Modulation of phrenic motoneuron excitability by ATP: consequences for respiratory-related output in vitro

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Miles, Gareth B., Marjorie A. Parkis, Janusz Lipski, and Gregory D. Funk. Modulation of phrenic motoneuron excitability by ATP: consequences for respiratory-related output in vitro. J Appl Physiol 92: 1899–1910, 2002. First published December 21, 2001; 10.1152/japplphysiol.00475.2001.—On the basis of the high level of P2X receptor expression found in phrenic motoneurons (MN) in rats (Kanjhan et al., J Comp Neurol 407: 11–32, 1999) and potentiation of hypoglossal MN inspiratory activity by ATP (Funk et al., J Neurosci 17: 6325–6337, 1997), we tested the hypothesis that ATP receptor activation also modulates phrenic MN activity. This question was examined in rhythmically active brain stem-spinal cord preparations from neonatal rats by monitoring effects of ATP on the activity of spinal C4 nerve roots and phrenic MNs. ATP produced a rapid-onset, dose-dependent inhibition associated with hydrolysis of ATP to adenosine (11, 21), which, through activation of pre- (3, 10, 31) and postsynaptic (49) P1 receptors (A1 adenosine receptors) has widespread inhibitory actions.

Our understanding of the properties of purinergic receptors in expression systems is growing considerably. However, unravelling the physiological significance of ATP signaling in the CNS lags behind. A role for ATP in synaptic transmission and modulation of synaptic activity is best established in sensory systems (9, 59). The ubiquitous expression of P2 receptors in motoneurons (MNs) (7, 23) suggests an important role for ATP in controlling motor activity, but physiological evidence is limited. In Xenopus, an interaction between the excitatory actions of ATP and the inhibitory actions of adenosine has been implicated in control of episodic motor patterns (8). In mammalian CNS, ATP receptors are distributed throughout respiratory regions of the ventrolateral medulla (63), and their activation modulates the activity of some respiratory neurons. In addition, by virtue of the pH sensitivity of the P2X2 subunit, ATP receptors may contribute to the central chemosensitivity of the respiratory motor network (55, 58). At the level of motor outflow, evidence is limited to the ATP-mediated excitation of hypoglossal (XII) MNs and potentiation of XII nerve inspiratory activity (15).

To further investigate the role of ATP acting at P2 receptors in controlling motor outputs, this study examined its effects on the activity of phrenic MNs and their inspiratory-related output in rhythmically active brain stem-spinal cord preparations from neonatal rats. We focussed on phrenic MNs, which innervate the

ADENOSINE-5'-TRIPHOSPHATE (ATP) acts on neurons through activation of two classes of purinergic receptors designated P2X and P2Y. P2X receptors, comprising seven subtypes (P2X1–7) and several splice variants, are ionotrophic, ligand-gated ion channels that mediate cation-selective inward currents involved in fast neurotransmission (25). P2Y receptors, comprising at least 11 subtypes (P2Y1–11), are G protein-coupled receptors that signal through slower second messenger systems (25). After initial observations that ATP contributes to fast synaptic transmission at some central synapses (12, 13), interest in the role of extracellular ATP in signaling within the central nervous system (CNS) has grown almost exponentially. ATP receptors are widely distributed throughout the CNS (7, 23, 50, 61, 63), and due to receptor diversity, their activation produces a variety of effects. Purinergic signaling is further complicated by the fact that ATP signaling is normally associated with rapid hydrolysis of ATP to adenosine (11, 21), which, through activation of pre- (3, 10, 31) and postsynaptic (49) P1 receptors (A1 adenosine receptors) has widespread inhibitory actions.

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main inspiratory pump muscle, the diaphragm, because purines acting at presynaptic P1 receptors profoundly inhibit glutamatergic inspiratory inputs to these MNs (10), but the effects of purines at P2 receptors are not known. In addition, this analysis allowed comparison of the effects of ATP on phrenic MNs with those previously described by our laboratory on XII MNs (15). Cervical spinal MNs and XII MNs express different P2X transcripts (7). Thus differential modulation of MNs controlling pump and airway muscles by ATP could lead to a mismatch in inspiratory output of these two pools and contribute to the pathology of sleep-related disorders of breathing (19). This work has previously been presented in abstract form (44).

METHODS

Electrophysiology

Brain stem-spinal cord preparation. Experiments were performed on brain stem-spinal cord preparations from neonatal Wistar rats ranging in age from postnatal day 0 to day 3 (P0 to P3, n = 39). Preparations were produced by using methods described previously (43, 52). Briefly, animals were anesthetized with ether and decerebrated. The brain stem-spinal cord was isolated in artificial cerebrospinal fluid (aCSF) containing (in mM) 120 NaCl, 3 KCl, 1.0 CaCl2, 2.0 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, and 20 d-glucose, pH 7.4, equilibrated with 95% O2-5% CO2 at 20–22°C. Preparations extended from the caudal-ponine level rostrally to approximately the 7th cervical segment caudally. Once dissected free, the preparation was pinned down with the ventral surface up on Sylgard resin in a recording chamber perfused with aCSF. The volume of the recording chamber was 10 ml for C4 nerve root recording experiments (flow rate of ~15 ml/min) and 2 ml for whole cell recording experiments (2 ml/min). After the removal of dura mater, pia mater was scraped from the ventral surface of the spinal cord just lateral to midline at the level of the C4 roots to facilitate drug diffusion and to allow access for patch pipettes. Bath temperature was gradually increased to 26–27°C before recording.

Nerve root recordings. Inspiratory MN activity was recorded from severed ends of C4 roots by using suction electrodes (80- to 100-μm internal diameter). Signals were amplified, band-pass filtered (100 Hz to 3 kHz), full-wave rectified, integrated by using a leaky integrator (τ = 25 ms), and displayed on a chart recorder and an oscilloscope. Signals were also recorded on videotape via pulse code modulation (Vetter model 402 or 3000A) for storage and off-line analysis.

Whole cell recording. Intracellular recordings from phrenic MNs (n = 22) were made with the blind whole cell patch-clamp recording technique (4). Patch pipettes (4.0–5.5 MΩ; 1.5- to 2-μm tip size) were pulled on a horizontal puller (Sutter P-97) from filamented borosilicate glass (Clark/WPI; 1.2-mm outside diameter) and filled with solution containing (in mM) 125 potassium-glucuronate, 5 NaCl, 1 CaCl2, 10 HEPES, 10 BAPTA, and 2 ATP (Mg2+ salt). Intracellular solution pH was adjusted to 7.3 with 5 M KOH. Signals were amplified and filtered with a patch-clamp amplifier (low-pass 5-kHz Bessel filter, Axopatch 1D, Axon Instruments).

Series resistance and whole cell capacitance were estimated under voltage-clamp conditions by using short voltage pulses (100 Hz, ~10 mV, 3.0 ms). Series resistance (mean of 15 ± 5 MΩ) was monitored throughout the experiment. Data were discarded if they changed by >5% between control and test conditions. Voltage-current (V-I) relationships were obtained in current clamp by applying a series of current steps (500 ms, +50 to −300 pA). In voltage clamp, current-voltage (I-V) relationships were obtained by applying a series of 5-mV voltage steps (+15 to −35 mV from resting membrane potential). Cell input resistance (RIN) was calculated from the slope or the inverse of the slope of a least-squares regression line fitted to V-I or I-V curves, respectively.

Neurons included in the database satisfied the criteria previously described for phrenic MNs (10, 34). Briefly, they had resting membrane potentials of at least −60 mV, received rhythmic synaptic drive currents/potentials in phase with inspiratory burst activity recorded from C1 or C4 ventral roots, produced action potentials that overshot 0 mV, and were in the C4 segment 110–260 μm below the ventral surface.

Drug application. Effects of P2X receptor agonists and antagonists on C4 root output were investigated by locally applying drugs over the phrenic MN column by pressure injection (controlled by solenoid valves) from triple-barreled pipettes. Drugs used included ATP disodium salt (ATP-Na+, Sigma Chemical), ATP Mg2+ salt (ATP-Mg2+, Sigma Chemical), adenosine-5′-O-(3-thiotriophosphate) (ATP-S, hydrolysis resistant P2 receptor agonist; Boehringer Mannheim), α,β-methylene ATP [P2X1,3 receptor agonist (7); Sigma Chemical], GABA (Sigma Chemical), pyridoaz-phosphate-6-azophenyl-2,4-disulfonic acid [PPADS, P2X1,3 receptor antagonist (7); RBI], and suramin hexaamide (general P2 receptor antagonist; among P2X receptors, it affects P2X1,3,5 (7); RBI).

All drugs were dissolved in standard extracellular solution. Osmolarities of containing solutions (1.0 mM, 309 ± 2 mosM; 10 mM, 312 ± 2 mosM), measured with a vapor pressure osmometer (Wescor), were not significantly greater than extracellular solution (vehicle, 306 ± 2 mosM).

The phrenic MN column extends rostrocaudally from the C3 to C5 segments but is very narrow (~100 μm) (32). Thus, to establish correct positioning of the ejection pipette over the phrenic nucleus, P2X receptor agonist/antagonist applications were preceded by injections of 1 mM GABA (60 s). Significant inhibitory effects of GABA on C4 inspiratory burst amplitude have previously been demonstrated (43, 56), and a reduction in burst amplitude of >50% was used as the criterion that the injection pipette was positioned correctly. Movement of the injection pipette 50 μm lateral from this point was typically sufficient to abolish the response. P2X receptor agonists (ATP: 1–10 mM; ATP·S: 10 mM) were applied for 60 s. For experiments investigating antagonism by the general P2 receptor antagonist suramin (100 μM) or the P2X selective antagonist PPADS (50 μM), 2-min local applications of antagonists preceded 60-s applications of 5 mM ATP.

Similar procedures were used for application of drugs during whole cell recording experiments, with the exception that the injection pipette was placed as close as possible to the site where the patch electrode entered the spinal cord (i.e., choice of injection site was not based on inhibition of C4 output by GABA). Experiments designed to test the effects of ATP on repetitive firing behavior of phrenic MNs required prolonged application of ATP (2–5 min).

A minimum of 15 min was allowed between consecutive, 60-s agonist applications. Preliminary experiments established that this delay ensured response reproducibility. Shorter recovery periods were often associated with response attenuation, perhaps reflecting incomplete recovery from receptor desensitization. For experiments designed to examine
repetitive firing behavior that required longer agonist application, a single trial was performed.

**Data analysis.** Effects of applied drugs on peak amplitude of integrated C4 inspiratory bursts were assessed with custom-written LabVIEW acquisition and analysis protocols. Response time course was calculated by averaging inspiratory burst amplitudes in 1-min bins before drug applications, in 30-s bins for the first 2 min after drug onset, in 1-min bins for the next 4 min, and in 2-min bins for the following 8 min. In order not to overestimate the effects of ATP on inspiratory burst amplitude, measurements were corrected for shifts in baseline associated with increased tonic discharge (51).

A separate analysis procedure was used to assess the maximum potentiating and inhibitory effects of ATP on burst amplitude. The short duration of the peak responses made it unlikely that the largest (or smallest) bursts would all fall within the same 30-s time bin. The time of peak response relative to drug onset was also likely to vary between animals due to small differences in pipette position relative to the phrenic MN column. Thus the time binning procedure used to calculate average time course was likely to underestimate the peak magnitude of these responses. Maximum potentiation and maximum inhibition of burst amplitude were therefore calculated relative to control from a moving average of the burst amplitude of five consecutive bursts. Maximum potentiation was taken as the largest value occurring within 2 min of the onset of drug application. Maximum inhibition was taken as the lowest value occurring up to 7 min after the onset of drug application.

To investigate the effects of ATP on inspiratory synaptic currents recorded in phrenic MNs, the total charge transfer per individual inspiratory cycle was measured and averaged before, during, and after drug application by integrating the current associated with each consecutive inspiratory cycle (AxoGraph 4.4 software). Response time course for changes in total charge transfer per inspiratory cycle was calculated as described above for calculating time course of changes in inspiratory burst amplitude. Maximum potentiating effects of ATP on inspiratory synaptic inputs were assessed by comparing currents averaged from three consecutive inputs in the control period, to those averaged over the first three bursts occurring immediately after the application of ATP. Initial whole cell recording experiments indicated that the reduction in inspiratory synaptic current occurred more rapidly than the reduction in C4 root output. Thus inspiratory currents occurring after this brief, three-cycle window were excluded due to the potentially confounding effects of adenosine. Maximum inhibition of synaptic currents during the post-ATP period was calculated from a moving average of three consecutive synaptic currents between 1 and 5 min after the onset of drug application.

The effects of ATP on repetitive firing of phrenic MNs were investigated by comparing steady-state firing frequencies before, during, and after drug applications. Steady-state firing frequencies were calculated from the last 400 ms of 1-s current steps (at the time when most spike frequency adaptation had occurred).

For comparison between experiments, parameters are reported relative to preinjection levels as means ± SE. Analysis of variance was performed on response time courses for C4 root recording experiments using raw data. Differences were identified with mutually orthogonal contrast coefficients to partition the treatment sum of squares. All other statistical analyses were performed on raw data or normalised data after arc sine transformation, and differences in means were tested with Student t-tests. Values of $P < 0.05$ were assumed significant.

**RESULTS**

Effects of ATP on C4 Inspiratory-Related Nerve Output Are Biphasic

To test the effects of ATP on inspiratory-related activity recorded from C4 roots, ATP was applied locally over the phrenic MN column. The C4 root response was typically composed of 1) tonic excitation, 2) potentiation of inspiratory burst amplitude, and 3) a post-ATP decrease in burst amplitude. Contralateral root output was never affected.

The increase in tonic activity was apparent as the thickening of the baseline in the raw C4 root recording (Fig. 1A) and the rise in the baseline of the integrated signal (Fig. 1B). Both raw and integrated traces demonstrated a rapid rise in tonic activity within 5 s of application of 10 mM ATP. Tonic activity peaked within 30 s of the 60-s application before steadily declining to baseline level. The decline in tonic root activity in the continued presence of drug was suggestive of receptor desensitization. Tonic discharge typically disappeared 80 s after the onset of 10 mM ATP applications. It was not apparent during applications of 1 mM ATP.

The potentiation of inspiratory burst amplitude was superimposed on the increase in tonic root activity and was most apparent in the rectified, integrated recording of C4 root output (Fig. 1B). Applications of both 1 and 10 mM ATP caused significant increases ($11 \pm 2.9$ and $22 \pm 7.3\%$, respectively) in inspiratory burst amplitude (Fig. 1C). The maximum potentiation typically occurred between 30 and 60 s after the onset of drug application. Burst amplitude was potentiated for ~30 s after application of 1 mM ATP compared with 90 s after 10 mM ATP.

Potentiation was followed by a significant post-ATP decrease in burst amplitude ($10 \pm 5.0\%$) that peaked 5

**Immunohistochemistry**

To retrogradely label MNs (1, 37), neonatal rats (P3 and P7, n = 2) received intraperitoneal injections of 0.04 mg/g body wt of Fluoro-Gold. One to two days after injection, rat pups were anesthetized with ether and decerebrated, and the brain stem-spinal cords were isolated in oxygenated aCSF before fixing the tissue in 5% formaldehyde solution (4°C for 16 h). The tissue was cryoprotected in 10 and 30% sucrose PBS solutions (4°C for 12 h each). The C4 segment was then isolated, frozen in an embedding medium, and sectioned at 30 μm with the use of a cryostat. Sections were processed for P2X2 receptor immunoreactivity by sequential incubation in 1.5% goat serum for 40 min at room temperature, P2X2-Rab anti serum (1:100; Refs. 23, 24) at 4°C for 16 h, and Alexa 568-conjugated goat anti-rabbit secondary antibody (1:800) at 4°C for 5–6 h. Sections were wet mounted by using Citifluor medium (Alltech). Aside from the initial incubation in goat serum, sections underwent three consecutive 5-min washes in 0.1 M PBS between each incubation step. To assess the specificity of labeling, control sections were concurrently processed in the absence of primary antibody. Sections lacking primary antibody were devoid of labeling. Images were obtained by using standard epifluorescence microscopy (Leica filter blocks D for Fluoro-Gold and N2 for Alexa 568).

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The post-ATP inhibitory effect was not induced by ATPγS. Burst amplitude gradually fell to, but never below, control levels.

To confirm the involvement of P2 receptors in mediating the effects of ATP on inspiratory-related output, ATP applications were preceded by 2-min local applications of the nonselective P2 antagonist, suramin, or the P2X receptor antagonist PPADS (Fig. 3). The inhibitory effects of both antagonists on responses to 5 mM ATP are shown in Fig. 3, A and C. Pooled data indicate that local application of suramin (100 μM) over the phrenic MN column significantly reduced the maximum potentiation of burst amplitude caused by 5 mM ATP from 30 ± 1.7% under control conditions to 11 ± 3.0% (Fig. 3B, n = 3). ATP responses did not return to control levels after suramin washout. PPADS (50 μM) significantly reduced responses to 5 mM ATP from a maximum potentiation of 28 ± 5.5% under control conditions to 12 ± 6.0% (Fig. 3D, n = 4). Again, responses did not return to control levels after washout of PPADS. This did not reflect a simple rundown or desensitization of the actions of ATP because consecu-

Fig. 1. A: bilateral recording of left (L) and right (R) C4 root activity from a rat brain stem-spinal cord preparation showing effects of 10 mM ATP locally applied over the left phrenic motoneuron (MN) column for 60 s. B: rectified and integrated signal of the raw data represented in A, demonstrating tonic excitation, inspiratory burst amplitude potentiation, and delayed inhibitory effects of ATP on C4 output (note different time scales in A and B). C: time course of changes in C4 root inspiratory burst amplitude in response to 60-s applications of 1 (○) and 10 mM (▲) ATP to the phrenic MN column. Values are means ± SE (n = 5). *Significantly different from control values.

Fig. 2. A: integrated C4 root output during a 60-s application of 10 mM ATP-γS over the phrenic MN column. B: time course of changes in C4 root inspiratory burst amplitude produced by 60-s local applications of 10 mM ATP or 10 mM ATP-γS to the phrenic MN column (n = 5). C: maximum potentiation or inhibition of C4 inspiratory burst amplitude 1 and 5 min after 60-s applications of 10 mM ATP or 10 mM ATP-γS. Values are means ± SE. *Significantly different from control values.
tive control applications at 15-min intervals produced consistent responses for up to 3 h (longest period tested; data not shown).

**Multiple Effects of ATP on Individual Phrenic MNs**

**Postsynaptic effects.** Whole cell recordings from individual phrenic MNs were used to further investigate the mechanisms by which ATP modulates inspiratory output. At resting membrane potential (−64 ± 4 mV), 10 mM ATP induced reversible (Fig. 4A), suramin-sensitive (Fig. 4B) inward currents (57 ± 39 pA, n = 15) or membrane depolarization (3.6 ± 1.1 mV, n = 11, see Fig. 6A) in all cells tested. Note that to increase speed of antagonism, the concentration of suramin used in the whole cell experiments (1 mM) was 10-fold higher than in the extracellular experiments (Fig. 3). At these higher concentrations and longer applications, suramin reversibly inhibited inspiratory synaptic inputs, consistent with the inhibitory effects of suramin on glutamatergic synaptic transmission at high concentration (40, 57). ATP-induced currents (n = 3) and depolarization (n = 1) were not blocked by bath application of 0.5 μM TTX (not illustrated). Action potential block was confirmed in all phrenic MNs before drug application. The P2X1,3 agonist α,β-methylene ATP (10 mM) also induced inward currents (51 ± 34 pA, n = 2; Fig. 4C). The reversal potentials of ATP-induced currents were highly variable, showing little or no voltage dependence in 10 of 15 phrenic MNs. In these 10 neurons, current magnitude varied little with changing membrane potential between approximately −95 and −45 mV. In the remaining 5 of 15 MNs, currents reversed between −78 and −125 mV, and one reversed at +15 mV. Changes in R_N were inconsistent and typically <10% (Fig. 4Ab). Increases >10% were observed in only 3 of 15 phrenic MNs. Averaged across all phrenic MNs, effects of ATP on R_N were not significant.

To verify postsynaptic receptor localization, we examined the distribution of P2X2 receptor protein in the region of the phrenic nucleus. The P2X2 subunit was chosen because PPADS, which acts at P2X1,2,3,5 receptors (7) attenuated the effects of ATP on C4 root output (Fig. 3), and a previous immunohistochemical study has demonstrated the presence of P2X2 receptor subunits in spinal MNs of the adult rat (23). We identified large cells in the medial (presumptive phrenic MNs) and lateral (presumptive shoulder MNs) (32) motor columns in the ventral horn of the C4 segment of the spinal cord as MNs by retrograde labeling with Fluoro-Gold (Fig. 5A). Both clusters of MNs showed labeling for the P2X2 subunit. Presumptive phrenic MNs from the medial group are shown (Fig. 5B).

To examine the effects of ATP on phrenic MN excitability and to test the hypothesis that increases in excitability contribute to ATP-mediated potentiation of phrenic MN output, the effects of this drug on repetitive firing under current clamp mode were examined using a protocol similar to that illustrated in Fig. 6, A and B. A steady-state level of ATP-mediated depolarization was established, and a small hyperpolarizing current was injected to return membrane potential to control levels. A series of variable amplitude depolarizing current pulses (1 s) was injected to elicit repetitive firing (Fig. 6B). Alternatively, short (200 ms) hyperpolarizing current pulses were injected to monitor
Injection of pulses was triggered with a 2-s delay from inspiratory bursts recorded from the ventral root to prevent overlap of inspiratory synaptic inputs and injected current pulses. ATP increased repetitive firing in response to injected current in four out of five cells, as shown by the response of a single phrenic MN to a current pulse before and during ATP application (Fig. 6B) and by the leftward shift in the plot of steady-state firing frequency vs. injected current in the same MN (Fig. 6Ca). Two cells showed responses similar to that depicted in Fig. 6Ca, whereas the remaining two cells showed responses similar to that depicted in Fig. 6Cb. This increase in MN excitability was not simply due to membrane depolarization because direct current was injected to offset the ATP-induced depolarization during all repetitive firing protocols. It also did not reflect an increase in neuronal $R_N$ because $R_N$ did not change significantly in the four cells that showed increased responses to depolarizing current pulses during ATP application.

**Modulation of inspiratory synaptic drive to phrenic MNs.** We next tested the hypothesis that ATP-mediated potentiation of glutamatergic inspiratory synaptic currents contributed to the increase in inspiratory burst amplitude recorded from the C4 root. In contrast to the effects of ATP on C4 burst amplitude, where a potentiation preceded an inhibition, ATP potentiation of inspiratory synaptic currents was never observed. In fact, a rapid-onset inhibition was seen. In some cells, this was apparent as a reduction in peak inspiratory current (Fig. 7A). In others, although a reduction in
peak current was minimal (Fig. 4A), the total charge transfer per inspiratory cycle was significantly reduced. Plots showing the time course of the ATP effect on total charge transfer per inspiratory cycle averaged over 30-s time bins revealed a maximum inhibition of 32 ± 11% (Fig. 7C; n = 7) that occurred between 1 and 2 min after the onset of ATP application (Fig. 7B). To ensure that the lack of an excitatory effect of ATP on inspiratory synaptic currents did not simply reflect the 30-s binning procedure, the charge transfer of the first three cycles that occurred immediately after the onset of ATP application was analyzed. By focussing on the first three cycles after ATP application, the intent was to assess changes in current before significant hydrolysis of ATP to adenosine could obscure any potentiating effect. This additional analysis, however, failed to reveal any ATP-mediated potentiation of inspiratory drive (Fig. 7C).

Thus the effects of ATP on C4 root inspiratory burst amplitude and inspiratory synaptic currents differed in two major ways. First, synaptic currents were not potentiated. Second, the inhibition of synaptic currents was much faster in onset than the inhibition of C4 burst amplitude. The rapid onset of the inhibition suggested that it was not dependent on ATP hydrolysis. To test this hypothesis, we compared the effects of ATP and ATPγS on inspiratory synaptic currents. In contrast to responses in C4 root, ATPγS was as effective as ATP in inhibiting inspiratory synaptic currents (Fig. 7D; n = 2). To test whether this might reflect direct activation of presynaptic P2Y receptors, as seen in hippocampal pyramidal neurons where ATP and ATPγS are similarly effective and more potent than α,β-methylene ATP (36), the effects of these three compounds on inspiratory synaptic currents in phrenic MNs were compared. In two cells to which all three drugs were applied, ATPγS and ATP caused similar reductions in total charge transfer per inspiratory cycle, whereas α,β-methylene ATP produced a much smaller current reduction in one and was without significant effect in the other (Fig. 7D).

**DISCUSSION**

ATP receptors are a diverse family of ligand-gated ion channels that are widely distributed throughout the nervous system. Their role in modulating motor outflow is poorly understood and in mammals is currently limited to analysis of vagal (39) and hypoglossal MNs (15). In this study, we describe a biphasic response to ATP similar to that observed in hypoglossal MNs, reflecting the diverse actions of ATP on membrane properties and inspiratory activity. An initial excitatory phase, comprising a tonic component and a potentiation of inspiratory burst amplitude, lasted 1–2 min and likely reflects the depolarizing actions of ATP on phrenic MNs and its potentiation of repetitive firing. A secondary inhibition followed that appears to be mediated by two mechanisms; a P2 receptor-mediated inhibition and a slower, possibly P1 receptor-mediated inhibition resulting from hydrolysis of ATP to adenosine. These results demonstrate not only the potential importance of ATP and its receptors in modulating inspiratory outflow but the importance of the balance between ATP and its catabolites in controlling phrenic MN excitability.
Mechanisms of ATP-Induced Modulation of C4 Root Activity

Tonic excitation. The rapid increase in tonic discharge evoked from the C4 root by ATP clearly demonstrated the excitatory nature of ATP on spinal MNs. Several pieces of evidence suggest that this effect is due to activation of postsynaptic P2 receptors and phrenic MN depolarization. Not only did MNs in the C4 segment of the spinal cord show immunolabeling for the P2X2 receptor, the increase in tonic discharge was sensitive to suramin and PPADS. In addition, the ATP-induced inward current or depolarization was sensitive to suramin and persisted in the presence of TTX. The ionic basis of this depolarization was not examined. Activation by ATP of a nonselective cationic conductance is likely to have contributed, as this action has been observed in a variety of neuron types (41), including hypoglossal MNs (15). Reduction of a voltage-gated K+ current by ATP, as seen in spinal MNs and pre-MNs of Xenopus (8), may also have contributed to the depolarization. We found both positive and negative reversal potentials for ATP-induced currents and inconsistent changes in R_N. One interpretation of the variability is that both ionic mechanisms are operating, but to varying degrees, in individual phrenic MNs, i.e., there may be differential expression of P2 receptor subtypes in phrenic MNs. However, it is also possible that the varied responses and the observation in some phrenic MNs that ATP-current amplitude did not change linearly with voltage reflect a site of current generation that is remote from the recording site and under poor voltage control.

Amplitude potentiation. We initially hypothesized that at least three mechanisms would contribute to the ATP-mediated potentiation of C4 inspiratory burst amplitude including 1) direct phrenic MN depolarization, 2) increased excitability of these MNs, and 3) potentiation of inspiratory synaptic currents. Just as direct ATP-mediated MN depolarization will have contributed to the tonic component of the response, it is also a likely mechanism contributing to the increase in inspiratory burst amplitude. ATP depolarized all phrenic MNs tested, which would bring them closer to firing threshold and result in a larger output for the same inspiratory input. The observation that ATP-mediated elevation of phrenic MN firing remained when membrane depolarization was offset via injection of direct current suggests that the potentiation of inspiratory output by ATP is also due to increased MN excitability (Fig. 6).

Fig. 7. A, top: whole cell recording of membrane current (I) in a phrenic MN in voltage clamp. After application of ATP (10 mM), there is a reduction in the amplitude of inspiratory currents. Top dashed line, level of holding current in control; lower dashed line, average amplitude of inspiratory currents during the control period. Bottom: fast time scale traces of inspiratory synaptic currents shown in the top trace, illustrating single inspiratory currents during the control period and after application of ATP. B: time course of the effects of ATP (10 mM) on total charge transfer (relative to control) of each inspiratory current (n = 7). C: total charge transfer per inspiratory current in control, during the first three bursts after the onset of 10 mM ATP application, and during the period of maximum inhibition of inspiratory currents (n = 7). D: time course of the effects of ATP (●), ATP-γ-S (○), and α,β-methylene ATP (●) (all 10 mM, 60-s applications) on total charge transfer (relative to control) of inspiratory currents. Values are means ± SE. *Significant difference.
This increased excitability may result from closure of channels (e.g., K⁺ channels) and increased \( R_N \) in response to ATP. However, \( R_N \), as measured via injection of current or voltage steps at the soma, did not change significantly in MNs that showed increased excitability. It is possible, therefore, that ATP-mediated changes in \( R_N \) occurred in dendritic processes and were not detected at the soma. It is also possible that ATP increased excitability through modulation of currents important in determining phrenic MN repetitive firing properties. Candidates include outward rectifier, A-type and Ca\(^{2+}\)-activated K⁺ currents that regulate the repetitive firing behavior of perinatal phrenic MNs (35). Evidence for ATP-mediated modulation of K⁺ conductances involved in repetitive firing responses is limited to sympathetic neurons where firing rates increase in response to UTP (14), likely through activation of P2Y\(_{1,2,6}\) receptors and inhibition of M-type K⁺ and N-type Ca\(^{2+}\) currents (5).

The possibility that potentiation of glutamatergic inspiratory inputs to phrenic MNs contributed to the ATP-mediated increase in \( C_4 \) inspiratory burst amplitude was also explored. ATP modulates glutamatergic transmission to neurons in the substantia gelatinosa (31), the CA3 region of the hippocampus (38), and potentiates glutamatergic inspiratory inputs to XII MNs. ATP, however, did not potentiate inspiratory-related synaptic currents in any phrenic MN tested. Thus ATP-mediated potentiation of inspiratory output does not involve enhancement of glutamatergic transmission. It most likely results from the combined actions of membrane depolarization and increased MN excitability.

**Receptor subtypes underlying excitatory actions of ATP.** Blockade of the ATP-mediated excitation by the general P2Y/P2X receptor antagonist suramin and the P2X receptor antagonist PPADS established that the excitation is mediated via P2 receptors. Furthermore, the similarity in the magnitude of the block by suramin and PPADS supports a primary role for P2X receptors in ATP-mediated potentiation of inspiratory output. The lack of selective ligands makes further pharmacological characterization difficult. To date the composition of most native P2X receptors remains unknown. Efforts to identify receptor subtype are further confounded by the vast receptor diversity that is associated with seven subtypes of P2X receptors (P2X\(_{1,7}\)) (25), the potential for heteromeric assembly of receptors (27, 29, 30, 60), and alternative splicing (42).

The time course of the PPADS inhibition and the actions of \( \alpha, \beta \)-methylene ATP, however, provide some insight into the subunit composition of P2X receptors involved in ATP-mediated potentiation of \( C_4 \) inspiratory output. As discussed by Collo et al. (7) and Buell et al. (6), PPADS has two main actions on homomeric P2X receptors. Inhibition of P2X\(_1\), P2X\(_2\), and P2X\(_5\) receptors by PPADS has a slow onset and slow, often only partial, recovery. In contrast, inhibition of P2X\(_2\) receptors by PPADS has a rapid onset and recovery. Although the speed of onset is difficult to assess when drugs are locally applied to tissue slices, the lack of recovery from PPADS in this study supports the presence of P2X\(_1\), P2X\(_2\), and P2X\(_5\) receptors. Generation of inward currents by the P2X\(_{1,7}\) agonist \( \alpha, \beta \)-methylene ATP further supports the presence of P2X\(_1\) receptors. RNA for five P2X receptor subunits, including P2X\(_1\), P2X\(_2\), and P2X\(_5\), has been detected in adult spinal cord MNs, with signals strongest for P2X\(_2\) and weaker for P2X\(_1\) and P2X\(_5\) probes (6, 7). Immunohistochemical data in adult (23) and neonatal rats (present study) localizing P2X\(_2\) receptor subunit protein to MNs in the C4 segment support the possibility that P2X\(_2\) receptors contribute to the potentiation of C4 inspiratory-related output. Thus ATP-mediated potentiation of phrenic MN activity is likely to be mediated, at least in part, by activation of P2X\(_1\), P2X\(_2\), and/or P2X\(_5\) receptors.

**Amplitude inhibition.** Whereas the excitatory actions of ATP result from membrane depolarization and increased MN excitability, the final magnitude of the ATP-mediated potentiation of burst amplitude results from an interaction between these potentiating effects and inhibitory mechanisms. In this study, inhibitory effects of ATP were evident in 1) recordings from the C4 root as a delayed, secondary inhibition of inspiratory burst amplitude and 2) whole cell recordings from phrenic MNs as a more rapid reduction in inspiratory synaptic currents.

Mechanisms remain to be defined, but data suggest two contributing factors. First, the secondary inhibition of \( C_4 \) inspiratory burst amplitude evoked in response to ATP, but not to ATP\(_\gamma S\), the hydrolysis-resistant analog (20), in conjunction with the prolonged excitatory actions of ATP\(_\gamma S\) relative to ATP, suggests that the post-ATP inhibition results from the actions of adenosine at P\(_1\) receptors. Extracellular ATP is rapidly degraded to adenosine by membrane-bound ectonucleotidases (11, 21), which are widespread throughout the brain and spinal cord (64). Indeed, adenosine, derived from the hydrolysis of ATP, has widespread inhibitory effects on synaptic transmission (11, 21, 31). Most relevant to the present study, ATP-mediated potentiation of XII nerve inspiratory output in rhythmically active medullary slice preparations is followed by an inhibition similar in magnitude and time course to that described here for \( C_4 \) inspiratory output. Although not tested in phrenic MNs, this inhibition in XII is blocked by the general P1 receptor antagonist theophylline (15). Endogenous adenosine also inhibits inspiratory drive to both phrenic and XII MNs via activation of presynaptic A\(_1\) adenosine receptors (3, 10). Taken together, data are consistent with the possibility that hydrolysis of ATP to adenosine and activation of presynaptic A\(_1\) receptors contributes to the secondary inhibitory action of ATP on \( C_4 \) inspiratory output.

In contrast to the delayed inhibition of \( C_4 \) burst amplitude, the ATP-mediated inhibition of inspiratory synaptic currents had a rapid onset and was also evoked by ATP\(_\gamma S\). Thus, in addition to the putative adenosine-mediated inhibition of inspiratory activity, a more rapid P2 receptor-mediated inhibitory mechanism may contribute. ATP-mediated activation of local inhibitory interneurons, including Renshaw cells, is...
unlikely to participate in the inhibition. First, recurrent inhibition of phrenic MNs is minimal relative to other spinal MN pools (18, 33, 43). Second, ATP-mediated excitation of Renshaw cells, or any other local inhibitory neuron, would lead to tonic release of glycine/GABA, which would manifest in an outward current (43, 47). This was not apparent in any recording.

Inhibition of inspiratory input to phrenic MNs by presynaptic P2X receptors is also unlikely. Although presynaptic P2X receptors increase the spontaneous release of neurotransmitter (including glutamate) (for review, see Ref. 26), evidence for P2X receptor-mediated inhibition of glutamatergic transmission is lacking. A P2Y receptor-mediated inhibition of glutamatergic excitatory postsynaptic potentials, as seen in hippocampal pyramidal cells (36), may contribute. This presynaptic P2Y inhibitory mechanism is activated by ATPγS and ATP and only partially by α,β-methylene ATP. It therefore has a similar pharmacology to the response observed here in two phrenic MNs. Additional experiments are required to verify this putative mechanism.

In summary, we propose that the biphasic time course of the response to ATP recorded from the C4 root is determined through an interaction of multiple effects. In the initial phase, membrane depolarization and increased MN excitability predominate to produce burst amplitude potentiation, although the inspiratory synaptic input to phrenic MNs actually diminishes as a result of a presynaptic, possibly P2Y receptor-mediated, inhibition. The potentiation then decreases as ATP is hydrolyzed to adenosine, which activates a slower time course inhibition mediated through presynaptic A1 receptors.

Functional Significance of ATP as a Modulator of Phrenic MN Activity

Modulation of phrenic and XII MN (15) excitability by extracellular ATP highlights the potential importance of this compound and the diversity of P2 receptors to respiratory control. A complete understanding of the functional significance of this signaling system will ultimately require several issues to be addressed. Most urgent is the need for development of specific P2 receptor antagonists, as these are required to test for endogenous actions of ATP, establish the source of ATP inputs, and define the factors affecting release. Direct support for endogenous modulation of inspiratory MN activity is lacking. However, electrophysiological analyses in vivo suggest that ATP receptors on respiratory neurons in the ventrolateral medulla (54, 55, 58), in particular P2X2 receptors whose currents are potentiated by low pH (28), may contribute to central respiratory chemosensitivity. The most likely source of ATP inputs to respiratory networks is from noradrenergic neurons of the brain stem. Not only do these neurons project widely to respiratory neurons and MNs (17, 47), they corelease ATP and norepinephrine (46, 53). The state-dependent activity of noradrenergic locus coeruleus neurons (2) raises the additional possibility that ATP-mediated signaling contributes to diurnal modulation of breathing pattern and MN excitability.

The opposing effects of ATP and adenosine and the fact that ATP is hydrolyzed to adenosine at many synapses (11, 21, 31) also suggest that controlling the rate of ATP hydrolysis to adenosine may provide an additional mechanism for modulating ATP-induced excitation. *Xenopus* embryos, for example, display rhythmic swimming movements that are lengthened by ATP and reduced by adenosine (8). A delicate balance between ATP and adenosine, presumably derived from the breakdown of ATP, has been proposed to form a feedback mechanism that controls duration of swimming episodes and possibly other episodic motor patterns.

Respiratory activity may be another motor pattern under complex purinergic control. Potentiation followed by inhibition of inspiratory output has been recorded in response to exogenous ATP for phrenic and airway MNs (15) in the neonatal rat. Under physiological conditions, alterations in the normal balance between synaptically released ATP and the resultant adenosine may contribute to the biphasic hypoxic ventilatory response. Although elevations in extracellular adenosine during hypoxia are largely believed to reflect release from nonsynaptic sources (45), altered activity of ectoATPases, adenosine transporters, and purinergic neurons must also be considered.

Although progress in establishing the physiological significance of ATP signaling in controlling motor outflow has been slow, interest in the pharmacological actions of ATP receptor activation on motor outflow is high due to the diversity of receptors and the potential for developing drugs to selectively manipulate neuronal and network activity. In this context, understanding the differential modulation of phrenic and airway MNs by P2 and P1 receptors and the mechanisms that control ATP hydrolysis are of interest as a mismatch between activity of phrenic and airway MNs has been implicated in some cases of sudden infant death syndrome as well as obstructive sleep apnea (19, 48). The ATP-mediated excitation of C4 inspiratory output demonstrated in this study was similar in magnitude to that observed previously for XII inspiratory output (15). The concentration of ATP required to produce these effects, however, was an order of magnitude higher for phrenic compared to XII MNs. Thus either phrenic MNs have reduced sensitivity to ATP compared with XII MNs or the inhibitory effects are more potent in phrenic MNs. It is also possible that the reduced sensitivity of phrenic MNs is due in part to reduced drug diffusion to the phrenic vs. XII MN pools. In the neonatal brain stem-spinal cord preparation, the phrenic motor nucleus is located ~200 μm below the ventral surface (32). In the rhythmic slice preparation previously used to study XII MNs, MNs are present near the cut surface of the slice. We minimized diffusion barriers to phrenic MNs in the present study by removing pia mater. The success of this procedure was confirmed by the rapid inhibitory actions of GABA on C4 inspiratory output. Thus different sensitivities of
phrenic and XII MNs to ATP may represent real physiological differences.

Whether the effects described here in neonates apply to older animals is not known. P2 receptor expression and physiological actions do change developmentally in some regions (22, 62). Within respiratory networks, however, developmental measurements are limited to the purinergic modulation of XII inspiratory activity where the biphasic effects of ATP at P0 and P10 are virtually indistinguishable (16).

In summary, the complex effects of ATP on respiratory MN output, in conjunction with the ubiquitous expression of ATP receptors on MNs, suggest that ATP receptors and purinergic signaling play an important role in controlling motor outflow from the CNS.

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