and frequency of phrenic bursts are shown in Fig. 6 for all seven of these experiments. As described in the preceding text, perfusion with ACSF/CBX decreased the peak amplitude of integrated phrenic nerve discharge and increased frequency of phrenic bursts. This response was maintained for the entire duration of CBX exposure, and no statistically significant differences were observed between the 15- and 30-min measurements for the CBX-induced effects on burst amplitude, frequency, \( T_1 \), or \( T_E \). It should be noted, however, that in four of these experiments (including the example provided in Fig.
the amplitude and frequency effects were slightly more pronounced at the 30-min measurement. Exposure to CBX for 40–45 min was performed in three experiments, and the results were similar at the 40- or 45-min measurement during CBX exposure in these experiments.

In seven experiments, spectral composition of phrenic nerve discharge was also determined before, during, and after perfusion with ACSF/CBX. Under control conditions, spectral analyses revealed peaks in the power spectrum at 40–50 Hz (corresponding to the medium frequency oscillations; MFO) (Cohen et al. 1987; Richardson and Mitchell 1982) and at 90–110 Hz (corresponding to the high-frequency oscillations; HFO) (Cohen et al. 1987); in some cases, a peak was also responding to the medium frequency oscillations; MFO) analyses revealed peaks in the power spectrum at 40 Hz. An example depicting the spectral composition of phrenic nerve activity under control conditions is provided in Fig. 7. Spectral analyses of the right and left phrenic nerve discharges revealed peaks in the power spectrum at common frequencies (Fig. 7B). These peaks were associated with peaks at the same frequencies in cross-spectrum power and in the coherence spectrum between the right and left phrenic nerve discharges (Fig. 7, C and D, respectively). The highest coherence was observed at the 90- to 110-Hz frequency (i.e., squared coherence value of ~0.5), and very low coherence was noted at the 40- to 50-Hz frequency (i.e., squared coherence value of ~0.1). During perfusion with ACSF/CBX, the spectral composition of phrenic nerve discharge was modified. An example demonstrating this modulation of spectral composition during perfusion with ACSF/CBX is provided in Fig. 8. In general, there was a reduction in the power of both the 40- to 50-Hz peak and the 90- to 110-Hz peak and an increase in activity ≥130 Hz. Some variability in the magnitude of the reduction in power of the 40- to 50-Hz peak was observed, and in one experiment, there was a small increase in power at this frequency. In contrast, there was a marked reduction in the power of the 90- to 110-Hz peak in each of these experiments, leading to the elimination of a distinct peak in the power spectrum at this frequency (i.e., HFO activity appeared broadly dispersed). The spectral area associated with the 40- to 50-Hz peak and the 90- to 110-Hz peak was also reduced at both frequencies. It should be noted that although both the power and area of the spectral peaks were reduced, there was no detectable shift in the frequency ranges of either the 40- to 50-Hz peak or the 90- to 110-Hz peak during perfusion with ACSF/CBX. Summary data illustrating the effects of CBX-induced blockade of gap junctions on the power spectral density and spectral area for the 40- to 50-Hz and 90- to 110-Hz peaks are shown in Fig. 9 for all seven of these experiments. These summary data also show that the CBX-induced alterations in spectral composition were (either completely or partially) reversed with washout of CBX (i.e., return to normal ACSF).

Control experiments

Although the applied concentration(s) of each uncoupling agent employed in our current experiments has been previously demonstrated to selectively block gap junction coupling, two series of control experiments were conducted to determine whether any potential nonspecific (i.e., nonjunctional) effects may have contributed to the observed responses. In the first series of control experiments, the preparation was perfused with ACSF containing GZA (ACSF/GZA; n = 7), the inactive analogue of glycyrrhetinic acid derivatives (i.e., CBX, 18α-GA, and 18β-GA). In the second series of control experiments, the preparation was perfused with ACSF containing hexanol (ACSF/HEX; n = 4), an inactive analogue of the higher-order alcohol gap junction inhibitors (i.e., heptanol and octanol). An example of data obtained from a control experiment using ACSF/GZA is provided in Fig. 10A, and summary data illustrating the effects of GZA application on the timing, amplitude, and frequency of phrenic bursts are shown in Fig. 10B for all seven of the GZA control experiments. In contrast to the effects of perfusion with glycyrrhetinic acid derivatives, perfusion with ACSF/GZA was ineffective in altering the ampli-
tude, frequency, or patterning of phrenic nerve discharge (Fig. 10). It should be noted that although no significant effects on phrenic nerve discharge were observed during perfusion with ACSF/GZA, in two of these experiments (using 25 μM GZA and 100 μM GZA), a short-duration (2–3 min) transient increase (15–20% above baseline) in both the peak amplitude of integrated phrenic nerve discharge and frequency of phrenic bursts was seen (not shown). This transient increase was followed by a small reduction (3–12% below baseline) in both amplitude and frequency of phrenic bursts that persisted for the remainder of GZA exposure (10–12 min). In addition, in one of these experiments (using 100 μM GZA), there was an increase in $T_I$. Return to normal ACSF reversed these effects. Similar to the overall effects of GZA, perfusion with ACSF/HEX was also ineffective in altering the amplitude, frequency, or patterning of phrenic nerve discharge in any of these experiments (Fig. 10B).

In nine of the control experiments, spectral composition of phrenic nerve discharge was also determined before and during perfusion with either ACSF/GZA ($n = 5$) or ACSF/HEX ($n = 4$). An example of data obtained from a control experiment using ACSF/GZA is provided in Fig. 11A, and summary data illustrating the effects of perfusion with ACSF/GZA and ACSF/HEX on the power spectral density and spectral area for the 40- to 50-Hz and 90- to 110-Hz peaks are shown in Fig. 11B. In contrast to the modulation of spectral composition...
observed during blockade of gap junctions (i.e., during perfusion with ACSF/CBX), perfusion with ACSF/GZA and ACSF/HEX were ineffective in altering spectral composition. Under these conditions, neither the power nor the spectral area associated with the 40- to 50-Hz peak and the 90- to 110-Hz peak were reduced. Further, no detectable shift in the frequency ranges of either the 40- to 50-Hz peak or the 90- to 110-Hz peak during perfusion with ACSF/GZA and ACSF/HEX was observed.

DISCUSSION

In the present study, we have demonstrated that pharmacological blockade of brain stem gap junctions alters the patterned, timing, and spectral composition of phrenic nerve discharge in adult rat in vitro. The primary effects of gap junction uncoupling included a decrease in the peak amplitude of integrated phrenic nerve discharge, an increase in the frequency of phrenic bursts, a shift from an augmenting (i.e., ramp-like) phrenic burst pattern to a bell-shaped or square-wave phrenic burst pattern, and a reduction in power of the dominant peaks in the frequency domain. The effects on amplitude, frequency, and pattern were consistently observed for each of the uncoupling agents employed in the current investigation, although the magnitude of these effects somewhat varied among the uncoupling agents. Further, the effects on spectral composition were observed in each of the experiments in which these analyses were performed. Taken together, we interpret these findings to indicate that gap junction coupling plays a role in the regulation of respiratory rhythm and pattern and in inspiratory motoneuron synchronization in adult rodents in vitro.

Role of gap junctions in inspiratory motor activity: neonatal versus adult rodents

Our findings are not the first to suggest a role for gap junction communication in the generation or modulation of respiratory rhythm and inspiratory motoneuron synchronization, as two recent investigations conducted using in vitro transverse medullary slice and en bloc brain stem-spinal cord preparations obtained from neonatal mice have addressed this issue (Bou-Flores and Berger 2001; Rekling et al. 2000). These previous investigations reported that bath application (or local perfusion over the preBötzinger complex) of gap junction blockers elicited a reversible reduction in respiratory frequency (Bou-Flores and Berger 2001; Rekling et al. 2000) with little or no effect on the...
Our current understanding of gap junction coupling.

During block-burst amplitude and a shift from an augmenting to a bell-alteration in phrenic burst pattern (i.e., a decrease in phrenic activity) was observed an increase in phrenic burst frequency and a marked alteration in phrenic burst pattern (i.e., an increase in activity $\leq 130$ Hz was also observed.

Patterning (i.e., amplitude or area) of inspiratory bursts recorded from either the hypoglossal and/or phrenic nerve roots (Bou-Flores and Berger 2001), suggesting that gap junction coupling is rhythm-promoting in these preparations. Our current findings in the adult rodent, however, are not consistent with those obtained in these recent reports as we consistently observed an increase in burst frequency and a marked alteration in phrenic pattern (i.e., a decrease in phrenic burst amplitude and a shift from an augmenting to a bell-shaped and/or square-wave phrenic burst pattern) during blockade of gap junction coupling.

Although the exact reason(s) for the discrepancies between our current findings and those of Rekling et al. (2000) and Bou-Flores and Berger (2001) is not known, a number of possibilities exist. Among these are developmental changes associated with both functional gap junction coupling and the structural composition of gap junctions in the respiratory control system, a contribution of neuroanatomical regions present in the arterially perfused adult rat preparation that are not present in medullary slice and en bloc brain stem-spinal cord preparations (e.g., midbrain structures; cerebellum; peripheral chemoreceptors; etc.), the method of application of uncoupling agents employed in these investigations (i.e., arterial perfusion vs. superfusion or local injection), and the animal models used in these studies (i.e., rat vs. mouse). We cannot exclude any of these possibilities; however, we believe that the primary reasons for the discrepancies observed are the developmental differences associated with gap junction neurobiology. For example, both electrotonic and anatomical (dye- and/or tracer-) coupling, which occur via gap junctions, have been demonstrated in multiple respiratory-related brain stem regions between some populations of neurons (and/or astrocytes) in preparations obtained from embryonic and early postnatal rodents (for a recent review, see Solomon and Dean 2002). Similar findings, however, have yet to be demonstrated in these regions in preparations obtained from adult rodents with the exception of the locus coeruleus, which typically requires the addition of BaCl$_2$, tetraethylammonium chloride (TEA), and tetrodotoxin (TTX) to the superfusate (Dean et al. 2001; Ishimatsu and Williams 1996; Travagli et al. 1995). Thus differences in coupling strength between electrically coupled neurons in an oscillatory or rhythmic network (i.e., respiratory network) may account for the differences, at least in part, observed between our current findings in the adult rodent and those of Rekling et al. (2000) and Bou-Flores and Berger (2001) in the early neonatal rodent. In support of this mechanism, computational models of electrically coupled oscillatory and/or nonoscillatory (silent or tonically firing) neurons have demonstrated that coupling strength may play a role in the modulation of oscillatory network behavior, leading to either an increase or a decrease in the frequency of network oscillations (Kepler et al. 1990; Moortgat et al. 2000). Thus electrical coupling via gap junctions may interact with intrinsic conductances to influence the length of the interval between each respiratory burst. Further, developmental regulation of connexin (Cx; the structural proteins that form gap junctions) expression in brain stem regions associated with respiratory control, including the presence and structural composition of gap junctions in the respiratory control system.

FIG. 8. Example demonstrating the effects of pharmacological blockade of brain stem gap junctions using CBX on the spectral composition of phrenic nerve discharge in the in vitro arterially perfused adult rat preparation. Top: original records of single inspiratory bursts recorded under control conditions and during perfusion with ACSF containing CBX. Bottom: spectral composition of phrenic nerve discharge under control conditions (---) and during perfusion with ACSF containing CBX (---). During perfusion with ACSF/CBX, a reduction in power of both the 40- to 50-Hz peak and the 90- to 110-Hz peak was observed. The marked reduction in the 90- to 110-Hz peak resulted in the elimination of a distinct HFO peak, such that activity in this frequency range appeared broadly dispersed. During perfusion with ACSF/CBX, an increase in activity $\leq 130$ Hz was also observed.

FIG. 9. Summary data showing the effects of pharmacological blockade of brain stem gap junctions using CBX on the power spectral density and spectral area of phrenic nerve discharge. During perfusion with ACSF containing CBX, there was a significant reduction in the power of both the 40- to 50-Hz and the 90- to 110-Hz spectral peaks (A) and in the spectral area associated with both of these peaks (B). These CBX-induced alterations in spectral composition were reversed with washout of CBX (i.e., return to normal ACSF). *, a statistically significant difference ($P < 0.05$) from control levels and , a statistically significant difference ($P < 0.05$) between the CBX-induced effect and recovery value.
BoC, has recently been reported (Solomon et al., 2001a,b; also see Solomon and Dean 2002). Because the physiological properties (i.e., gating, permeability/selectivity, etc.) of the gap junction channel are defined by the Cx isoforms incorporated (Bruzzone and Ressot 1997; Kumar and Gilula 1996), gap junction channels composed of different Cx’s may subserve different functions. In addition to electrotonic coupling, for example, gap junctions have been suggested to regulate various signaling pathways via the exchange of second messengers [i.e., Ca^{2+}, cAMP, cGMP, inositol trisphosphate (IP3)], which in turn can affect neuronal excitability and synchronization (Bruzzone and Ressot 1997). The exact contributions of electrotonic and metabolic (i.e., biochemical) coupling in the neonatal versus adult CNS (and in respiratory control), however, have not yet been elucidated, and neither our data nor that of Rekling et al. (2000) and Bou-Flores and Berger (2001) resolves this issue. Further, gap junction coupling is not limited to neurons, and astrocytic gap junctions as well as functional heterocellular coupling between neurons and astrocytes may also be important in the modulation of neuronal excitability (see Solomon and Dean 2002). Thus although the precise functions of gap junctions in respiratory control require further investigation, our current findings in conjunction with the recent work of Rekling et al. (2000) and Bou-Flores and Berger (2001) support a role for gap junction coupling in the regulation of respiratory rhythm in both adult and neonatal rodents in vitro, albeit, the role of gap junction coupling in the regulation of respiratory rhythm appears to be quite different.

**Gap junctions and spectral composition**

Blockade of gap junctions has also recently been shown to modify the spectral composition of hypoglossal and phrenic nerve discharges in medullary slice and en bloc brain stem-cord preparations obtained from neonatal mice, suggesting a possible role for gap junctions in synchronization within an inspiratory burst (Bou-Flores and Berger 2001). Although the effects on temporal domain characteristics during superfu-
sion with gap junction blockers were similar between these two preparations, some differences in modulation of spectral composition were observed between the preparations during superfusion with gap junction blockers. In the medullary slice preparation, the power of the 10- to 20-Hz spectral peak observed in hypoglossal nerve discharge was enhanced during superfusion with gap junction blockers while the power of the 30- to 40-Hz spectral peak also seen in hypoglossal nerve discharge remained unaffected. In contrast, in the en bloc brain stem-spinal cord preparation, the power of both the 10- to 20-Hz spectral peak observed in hypoglossal nerve discharge and the 30- to 40-Hz spectral peak observed in phrenic (not hypoglossal) nerve discharge was enhanced during superfusion with gap junction blockers. Thus these data indicate that gap junctions may reduce the magnitude of neuronal synchronization in these preparations, perhaps by acting as an electrical load on the rhythmic neuronal network (Bou-Flores and Berger 2001).

The spectral composition of phrenic nerve discharge in the arterially perfused adult rat preparation has not previously been described; therefore a brief description (and comparison to the literature) is provided before discussing our findings regarding the effects of gap junction uncoupling on spectral composition. In the current experiments, spectral analyses revealed distinct peaks in the power spectrum at 40–50 Hz (i.e., MFO) (Cohen et al. 1987; Richardson and Mitchell 1982) and at 90–110 Hz (i.e., HFO) (Cohen et al. 1987) in each of the preparations examined; an additional peak was also observed at 130–150 Hz in some preparations. The identification of two distinct peaks is in contrast to the spectral composition previously reported in the anesthetized adult rat in which a single peak located between 45 and 120 Hz (corresponding to the HFO) has been observed in most cases (Kocis and Gyimesi-Pelczer 1997). It should be noted that when double peaks were observed in the (barbiturate- but not urethan-) anesthetized adult rat, the peaks were separated by 45–70 Hz with the lower peak seen between 35 and 78 Hz and the higher peak seen between 97 and 160 Hz (Kocis and Gyimesi-Pelczer 1997). Whether the differences observed in our study reflect the difference between the unanesthetized (i.e., decerebrate) and anesthetized adult rat or whether the differences are due to the lower temperature (i.e., 31°C) in our perfused rat preparation is unclear. The frequencies associated with the spectral peaks observed in phrenic nerve discharge in our current experiments, however, are similar to those previously reported (MFO, 20–50 Hz; HFO, 50–150 Hz) for phrenic nerve discharge (and other inspiratory motor outputs) in other decerebrate adult mammals (e.g., cat) (Cohen et al. 1987; Richardson and Mitchell 1982).

With respect to blockade of gap junctions, our current findings regarding changes in the magnitude of spectral composition are in contrast to those of Bou-Flores and Berger (2001). Although Bou-Flores and Berger (2001) observed a generalized increase in the power of the spectral peaks (including the 30- to 40-Hz spectral peak seen in phrenic nerve discharge) during superfusion with gap junction blockers, in the current...
investigation, blockade of gap junctions elicited a marked reduction in the power of both the 40- to 50-Hz (MFO) and the 90- to 110-Hz (HFO) spectral peaks observed in phrenic nerve discharge and an increase in spurious activity ≥130 Hz. We interpret these findings to suggest that blockade of gap junctions produced an overall reduction or loss of synchronization within an inspiratory burst. This explanation seems reasonable since neuronal gap junctions are proposed to play a role in synchronization of neuronal activity (Bennett 1997; Christie et al. 1989; Jefferys and Haas 1982; Llinás et al. 1974) as well as in the generation of oscillatory neuronal activity and synchronization of spontaneously produced high-frequency oscillations (Bleasell and Pettigrew 1992; Buzański et al. 1992; Drago et al. 1998; Llinás and Yarom 1986). It should be noted, however, that computational models of oscillatory neuronal networks have also shown that although strong electrotonic coupling between neurons synchronizes electrical oscillations between cells, weak electrotonic coupling can phase-lock cells (Moortgat et al. 2000; Sherman and Rinzel 1992); thus the difference between the observed modulation of spectral composition in our current investigation and those of Bou-Flores and Berger (2001) may be explained in the differences in the strength of coupling under the experimental conditions employed.

Although we observed a loss of synchronization within an inspiratory burst, it was accompanied by an increase in respiratory burst frequency in each experiment. We believe that this finding lends additional support to the idea that the mechanisms responsible for the production of MFO and HFO activity, which appear to arise from interactions within motoneuron pools and medullary respiratory neuronal populations, respectively, are separate from the mechanisms by which respiratory rhythm is generated (for a recent review, see Funk and Parkis 2002). These data also provide additional support for separate roles of gap junction coupling in the regulation of respiratory rhythm and the generation of short-time-scale synchrony as proposed by Bou-Flores and Berger (2001). We believe that this loss of synchronization within an inspiratory burst produced, at least in part, the modulation of phrenic burst pattern observed in the current investigation. We cannot distinguish, however, the contribution of gap junction coupling within the (phrenic) motoneuron pool versus medullary inspiratory neurons on the observed patterning changes since gap junction proteins and electrotonic and/or anatomical coupling have been demonstrated in some presumptive respiratory-related (medullary) neurons and spinal (phrenic) motoneurons (Cardone et al. 2002; Dean et al. 1997; Greer et al. 1999; Huang et al. 1997; Solomon et al. 2001a; also see Solomon and Dean 2002) and blockade of gap junction coupling produced a reduction in the spectral peaks associated with both MFO and HFO activity (our current findings).

**Gap junction uncoupling agents**

In the current investigation, a variety of gap junction uncoupling agents were employed to assess the effects of pharmacological blockade of gap junctions on phrenic nerve discharge. The uncoupling agents selected included both glycyrrhetinic acid derivatives and higher-order (i.e., long-chain) alcohol gap junction inhibitors as these types of agents are among those most commonly used and have all been demonstrated to rapidly reduce functional gap junction coupling in various cells (Burt and Spray 1989; Christ et al. 1999; Davidson and Baumgarten 1988; Davidson et al. 1986; Delèze and Hervé, 1983; Johnston et al. 1980; Pappas et al. 1996; Rozental et al. 2001; Weingart and Bukauskas 1998; Yamane et al. 2002) although the precise mechanisms of action are not known (recently reviewed by Rozental et al. 2001). Although each of the uncoupling agents employed similarly modified phrenic nerve discharge (although magnitude differences were noted), it is unclear from the current experiments which respiratory-related brain stem regions were responsible for the modulation observed. The method of application did not specifically target a single respiratory-related brain stem region because gap junction coupling was blocked by arterial perfusion of the preparation with the gap junction uncoupling agents. Thus using this method of application, all gap junctions in brain stem and spinal cord regions implicated in respiratory control (as well as those in the entire preparation) would presumably be uncoupled simultaneously. Further, the gap junction blockers employed were not specific for gap junctions in a particular cell type (i.e., neuron, glia) nor were they specific for gap junctions composed of a specific Cx isoform as there are currently no cell type- or Cx isoform-specific blockers available (Rozental et al. 2001). Previous work from our laboratory has demonstrated neuronal and astrocytic expression of Cx26 and neuronal expression of Cx32 in the preBoC (Solomon et al. 2001b) as well as in putative CO₂-chemosensitive brain stem regions (Solomon et al. 2001a) and presumptive phrenic and hypoglossal motoneurons (Cardone et al. 2002) in both neonatal and adult rat. In addition, Parenti et al. (2000) have reported the presence of the neuron-specific Cx36 (Condorelli et al. 1998; Sohl et al. 1998) mRNA in the nucleus tractus solitarius and preBoC in adult mouse brain, and preliminary data from our laboratory confirm the expression of Cx36 protein in these regions as well as in other brain stem regions implicated in CO₂ chemoreception (Solomon, unpublished observations). It should also be noted that morphological and functional heterocellular coupling between neurons and astrocytes in the locus coeruleus of neonatal rats has been shown (Alvarez-Maubecin et al. 2000). Thus from the current experiments, we cannot distinguish the exact contributions of neuronal versus astrocytic (or heterocellular) gap junctional coupling or the individual Cx isoforms, some of which are also known to form functional heteromeric and/or heterotypic channels (White and Bruzzone 1996), in these regions in the modulation of amplitude, frequency, and patterning of phrenic nerve discharge observed in our experiments.

For each uncoupling agent used, we attempted to minimize the potential for nonspecific effects by selecting concentrations that have previously been demonstrated to be selective for gap junction uncoupling with little or no effects on nonjunctional parameters. It should be noted, however, that in some cells, similar concentrations of glycyrrhetinic acid derivatives have been shown to exert adverse nonjunctional effects, including cytotoxic cell damage (see Rozental et al. 2001). Further, in a recent report, Rekling et al. (2000) found that ≥20–30 min of exposure to 100 μM CBX reduced input resistance as well as the number of action potentials elicited by depolarizing current in presumptive rhythmogenic type-1 preBoC neurons of neonatal mice. In contrast to this report, exposure to 100 μM CBX has also been demonstrated to be ineffective in altering the
resting conductance, action potential waveform, spontaneous firing, and evoked action potentials in locus coeruleus neurons of both neonatal and adult rats (Dean et al. 2001; Ishimatsu and Williams 1996; Travaglì et al. 1995). We believe that the results of our experiments were mediated by blockade of gap junctions and not by nonspecific effects because both glycyrrhetinic acid derivatives and higher-order alcohol gap junction inhibitors (i.e., 2 classes of chemically distinct gap junction blockers) elicited similar modulation of phrenic nerve discharge, the concentrations of the gap junction blockers used were generally lower than those known to cause nonspecific effects, and the control agents (i.e., GZA and hexanol) employed were ineffective in altering phrenic nerve discharge in a manner similar to that observed by application of uncoupling agents. Further, in our experiments, the effects of all uncoupling agents used were reversible even in experiments in which a longer duration of exposure (i.e., 30–45 min) to CBX was employed, which we interpret to suggest that cytotoxic cell damage was minimal or absent.

Although multiple uncoupling agents were used, the effects of these agents on phrenic nerve discharge were somewhat different in magnitude. This might be expected because inhibition of gap junction coupling by these agents is not identical and in some cases, only partially reduces channel conductance (reviewed by Rozental et al. 2001). Glycyrrhetinic acid derivatives, for example, have been shown to functionally block ≤80% of electrical coupling (Davidson and Baumgarten 1988; Davidson et al. 1986; Goldberg et al. 1996; Guan et al. 1996; Rozental et al. 2001; Yamamoto et al. 1998) even though dye-coupling may be completely eliminated (Davidson and Baumgarten 1988; Davidson et al. 1986; Martin et al. 1991; also see Rozental et al. 2001). Additionally, CBX appears to be a more potent blocker of gap junction coupling than the other glycyrrhetinic acid derivatives (Martin et al. 1991; Rozental et al. 2001). In contrast, both octanol and heptanol (at concentrations of 1–3 mM) have been reported to reversibly reduce junctional conductance to zero (i.e., completely close the channel) (Burt and Spray 1989; Rozental et al. 2001); however, the high concentrations of these agents (i.e., 1–3 mM) have also been shown to lack specificity and exert nonspecific adverse effects on neuronal excitability independent of the effects on gap junction coupling (Christ et al. 1999; Rekling et al. 2000).

In our experiments, the effects of heptanol were the least robust, which we attribute to the concentration used in our study (i.e., 200 μM vs. 1–3 mM used in some other investigations). Although the higher-order alcohol gap junction inhibitors can be more effective in inhibiting functional gap junction coupling than the glycyrrhetinic acid derivatives, the potency (i.e., concentration corresponding to half-maximal effectiveness) of glycyrrhetinic acid derivatives in inhibiting gap junction permeability has been reported to be much higher than that of the higher-order alcohol gap junction inhibitors (see Rozental et al. 2001). Based on the previously described characteristics of these uncoupling agents, the magnitude of the effects on amplitude, frequency, and pattern of phrenic bursts observed in our experiments appear to be consistent with what would be predicted in that, at the applied concentrations (which were selected to minimize the potential for nonspecific effects), the glycyrrhetinic acid derivatives appeared to elicit a more pronounced modulation of phrenic nerve discharge than that seen with the higher-order alcohol gap junction inhibitors.

Conclusions

It is clear from our current findings and the recent work of Rekling and co-workers (2000) and Bou-Flores and Berger (2001) that gap junction coupling plays a role in the regulation of respiratory rhythm as well as in inspiratory motoneuron synchronization in both adult and neonatal rodents in vitro. Although the precise functions of gap junctions in respiratory rhythm generation, pattern formation, and inspiratory motoneuron synchronization require further investigation, gap junction coupling appears to play a role in establishing (or maintaining) the frequency, pattern, amplitude, and spectral composition of phrenic nerve discharge, although the role of gap junction coupling in this regulation appears to be different between neonatal and adult rodents in vitro.

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