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P2 Receptor Excitation of Rodent Hypoglossal Motoneuron Activity *In Vitro* and *In Vivo*: A Molecular Physiological Analysis

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The role of P2 receptors in controlling hypoglossal motoneuron (XII MN) output was examined (1) electrophysiologically, via application of ATP to the hypoglossal nucleus of rhythmically active mouse medullary slices and anesthetized adult rats; (2) immunohistochemically, using an antiserum against the $P2X_2$ receptor subunit; and (3) using PCR to identify expression of $P2X_2$ receptor subunits in micropunches of tissue taken from the XII motor nucleus. Application of ATP to the hypoglossal nucleus of mouse medullary slices and anesthetized rats produced a suramin-sensitive excitation of hypoglossal nerve activity. Additional *in vitro* effects included potentiation of inspiratory hypoglossal nerve output via a suramin- and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS)-sensitive mechanism, XII MN depolarization via activation of a suramin-sensitive inward current, decreased neuronal input resistance, and a slow-onset theophylline-sensitive reduction of inspiratory output likely resulting from hydrolysis of extracellular ATP to adenosine and activation of P1 receptors. Immunohistochemically, $P2X_2$ receptors were detected in inspiratory XII MNs that were labeled with Lucifer yellow. These data, combined with identification of mRNA for three $P2X_2$ receptor subunit isoforms within the hypoglossal nucleus (two of which have not been localized previously in brain) and the previous demonstration that P2X receptors are ubiquitously expressed in cranial and spinal motoneuron pools, support not only a role of P2 receptors in modulating inspiratory hypoglossal activity but a general role of P2 receptors in modulating motor outflow from the CNS.

Key words: respiration; P2 receptor; hypoglossal motoneuron; ATP; suramin; PPADS; adenosine; medullary brain slice; rat; mouse

ATP clearly has been established as a transmitter within the CNS (Edwards et al., 1992; Evans et al., 1992; Salter et al., 1993; Zimmerman, 1994; Burnstock, 1995). Its actions are mediated by two major receptor families (Abbracchio and Burnstock, 1994; Fredholm et al., 1994; Burnstock, 1995). P2X receptors are ligand-gated ion channels that mediate fast excitatory responses (Surprenant et al., 1996). P2Y receptors mediate slower responses via G-proteins (Dubyak and El-Moatassim, 1993; Burnstock, 1995). Purinergic synaptic signaling in the CNS is complicated further by the extracellular hydrolysis of ATP to adenosine, which modulates synaptic transmission via activation of P1 receptors (Burnstock, 1995).

Widespread distribution of P2 receptors (Burnstock, 1995) in the brain, indicated by autoradiographic (Bo and Burnstock, 1994; Balcar et al., 1995), *in situ* hybridization (Kidd et al., 1995; Collo et al., 1996; Kanjhan et al., 1996; Séguéla et al., 1996), and immunohistochemical studies (Kanjhan et al., 1996; Vulchanova et al., 1996), suggests involvement of ATP in many neuronal systems. It clearly is involved in sensory transduction (Thorne and Housley, 1996), fast excitatory neurotransmission (Edwards et al., 1992; Evans et al., 1992; Harms et al., 1992; Tschöpl et al., 1992), modulation of glutamatergic synaptic transmission (Li and Perl, 1995; Motin and Bennett, 1995; Nakazawa et al., 1995), and modulation of norepinephrine (NE) release (Burnstock and Sneddon, 1985; von Kügelgen et al., 1994; Zimmerman, 1994; von Kügelgen, 1996) and is implicated in central control of blood pressure (Sun et al., 1992; Day et al., 1993).

An important role for ATP in motor control is suggested by the presence of mRNA for several P2X receptor subunits within cranial and spinal motor nuclei (Collo et al., 1996). As an initial step in elucidating the role of ATP in controlling motoneuron activity, we examined the effects of ATP on inspiratory hypoglossal nerve output in rhythmically active neonatal mouse medullary slices and anesthetized adult rats. Hypoglossal motoneuron (XII MN) activity was examined because (1) the function of the XII nerve in maintaining upper airway patency is well established (Remmers et al., 1978), allowing interpretation of the effects of ATP in a well defined behavioral context; (2) inspiratory drive to XII MNs is mediated primarily by glutamate (Greer et al., 1991; Funk et al., 1993), and ATP modulates glutamatergic transmission (Li and Perl, 1995; Motin and Bennet, 1995); (3) the XII motor nucleus receives NE innervation (Levitt and Moore, 1979; Aldes et al., 1992), and NE and ATP are colocalized in some synapses (Burnstock and Sneddon, 1985; Zimmerman, 1994); and (4) preliminary studies indicate an ATP excitation of XII nerve activity in medullary slices (Funk, 1996), but application of ATP to acutely dissociated adult XII MNs has no effect (Ueno et al., 1996). We also examined the localization of $P2X_2$ receptors within the XII motor nucleus, using immunohistochemical and PCR techniques. We focused on $P2X_2$ receptors, not to exclude involvement of other P2 receptor subtypes but because preliminary experiments (Funk, 1996) and previous pharmacological and *in situ* hybridization data (Buell et al., 1996; Collo et al., 1996)

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suggested that $P2X_2$ receptors most likely were expressed in the XII nucleus.

MATERIALS AND METHODS

In vitro *mouse experiments*

Rhythmic medullary slices. In vitro experiments were performed on transverse medullary slices from postnatal day 0–3 (P0–P3) neonatal Swiss CD mice ($n = 64$) at 27°C. Details of procedures for obtaining slices are described elsewhere (Funk et al., 1993, 1994). Briefly, animals were anesthetized with ether and decerebrated, and the brainstem–spinal cord were isolated in control solution containing (in mM): 128 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 21 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose equilibrated with $95\%O_2/5\%CO_2$. The brainstem–spinal cord was pinned down on a paraffin block and sectioned serially in the transverse plane with a vibratome. Sectioning started from the rostral medulla and continued to a point 150 μm rostral to the rostral boundary of the pre-Bötzinger complex (Smith et al., 1991; Funk et al., 1993). A 600 μm transverse slice was cut, transferred to a recording chamber, and superfused continuously (40–45 ml/min) with control solution containing 9 mM K^+.

Population inspiratory activity was recorded bilaterally with suction electrodes from XII nerve roots. These signals were amplified, bandpass-filtered (0.1–3 kHz), rectified, integrated ($\tau = 50$ msec), and recorded on chart recorder and video cassette via pulse code modulation for measurement of frequency and burst amplitude of XII MN population discharge.

Drug application. ATP–Na^+ salt [1.0 mM, Research Biochemicals (RBI), Natick, MA], ATP–Mg^{2+} salt (Sigma, St. Louis, MO), suramin (1.0 mM, RBI), adenosine (1.0 mM, RBI), 1,3,-dimethyl-8-phenylxanthine (theophylline, 100 μM, RBI), and control solution (vehicle) were applied by timed pressure injection (10 psi, 10–30 sec) from triple-barreled pipettes (6 μm per barrel outside diameter). Pipette tips were placed within 25 μm of the slice surface over the ventromedial aspect of the XII motor nucleus. Preliminary experiments indicated that appropriate electrode placement was critical for reducing response variability. ATP application over the dorsal aspect of the XII nucleus produced tonic excitation only. When it was injected over the ventromedial inspiratory (Remmers et al., 1978; Krammer et al., 1979) aspect of the XII nucleus, ATP consistently produced a significant increase of phasic inspiratory activity as well as tonic excitation.

All experiments examining the effects of ATP on XII nerve output used 1 mM ATP. At 10 and 100 μM, ATP produced minor excitation, whereas 10 mM ATP generated large increases in tonic activity that made inspiratory activity difficult to resolve. ATP applications of 30 sec encompassing five to seven inspiratory bursts were used to assess effects of ATP on inspiratory burst amplitude. ATP applications were reduced to 10 sec when suramin antagonism was tested to minimize suramin application and to facilitate recovery. ATP also was used at 1 mM during whole-cell recording experiments, with the exception of those experiments performed in the presence of 1 μM bath-applied TTX for which 10 mM ATP was used.

The concentrations and durations of drug application used in the present study cannot be compared with those in experiments in which similar agents were bath-applied or applied to isolated cells. First, concentration of drug decreases exponentially with distance from the pipette tip (Nicholson, 1985), and previous experiments with this preparation indicate that drug concentration in the pipette must be approximately one order of magnitude greater than the bath-applied concentration to produce similar effects (Liu et al., 1990). Second, diffusion barriers of thick slices slow response kinetics relative to isolated cells. To speed the concentration buildup at neurons within the slices, we used relatively high concentrations of ATP and suramin.

In addition to local application of drugs via pressure injection, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 5 μM; RBI), a putative P2X-selective receptor antagonist (Lambrecht et al., 1992; Trezise et al., 1994; Ziganshin et al., 1994; Connolly, 1995), was applied directly to the medium bathing the slices. PPADS was administered via bath application because of the limited diffusion of PPADS into the tissue during the relatively brief exposure periods associated with local application. In addition, the selectivity of PPADS for P2X receptors may be concentration-dependent (Windscheif et al., 1994), and bath application allows precise control over antagonist concentration.

Analysis. Response time course was calculated by averaging inspiratory burst amplitudes in 1 min bins for 2 min before drug application, in 30 sec bins for the first 2 min after onset of ATP application, and in 1 min bins for the next 8 min. To discriminate between the effects of ATP on inspiratory burst amplitude and tonic nerve activity, we corrected burst amplitude measurements for shifts in baseline associated with increased tonic discharge. Suramin block was assessed by comparing control/ATP responses with suramin/ATP responses. In control responses ATP was applied during the last 10 sec of a 30 sec control (vehicle) application. In suramin responses, ATP was applied during the last 10 sec of a 30 sec suramin application.

Data are reported relative to preinjection values as mean ± SD. Differences between means were tested with the statistical software package SYSTAT 5.1, with ANOVA and multiple comparison tests. Values of $p < 0.05$ were assumed significant.

Whole-cell recording. Intracellular recordings were made from XII MNs with whole-cell patch-clamp recording techniques (Blanton et al., 1989). Patch electrodes (resistance, 4.0–4.5 MΩ; 1.5–2 μm tip size) were pulled from borosilicate glass and filled with K^+-gluconate solution containing (in mM): 120 K^+-gluconate, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (Sigma), 10 BAPTA (tetra K^+ salt; Sigma), and 2 ATP (Mg^{2+} salt) pH-adjusted to 7.3 with KOH. The electrode solution also contained Lucifer yellow (dipotassium salt, ~0.1%; Sigma). Intracellular signals were amplified with a patch-clamp amplifier (5 kHz low-pass filter; Axopatch 1D, Axon Instruments, Foster City, CA).

Series resistance and whole-cell capacitance were estimated under voltage-clamp conditions by using short voltage pulses (100 Hz, −10 mV, 3.0 msec). Series resistance (70–90% compensation with 10–20 μsec time lag) and membrane capacitance compensation were used. Neuron input resistance (R_N) was calculated at a −60 mV holding potential from the current responses to 10 or 20 mV hyperpolarizing voltage steps (300 msec duration).

In vivo *rat experiments*

Adult Wistar rats ($n = 5$) were anesthetized with sodium pentobarbital (Nembutal, 80 mg/kg, i.p., followed by 4–6 mg/hr, i.v.), paralyzed with pancuronium bromide (Pavulon, 0.4 mg/hr), and artificially ventilated with O_2-enriched air. Tracheal pressure (peak, <10 cm H_2O), arterial blood pressure, end-tidal CO_2 (4.5–5.5%), and body temperature (35–36°C) were monitored. The dorsal surfaces of the medulla oblongata and cervical cord (C1–C7 segments) were exposed. Standard bipolar electrodes were used to record from phrenic (unilaterally) and XII nerves (bilaterally). These electrodes also were used to stimulate XII nerves electrically (0.1 msec, 1.0–2.0 V) for antidromic identification of XII nuclei.

The location of the XII motor nucleus was established by using glass microelectrodes filled with 2 M NaCl (resistance 4–8 MΩ). An antidromic field potential after stimulation of the ipsilateral XII nerve (see Fig. 6*Ci*) indicated electrode placement within the XII nucleus. In three experiments, series of tracks spaced 100 μm laterally were made at three rostrocaudal levels (obex, 500 μm rostral, and 1000 μm rostral) to establish the borders of the nucleus. After electrophysiological identification the NaCl electrode was removed, and a single-barrel drug ejection pipette containing ATP was lowered into the same location. Drugs were pressure-injected with 3.8 or 7.6 sec 10–16 psi pulses. Four to eight bursts of inspiratory activity occurred during the period of ATP application. After several ATP applications (see below), the ATP pipette was removed and replaced with a suramin-containing pipette. Suramin was applied, and then the suramin pipette was replaced with the ATP pipette. Proper placement was guided during each exchange by the use of surface landmarks and recording antidromic field potentials. With this method pipette tip placement could be reproduced within <100 μm. Duration of drug application was limited *in vivo* relative to *in vitro* to prevent pressure changes associated with large injections inside tissue.

The experimental paradigm involved electrophysiological identification of the XII motor nucleus (described above), repeated local injections of ATP (1.0–10 mM) to establish response reproducibility, suramin injection (1–10 mM), and repeated ATP injection to test the effects of suramin on ATP responses and monitor recovery. Successive ATP applications were performed at a minimum of 5 min intervals. The effects of ATP on XII nerve discharge *in vivo* were evaluated similarly to the analysis conducted *in vitro*, using correction for shifts in baseline associated with increased tonic discharge.

Recordings were made with AC amplifiers (Neurolog NL 104/125, bandwidth from 120 Hz to 6 kHz). All signals were monitored on a multichannel pen recorder and oscilloscopes. Nerve output signals were rectified, filtered ($\tau = 100$ msec), and recorded on a thermal array

recorder (Nikon Kohden, RTA-1100) and video cassette via pulse code modulation.

Immunohistochemistry

Adult rats were anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with heparinized saline, followed by 4% formalin and 0.5% glutaraldehyde in 50 mM PBS, pH 7.4. Brainstems were removed and post-fixed in the same fixative for 48 hr at 4°C. Free-floating transverse sections, cut with a vibratome (50 μm), underwent a 30 min preincubation with 1% bovine serum albumin (BSA; for details of $P2X_2R$ antiserum production and characterization, see Kanjhan et al., 1996). Then the sections were incubated with $P2X_2R$ 96 antiserum (1:2000–1:4000), which had been pretreated with keyhole limpet hemocyanin (50 μg/ml; Sigma). Subsequently, sections were incubated with biotinylated goat anti-rabbit immunoglobulin (1:400; Sigma) and ExtrAvidin–peroxidase conjugate (1:1000; Sigma). Antiserum was diluted in PBS containing 1% BSA. All incubations were carried overnight at 4°C and were separated by four washes in PBS. Sections were reacted with 0.5 mg/ml 3′,3-diaminobenzidine tetrahydrochloride, 6 mg/ml ammonium nickel sulfate, and 0.01% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.6, mounted on poly-L-lysine-subbed slides, dehydrated, cleared, and coverslipped with Histomount (Hughes and Hughes). Control experiments in which primary antiserum was excluded were run with each group. As an additional control for antiserum specificity, the $P2X_2R96$ antiserum was preadsorbed with a synthetic peptide (50 μg/μl; Chiron, Australia) corresponding to amino acids 96–113 of the putative extracellular domain of the $P2X_2R$ subunit of the ATP-gated ion channel (Brake et al., 1994). Then the immunoreactivity of the $P2X_2R96$ antiserum and peptide-blocked antiserum (both at 1:4000) were compared.

Neonatal mouse tissue was isolated as described below (*in vitro* preparations) and post-fixed in 4% formalin and 0.5% glutaraldehyde for 48 hr before cryosectioning (20 μm). $P2X_2$ receptor immunoreactivity was examined as described above, except that the primary antibody was diluted at 1:100 and swine anti-rabbit tetramethylrhodamine isothiocyanate (TRITC; 1:20; Dako, High Wycombe, UK) conjugate was used as the secondary antibody.

Molecular characterization of $P2X_2$ receptor gene expression in the hypoglossal nucleus

Adult Wistar rats were anesthetized by carbon dioxide inhalation and decapitated, and the brainstem was removed rapidly and frozen. Micropunches of the hypoglossal nucleus from frozen 180- to 240-μm-thick transverse medullary sections were made with a 23 gauge microdissection needle (Palkovits and Brownstein, 1988). Total RNA was extracted by TriReagent (Molecular Research Center, Cincinnati, OH) dissolved in 10 μl of H_2O and reverse-transcribed in a final volume of 20 μl containing 10 μl of RNA solution and (in mM): 20 Tris-HCl, pH 8.4, 50 KCl, 1.5 $MgCl_2$, and 1.25 dNTPs plus 1 μl of random hexamers, 40 U of RNA guard (Pharmacia Biotech, Piscataway, NJ), and 200 U of SuperScript II (Life Technologies, Gaithersburg, MD). A semi-nested PCR protocol was used to identify $P2X_2$ receptor subunits expressed in the tissue. First round PCR reactions were performed in 50 μl volumes containing (in mM): 20 Tris-HCl, pH 8.4, 50 KCl, 2 $MgCl_2$, and 0.25 dNTPs plus 10 nM $P2X_2756s$ (CAC AGA ACT GGC ACA CAA GG, relative to 5′ position 756 of GenBank accession number U14414) and $P2X_21558as$ (GGA CAT GGT TAC TGA AGA GCG, 5′ position 1558 of GenBank accession number U14414) primers and 1.25 U of *Taq* DNA polymerase (Life Technologies). Second round amplifications were identical, except that alternative sense primers were used and that the concentration of both primers was increased to 100 nM [sequences: $P2X_2S4$ was CAG GTA GGG AGT GGT TGG TAG, specific to the 85 bp insert splice variant of $P2X_2R$ (Housley et al., 1995) (5′ position 94 of GenBank accession number L43511); $P2X_2S1$ was GCA TGG ACA GGC AGG GAA AT (5′ position 990 of GenBank accession number U14414); and $P2X_2S3$ was CGG GGT GGG CTC CTT CCT GT (5′ position 1059 of GenBank number U14414)]. The thermal cycler (PTC-100, MJ Research, Watertown, MA) was programmed for 3 min at 94°C and then cycled through 30–35 cycles of 1 min denaturation (94°C), 2 min annealing (58–60°C), and 2.5 min extension (72°C). Aliquots of the PCR products were run on a 1% agarose (Life Technologies)/1% NuSieve (FMC Bioproducts, Rockland, ME) gel and visualized with ethidium bromide under UV transillumination. The remaining PCR products were purified either directly or from the agarose gel (QIAquick PCR purification kit or QIAquick gel extraction kit, Qiagen, Hilden, Germany) and sequenced.

RESULTS

ATP mediates tonic excitation of hypoglossal activity and potentiates inspiratory output *in vitro*

To test the effects of ATP on phasic (inspiratory) activity recorded from XII nerves, we applied ATP locally over XII motor nuclei of rhythmically active medullary slices. ATP application produced potent excitation of XII nerve output, the onset and termination of which coincided closely with the timing of the ATP application. The response was composed of three distinct components: (1) tonic excitation (present in 39 of 40 preparations), (2) potentiation of inspiratory burst amplitude (39 of 40), and (3) inhibition of inspiratory burst amplitude after the potentiation (29 of 40).

Tonic excitation, apparent as a thickening of the baseline in the raw XII nerve recording (Fig. 1*A*) or a shift in the baseline of the rectified, integrated trace (Fig. 1*B*), was exclusively unilateral (on the drug application side) and typically peaked within the first 10 sec of the application, decreasing slightly during the remainder of the application. Superimposed on the tonic component was a significant 40 ± 20% ATP-mediated potentiation of inspiratory burst amplitude (Fig. 1*B,D*) that was also exclusively unilateral and was followed in most cases by a slowly developing decrease in inspiratory burst amplitude. The inhibition peaked at 2 min after the start of the ATP application with a decrease in burst amplitude to 0.82 ± 0.05 of control. Single XII inspiratory bursts recorded before, during, and after ATP application are expanded in Figure 1*C*. To ensure that the ATP-mediated excitation was not attributable to chelation of divalent ions by ATP (Na^+ salts), we applied Mg^{2+} salts of ATP in four experiments. Mg^{2+} and Na^+ salts were equally effective at exciting XII nerve output (data not shown).

To confirm that the effects of ATP on XII nerve output were attributable to the activation of P2 receptors, we examined the effects of suramin (Fig. 2*A*). Suramin is a general P2 antagonist that inhibits responses mediated by P2Y and $P2X_2$ receptors. A 30 sec application of suramin starting 20 sec before a 10 sec ATP application completely ($n = 2$) or partially ($n = 3$; Fig. 2*A, middle*) blocked the tonic excitation and the potentiation of inspiratory amplitude. Suramin was ineffective at blocking the post-ATP inhibition (Fig. 2*A, middle*). Partial to complete recovery from suramin block was observed within 30 min of suramin application in all (Fig. 2*A, right*) but one preparation.

The rapid onset and termination of the ATP-induced excitation suggested involvement of P2X receptors in the ATP-induced excitation of XII nerve output. Thus, ATP was applied in the presence of bath-applied PPADS (5 μM, 15 min equilibration period) to examine the role of P2X receptors. PPADS virtually abolished the tonic excitatory component of the ATP response (Fig. 2*B, middle*) and significantly reduced the potentiation of inspiratory burst amplitude by more than one-half from 33 ± 22 to 15 ± 16% ($n = 5$; Fig. 2*B, middle*, *C*). The post-ATP inhibitory response was unaffected by PPADS. Recovery of the burst amplitude-potentiating component of the ATP response from PPADS block was complete but slow, taking between 70 and 120 min (Fig. 2*B, right*). The tonic excitatory component of the ATP response, however, recovered only partially.

Local application of ATP over the XII motor nucleus would affect both inspiratory as well as noninspiratory XII MNs and could produce its effects pre- or postsynaptically. We therefore established whole-cell recordings to test directly the effects of ATP on inspiratory XII MNs. Inspiratory XII MNs received

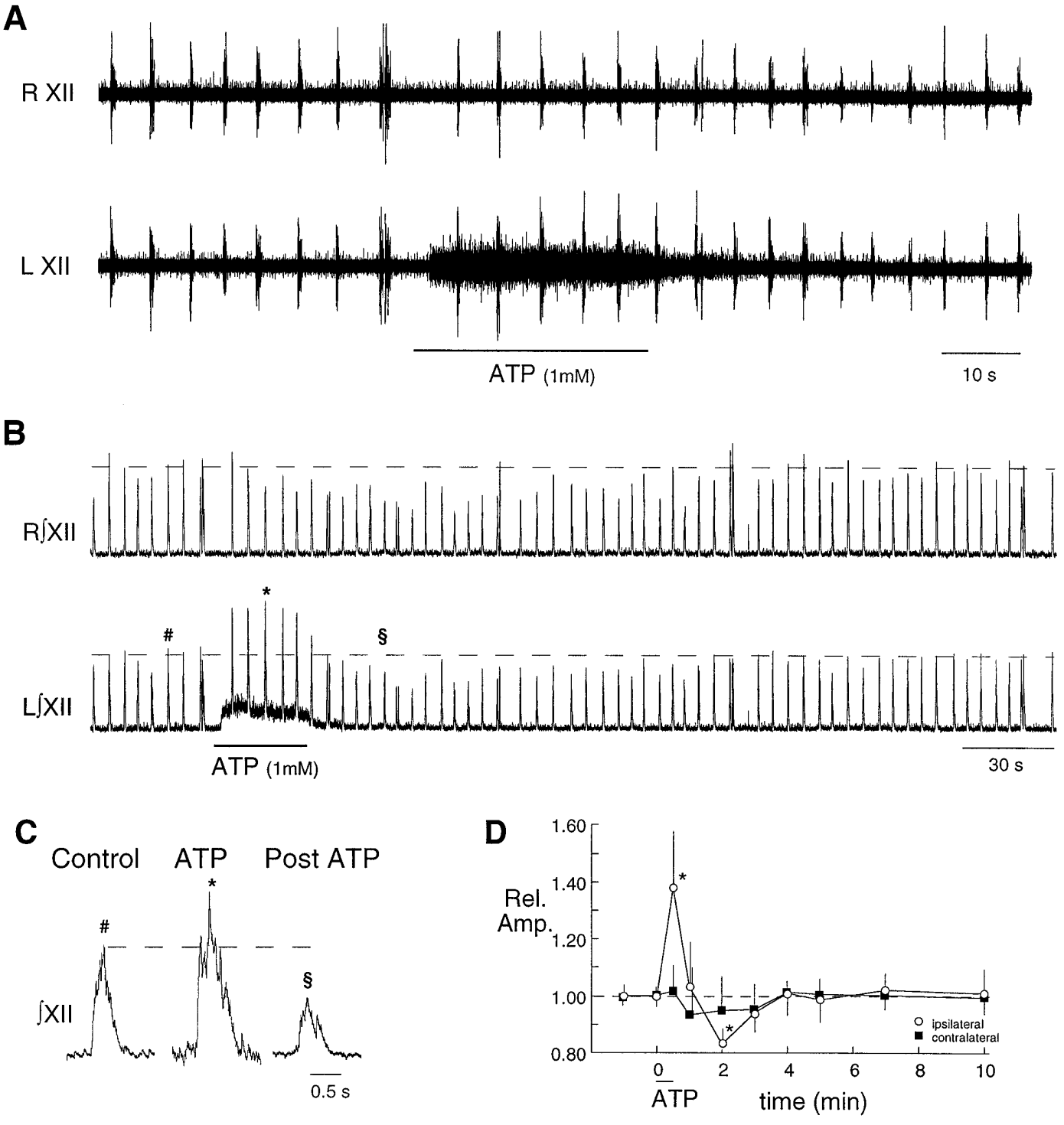

Figure 1. Excitation of XII nerve activity *in vitro* by ATP. *A*, Bilateral recordings of XII nerve activity of a medullary slice from a postnatal day 2 mouse showing the effects of 30 sec, 1 mM ATP injected unilaterally over the ventromedial portion of the LXII nucleus. Rhythmic bursts of activity represent inspiratory-related XII nerve activity. *B*, Rectified and integrated signal of the raw data traces and ATP response shown in *A* illustrates the ATP-mediated potentiation of burst amplitude and subsequent decrease in burst amplitude. Note the slower time scale in *B*. *C*, The individual control (#), ATP (*), and post-ATP (§) bursts indicated in *B* are shown in expanded form. The baseline shift present during ATP application has been removed to facilitate comparison of burst amplitude. *D*, Time course of the changes in XII nerve inspiratory amplitude after local application of 1 mM ATP to the ventromedial portion of the XII motor nucleus ($n = 20$). Drug application occurred at time = *0*. Effects on contralateral XII nerve output are documented also; *asterisk* indicates significant difference from control levels.

rhythmic synaptic inputs in phase with each burst of activity on the XII nerve. Peak inward inspiratory currents ranged from 25 to 300 pA and lasted 350–600 msec (Figs. 3, 4). ATP induced inward currents in all inspiratory XII MNs tested ($n = 9$; range −12 to −120 pA; mean = −65 ± 50 pA) and two noninspiratory XII MNs (−67 ± 42 pA). Similar to the XII nerve responses, the

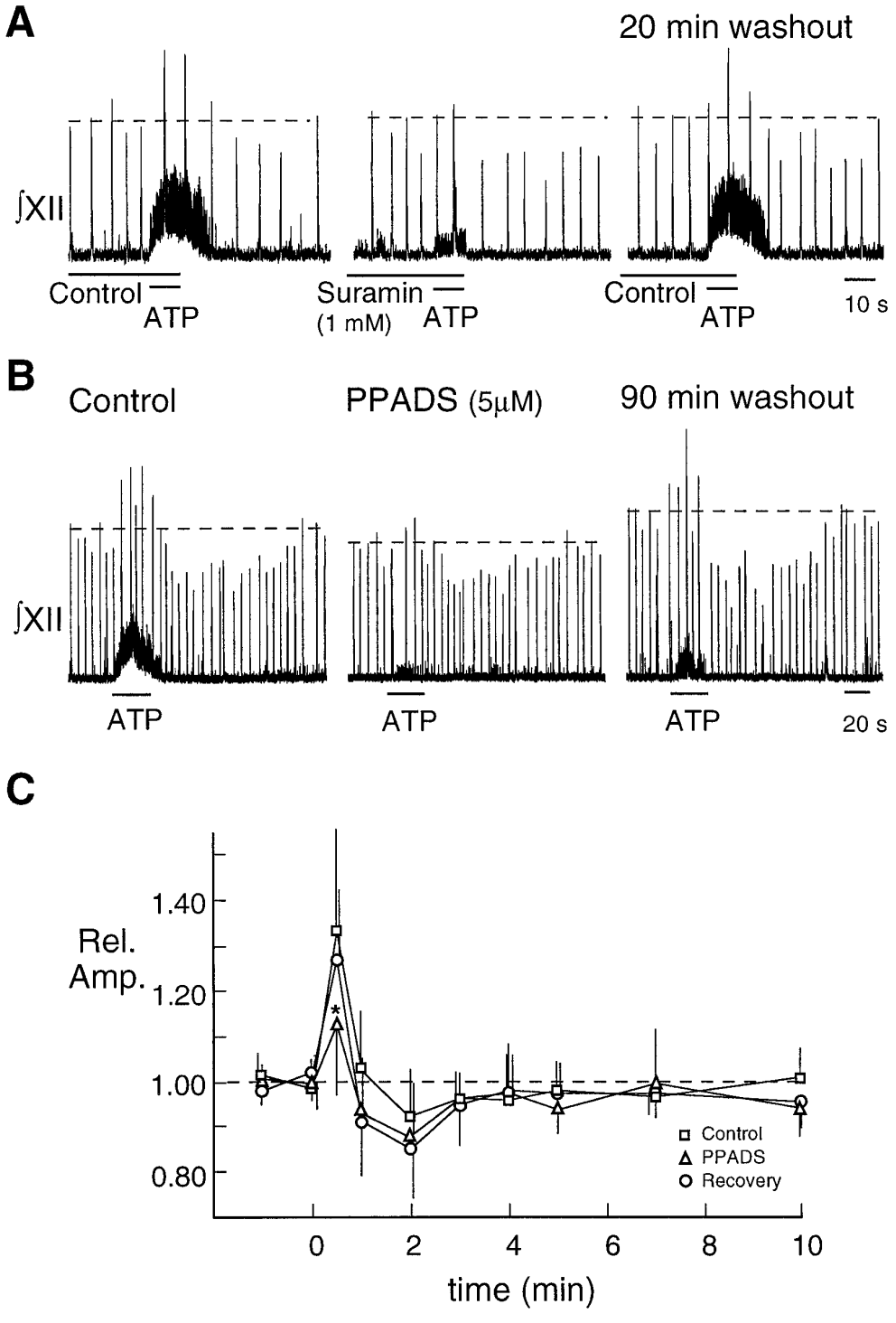

Figure 2. Antagonism of ATP excitatory responses by suramin and PPADS. *A*, The tonic excitatory and amplitude-potentiating effects produced by ATP were reduced significantly when ATP was applied for the last 10 sec of a 30 sec suramin (1 mM) application, although the post-ATP inhibition was not blocked (*middle panel*). Inhibitory action of suramin was removed after 20 min of washout (*right panel*). *B*, Bath application of PPADS caused a significant reduction in the excitatory effects of ATP without affecting the post-ATP decrease in inspiratory burst amplitude (*middle panel*). The PPADS block of the inspiratory burst amplitude-potentiating component of the ATP response was reversible, given sufficient recovery time, whereas the tonic excitatory effect did not show complete recovery even after 90 min of washout. *C*, Time course of the changes in XII nerve inspiratory burst amplitude after local application of ATP (1 mM) before, during, and after bath application of PPADS ($n = 5$). ATP application occurred at time = *0*; *asterisk* indicates significant difference from amplitude values observed at the same time during the control ATP application.

inward current developed rapidly, peaked early in the application, and decreased throughout the application in eight of nine cells. The ATP currents were associated with a significant 13 ± 5% decrease in neuronal input resistance from 108 to 94 MΩ ($n = 4$), were blocked by suramin ($n = 2$; Fig. 3*B*), and showed weak inward rectification ($n = 2$; Fig. 3*A*) in response to 2 sec voltage ramps between −100 and −45 mV.

Effects of ATP on inspiratory synaptic currents were variable and time-dependent. Thus, the responses were analyzed by comparing the peak inspiratory synaptic current averaged during control, during the first 20 sec of ATP application, and between 20 and 60 sec after onset of ATP application in six neurons, the inward inspiratory synaptic currents of which were >100 pA. Responses of one MN are shown at slow and expanded time scales in Figure 4, *C* and *D*. The tonic component of the ATP-induced current was subtracted in Figure 4*D* to facilitate comparison of the inspiratory synaptic currents before, during, and after ATP application. Four of the six inspiratory MNs behaved like the neuron in Figure 4*C*, showing an average 20 ± 10% increase in synaptic current during the first 20 sec of ATP application. The remaining two neurons showed no change. Between 20 and 60 sec, synaptic current decreased significantly in all cells

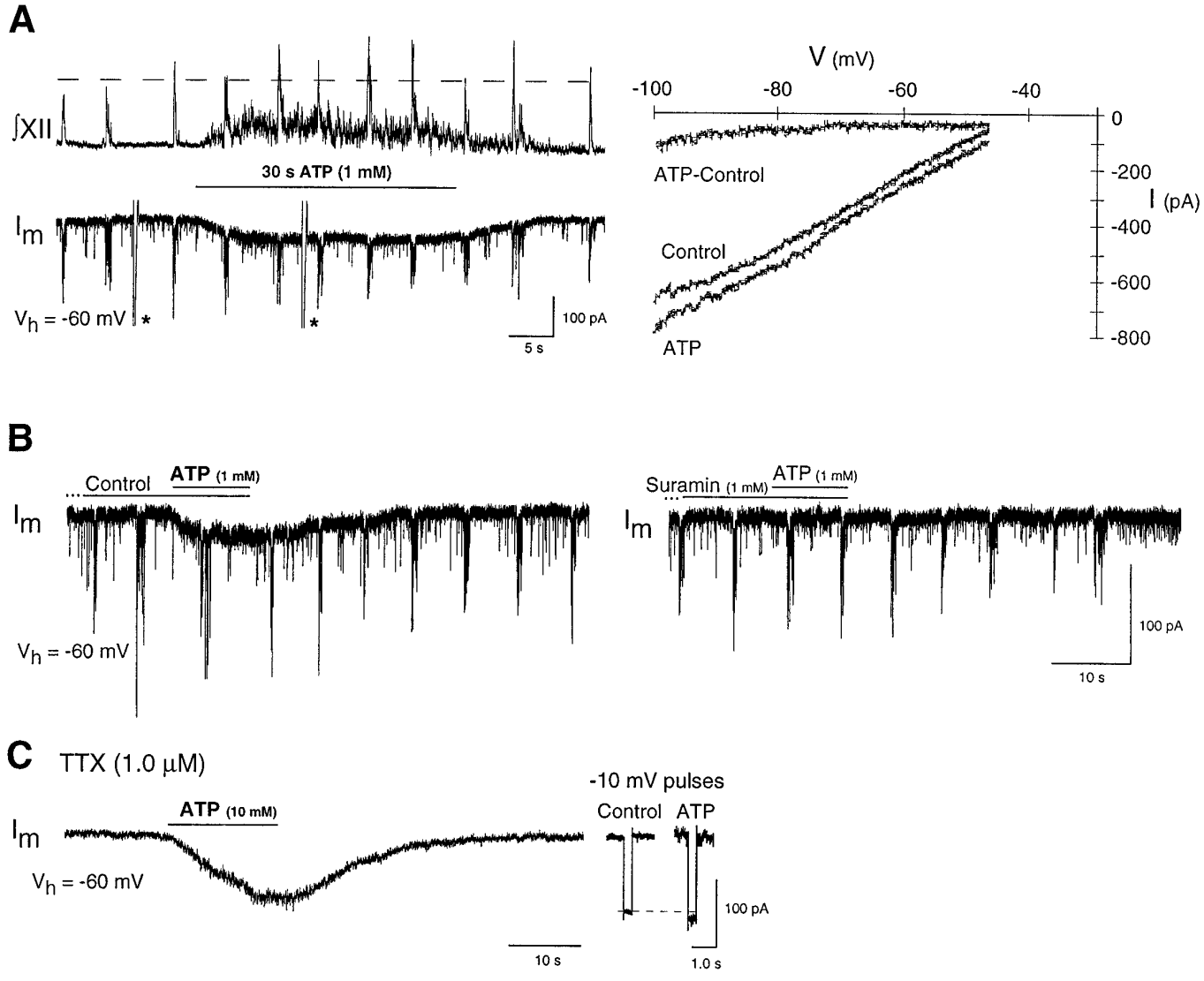

Figure 3. ATP-mediated inward currents in inspiratory XII MNs. *A*, A 30 sec application of 1 mM ATP over XII motor nucleus produced tonic excitation of the nerve, increased XII nerve inspiratory burst amplitude (*top trace*, ∫XII), and induced a 75 pA inward current in an inspiratory XII MN. Voltage ramps (−100 mV to −45 mV conducted over 2 sec) performed during control and ATP application (indicated by *asterisk*) are plotted versus current. The ATP-induced current, obtained by subtracting the control from the ATP curve (*ATP-Control*), shows weak inward rectification. *B*, Inward current induced by 10 sec of 1 mM ATP is blocked by 30 sec preapplication of 1 mM suramin. *C*, Inward current produced by 15 sec application of 10 mM ATP over inspiratory XII MN after bath application of TTX. The current responses to 10 mV hyperpolarizing voltage steps during control (*Control*) and during a subsequent ATP application (*ATP*) indicated a decrease in input resistance (*right panel*).

to 0.84 ± 0.16 of control. That the inhibitory response had an earlier onset in the whole-cell relative to whole-nerve recording likely reflects that MNs examined during whole-cell recording all were located near the surface of the slice, whereas MNs contributing to whole-nerve activity were located at a variety of depths. Because of the time required for diffusion, ATP and its metabolites would affect surface cells before deeper cells, and the inhibition would appear first in the whole-cell recording. Thus, in two of the six neurons a more rapid onset of the adenosine-mediated inhibition under whole-cell conditions may have obscured an ATP-induced potentiation of inspiratory synaptic currents. Alternatively, inspiratory synaptic currents may not be potentiated by ATP in all XII MNs. This latter possibility is not inconsistent with our observation that ATP consistently potentiates XII nerve inspiratory activity *in vitro*, because not all XII MNs need to respond with an increased inspiratory current to account for an increased nerve activity.

Five XII MNs (three identified as inspiratory) also were examined in the presence of 1.0 μM bath-applied TTX to confirm electrophysiologically the presence of postsynaptic ATP receptors. Neurons were identified as inspiratory or noninspiratory before application of TTX. Disappearance of XII nerve activity and inspiratory synaptic currents under voltage clamp and action potentials under current clamp confirmed adequate incubation in TTX. As shown for one inspiratory XII MN in Figure 3*C*, inward currents induced by ATP (10 mM) were similar in all MNs examined under TTX conditions. They averaged −89 ± 48 pA in amplitude and were associated with a significant 9.0 ± 6.0% decrease in input resistance.

As further confirmation of a postsynaptic effect of ATP on XII

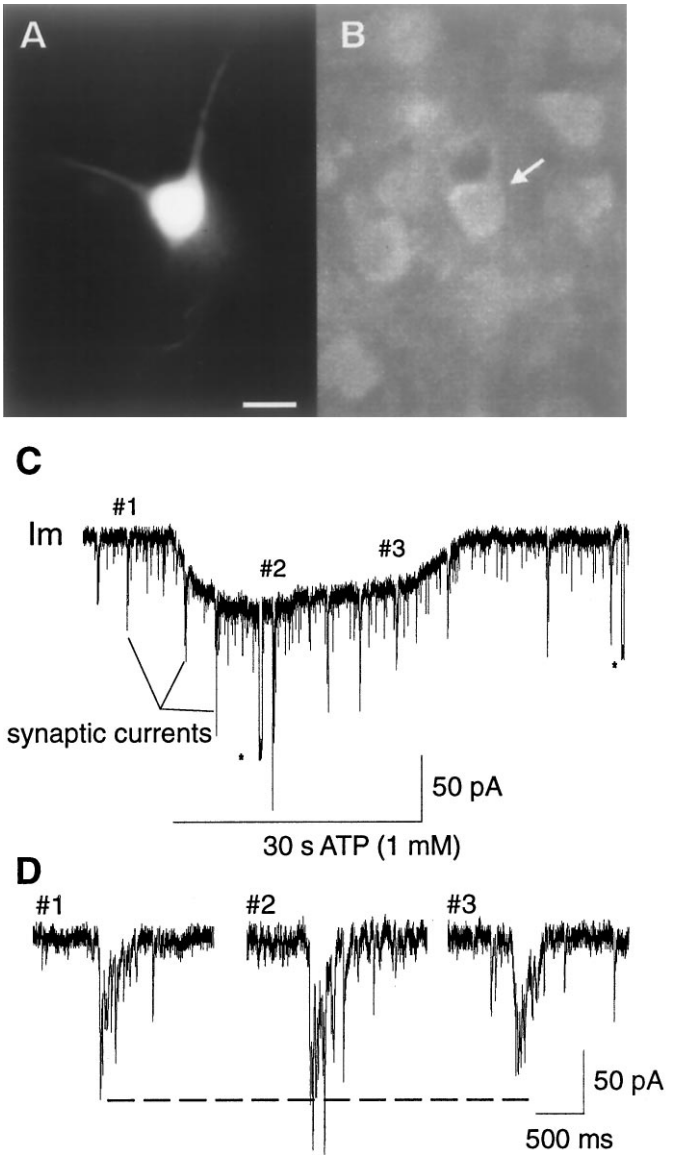

Figure 4. Lucifer yellow-labeled inspiratory MN (*A*) shows immunofluorescence for $P2X_2R$ (*B*, *arrow*). The neuron labeled in *A* and *B* responded to local application of 1 mM ATP with a 75 pA inward current and a decrease in input resistance (*C*). Inspiratory synaptic currents recorded during control (*#1*), early during ATP application (*#2*), and late in the ATP application (*#3*) are shown with an expanded time scale in *D*. Holding potential, −60 mV; peaks marked *synaptic currents* represent 3 of 11 inspiratory synaptic currents; *asterisk* equals −10 mV pulses for calculating neuronal input resistance. Scale bar in *A* (applies to *B*), 25 μm.

MNs, the distribution of $P2X_2$ receptors within the XII nucleus was examined immunohistochemically (see below, Fig. 7). In addition, three inspiratory XII MNs responsive to ATP were labeled with Lucifer yellow and examined immunohistochemically for $P2X_2$ receptor expression. We focused on $P2X_2$ receptors because (1) the relatively rapid time course of ATP effects suggested an ionotropic P2X receptor mechanism, (2) of the P2X receptors, only $P2X_2$ receptors are sensitive to suramin and PPADS antagonism (Buell et al., 1996; Collo et al., 1996), and (3) ATP excitation of XII nerve responses was suramin-sensitive (*in vitro* responses were also PPADS-sensitive). As illustrated for one MN (Fig. 4), all three ATP-sensitive inspiratory XII MNs were double-labeled, indicating $P2X_2$ receptor immunoreactivity.

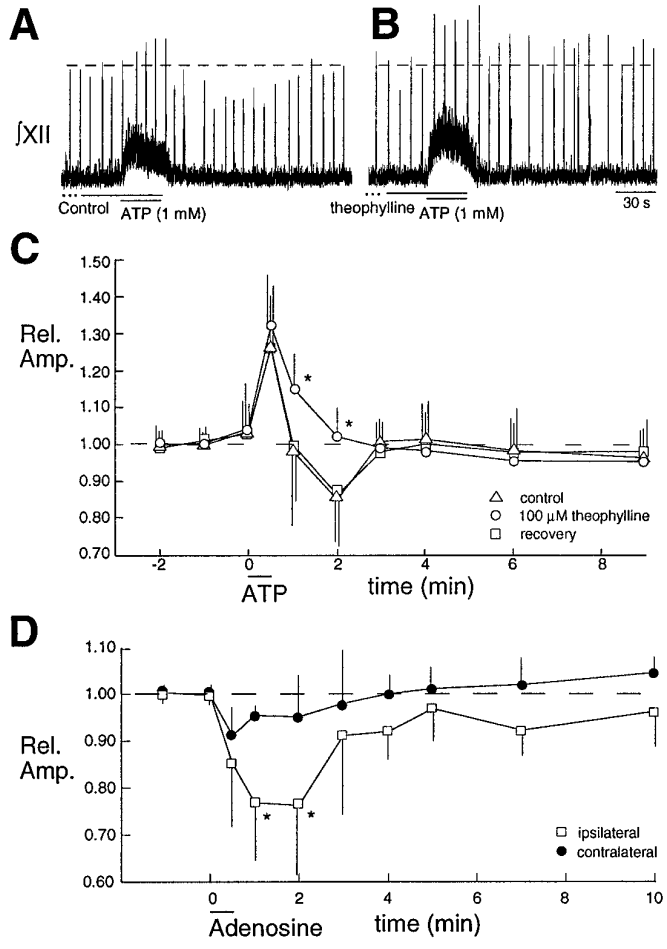

Figure 5. Post-ATP inhibition of inspiratory burst amplitude results from activation of A_1 adenosine receptors. *A*, Inhibition of inspiratory burst amplitude after 30 sec application of ATP is blocked (*B*) by 90 sec preapplication of theophylline (100 μM). *C*, Time course of the changes in XII nerve inspiratory burst amplitude after local application of ATP (1 mM) before, during, and after local application of theophylline (100 μM; $n = 8$); *asterisk* indicates significant difference from amplitude values observed at the same time during the control ATP application. *D*, Time course of the changes in XII nerve inspiratory burst amplitude after local application of adenosine (1 mM; $n = 6$); *asterisk* indicates significant difference from control, pre-ATP levels.

Adenosine mediates post-ATP inhibition of XII nerve burst amplitude *in vitro*

To test the hypothesis that the post-ATP inhibition of inspiratory burst amplitude results from the catabolism of ATP to adenosine and subsequent activation of P1 receptors, we applied ATP during the last 30 sec of 90 sec theophylline (A_1 adenosine antagonist) applications. The tonic excitatory and burst amplitude-potentiating components of the ATP response were enhanced only marginally by theophylline ($n = 8$; Fig. 5*A–C*). The post-ATP inhibition of burst amplitude, however, was abolished completely (Fig. 5*B,C*). At 2 min after the start of a 30 sec ATP application, burst amplitude in the absence of theophylline (0.85 ± 0.11) was significantly lower than in the presence of theophylline (1.02 ± 0.08), where it remained above control (pre-ATP) levels (Fig. 5*C*).

To verify that adenosine could mediate the post-ATP inhibition of burst amplitude, we applied adenosine locally (1 mM) over XII motor nuclei for 30 sec. Adenosine caused significant inhibi-

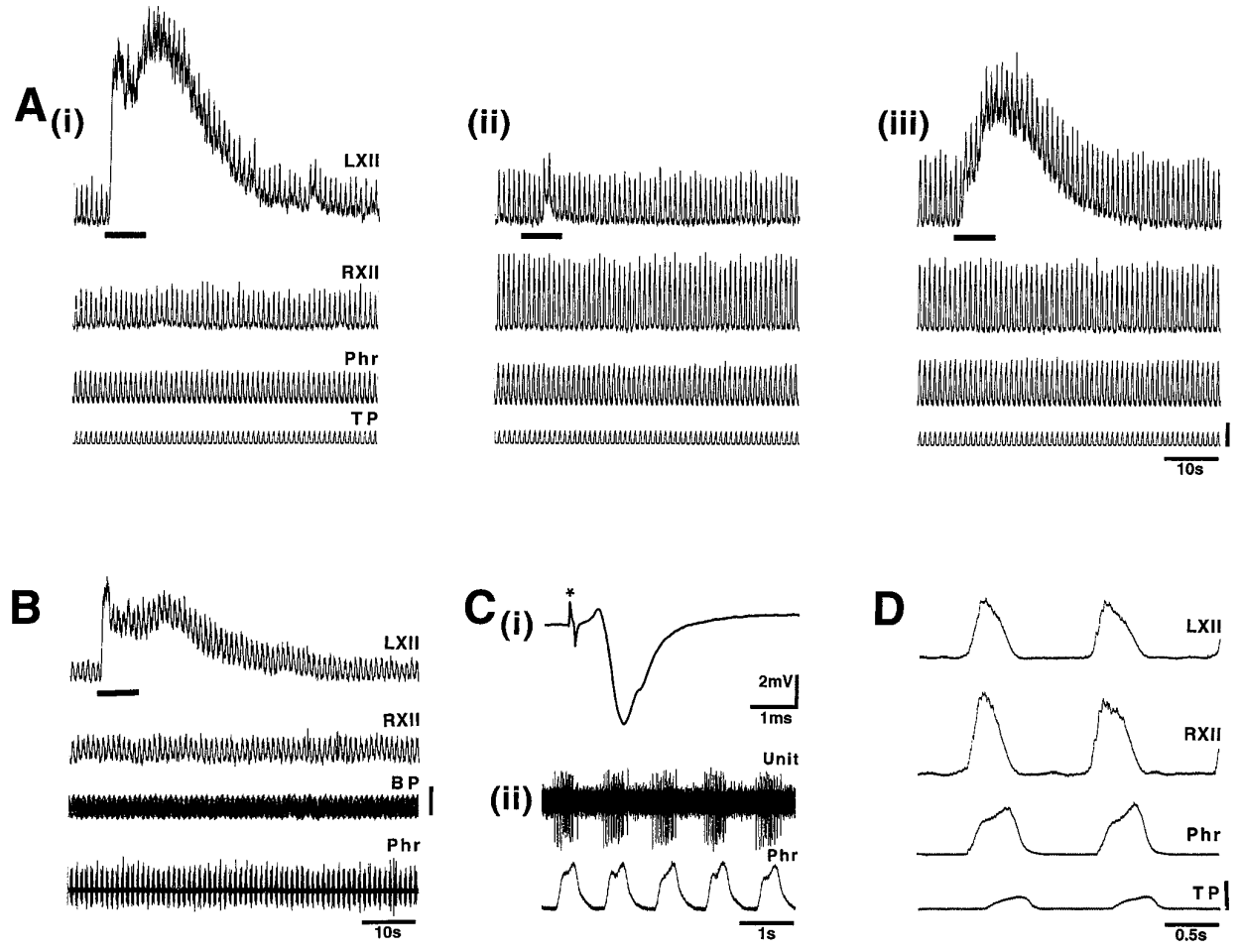

Figure 6. Excitation of XII nerve activity *in vivo*. *A*, Tonic excitation of the ipsilateral XII nerve activity after 7.6 sec application of 10 mM ATP into the left XII nucleus under control conditions (*i*), 10 min after a 7.6 sec application of 10 mM suramin (*ii*), and 30 min after suramin application (*iii*). *Traces* represent integrated activity from the left and right XII nerve (*LXII*, *RXII*), phrenic nerve (*Phr*), and tracheal pressure (*TP*; calibration, 0–10 cm H_2O). *B*, Application of 10 mM ATP into the rostral portion of the XII motor nucleus (1 mm rostral to obex) potentiated inspiratory burst amplitude. (Note that burst amplitude potentiation was observed in only 2 of 13 trials.) *Traces* represent integrated activity from the left and right XII nerve (*LXII*, *RXII*), arterial blood pressure (*BP*; calibration, 100–200 mm Hg), and raw phrenic nerve activity (*Phr*). *C*, Antidromic field potentials generated by stimulation of the ipsilateral XII nerve and recorded within the hypoglossal motor nucleus were used to indicate pipette placement (*i*). *ii*, An example of inspiratory-related extracellular action potentials (*Unit*) to verify ATP injection within the vicinity of inspiratory-modulated XII MNs. *D*, Expanded time scale recording showing the discharge envelope of inspiratory activity recorded bilaterally from XII nerves and unilaterally from the phrenic nerve (end-tidal CO_2, 5.5%).

tion of ipsilateral XII nerve inspiratory burst amplitude in all cases ($n = 6$; Fig. 5*D*). The maximum decrease in burst amplitude to 0.77 ± 0.12 of control was reached 1 min after onset of adenosine application. Burst amplitude then returned to control levels over the next 10 min.

ATP mediates tonic excitation of XII nerve activity *in vivo*

The effects of ATP on XII nerve output also were examined *in vivo*. At end-tidal $PCO_2 \geq 5\%$, robust inspiratory activity was present on XII and phrenic nerves (Fig. 6*A,B,D*). Similar to *in vitro* findings, application of ATP within the XII motor nucleus produced potent excitation of XII nerve output in 13 of 13 sites. The response was dominated by an increase in tonic discharge, apparent as a rapid shift in the baseline of the rectified, integrated XII nerve trace (Fig. 6*A,B*). In 11 of 13 sites a biphasic excitatory response was observed in which an initial rapid depolarization, which peaked within 1 sec, began to decay in 2–3 sec, giving way to a slower time course excitation that lasted ~30 sec (Fig. 6*Ai,B*). Injection into the two remaining sites elicited a rapid response in one case and a slower response in the other. Repetitive ATP applications presented at 5 min intervals produced consistent responses. The excitation was exclusively ipsilateral, indicating minimum diffusion of ATP away from the injection site. Blood pressure and heart rate were unaffected.

Suramin, which caused a brief increase in tonic discharge when injected alone, completely or partially blocked the excitatory effects of ATP in eight of nine trials (Fig. 6*Aii*). Full recovery from suramin block was observed in seven of eight trials (Fig. 6*Aiii*) after 20–60 min washout. PPADS antagonism of ATP responses was not examined *in vivo* because of the extended periods required for recovery *in vitro* and the inability to establish drug concentration within the tissue after local injection (see Drug Application in Materials and Methods).

In contrast to the uniform potentiation of burst amplitude observed *in vitro*, inspiratory burst amplitude was affected minimally by ATP *in vivo*, increasing in 2 of 13 trials (Fig. 6*B*).

Similarly, the post-ATP decrease in inspiratory burst amplitude observed *in vitro* was not observed *in vivo* (Fig. 6*A,B*).

In comparing *in vitro* versus *in vivo* results, it is important to consider that whole XII nerve activity reflects activity of inspiratory as well as noninspiratory MNs excited by ATP. Thus, the relative magnitude of the inspiratory potentiation versus tonic excitation should increase with an increase in the proportion of inspiratory XII MNs activated by ATP. Consistent with this hypothesis, application of ATP over the ventromedial inspiratory aspect of the nucleus, but not the dorsal aspect, *in vitro* potentiated inspiratory amplitude. Optimal placement of the drug pipette within inspiratory regions of the XII motor nucleus is more difficult *in vivo*. Thus, minimal burst amplitude potentiation and absence of post-ATP inhibition *in vivo* may reflect that ATP and its metabolite, adenosine, primarily activated noninspiratory MNs.

Therefore, to enhance activation of inspiratory versus noninspiratory MN pools, inspiratory-related XII MN discharge was recorded extracellularly before ATP injection in two experiments (Fig. 6*Cii*). Inspiratory-related XII MN discharge was observed in only a few recording positions at ~1 mm rostral to obex in the ventromedial part of the nucleus. Injection into the inspiratory regions identified in this way did not potentiate inspiratory amplitude.

In addition, end-tidal CO_2 was elevated to 5.5% in two trials to recruit inspiratory XII MNs and thus increase the relative size of the inspiratory MN pool. That elevated CO_2 recruited XII MNs during inspiration was suggested by an increase in integrated XII nerve inspiratory burst amplitude. However, this was not associated with ATP-induced increases in burst amplitude.

Immunohistochemical and RT-PCR detection of $P2X_2$ receptors in XII motoneurons

To verify further P2 receptor involvement in the XII nerve responses as well as a postsynaptic site of action, we examined the distribution of $P2X_2$ receptors within the XII motor nuclei of neonatal mice and adult rats. As described previously, we focused on $P2X_2$ subunit expression because the speed of the ATP responses and their suramin/PPADS sensitivity were consistent with $P2X_2$ receptor activation (Buell et al., 1996; Collo et al., 1996). A specific antiserum ($P2X_2R96$; Kanjhan et al., 1996) recognizing residues 96–113 of the $P2X_2$ receptor subunit was used.

Immunostaining was observed throughout the XII motor nuclei of both neonatal mice ($n = 3$; Fig. 7*A–C*) and adult rats ($n = 3$, Fig. 7*D–F*). Staining of individual XII MNs was variable, but, in general, XII MNs in all regions of the nucleus had densely stained cell bodies and proximal dendrites. There were no obvious subpopulations of MNs that failed to show immunoreactivity. Cytoplasmic staining was more intense than nuclear labeling (Fig. 7*C,E*). Control sections, incubated with PBS without $P2X_2$ receptor antiserum, showed no evidence of staining (data not shown). In addition, immunostaining was blocked significantly by preincubation of antiserum with the target peptide (Fig. 7*F,G*).

Expression of mRNA for the $P2X_2$ receptor subunit in micropunches of adult rat XII nucleus is also consistent with ATP acting via $P2X_2$ receptors (Fig. 8). Direct sequencing identified three isoforms of the $P2X_2$ subunit. In addition to the original isoform cloned from PC12 cells [Brake et al. (1994), GenBank U14414], two splice variants [Housley et al. (1995), GenBank L43511; Brändle et al. (1997), GenBank Y09910] were expressed in the hypoglossal nucleus. The two latter $P2X_2$ isoforms have not been localized previously in the CNS. Whether a single XII MN expresses all three isoforms remains unclear because the $P2X_2$ antiserum does not discriminate among these isoforms. The XII nucleus is a relatively homogeneous population of cells with few interneurons (Viana et al., 1990). Thus, although the source of the $P2X_2$ mRNA in these experiments remains equivocal, its most likely source is from XII motoneurons. Control reactions omitting first-strand cDNA synthesis before PCR failed to yield PCR products, confirming reaction specificity for $P2X_2R$ mRNA.

DISCUSSION

Mechanisms of ATP-induced excitation

Tonic depolarization

The tonic depolarizing effect of ATP on XII MNs may have several components, because P2 receptor agonists excite neurons via several actions. They inhibit (1) a resting K^+ current in central catecholaminergic neurons (Harms et al., 1992), (2) a calcium-sensitive K^+ current in myenteric ganglion cells (Katayama and Morita, 1989), and (3) an M-type K^+ current in sympathetic ganglion cells (Akasu et al., 1983). They also activate (1) a nonselective cationic conductance with a large Ca^{2+} permeability in cochlear hair cells (Thorne and Housley, 1996), (2) a cationic conductance in rat sensory neurons (Krishtal et al., 1988), and (3) Ca^{2+}-sensitive Na^+ channels and Ca^{2+}-sensitive nonselective cationic channels in locus coeruleus neurons (Harms et al., 1992). ATP activation of an inward current via increased conductance in XII MNs (Figs. 3, 4) (as well as substantia gelatinosa neurons; Li and Perl, 1995) is more consistent with a mechanism involving P2 receptor activation of a cationic conductance.

The effects of ATP on respiratory-related neurons is unclear. ATP, acting via P2 receptors, excites putative respiratory and cardiovascular neurons of the nucleus tractus solitarius (Ueno et al., 1992) as well as putative cardiovascular neurons of the rostroventrolateral medulla (Ralevic et al., 1996). MNs express mRNA for several subunits of P2X receptor (Collo et al., 1996), but direct evidence of P2 receptor-mediated excitation of MNs is limited to the activation of inward currents in MNs dissociated from the dorsal motor vagus of rat (Ueno et al., 1996) and XII MNs in slices from neonatal mouse (present study). That XII MNs acutely dissociated from rat are unresponsive to ATP (Ueno et al., 1996) is difficult to explain, given the powerful excitatory effects of ATP on XII MN activity observed in our study.

Potentiation of hypoglossal inspiratory activity

Inspiratory drive to XII MNs is almost entirely glutamatergic (Greer et al., 1991; Funk et al., 1993). Thus, the ATP potentiation of inspiratory XII nerve and MN activity (Fig. 1) *in vitro* supports P2 receptor potentiation of glutamatergic synaptic transmission. In the substantia gelatinosa ATP not only activates a fast inward current, it also potentiates glutamate-induced and synaptically evoked glutamate currents (Li and Perl, 1995). Inhibition of evoked glutamatergic EPSCs and glutamate-induced currents in CA3 pyramidal cells by P2 antagonists also supports purinergic potentiation of glutamatergic synaptic transmission (Motin and Bennett, 1995).

Pre- and postsynaptic mechanisms could contribute to this potentiation. A postsynaptic action of ATP on XII MNs is indicated by depolarization and decreased input resistance under TTX (Fig. 3). Reduction of evoked EPSCs and glutamate-evoked currents by suramin and reactive blue 2 in hippocampal CA3 neurons (Motin and Bennett, 1995) and ATP potentiation of glutamate currents and glutamate-evoked EPSCs in neurons of the substantia gelati-

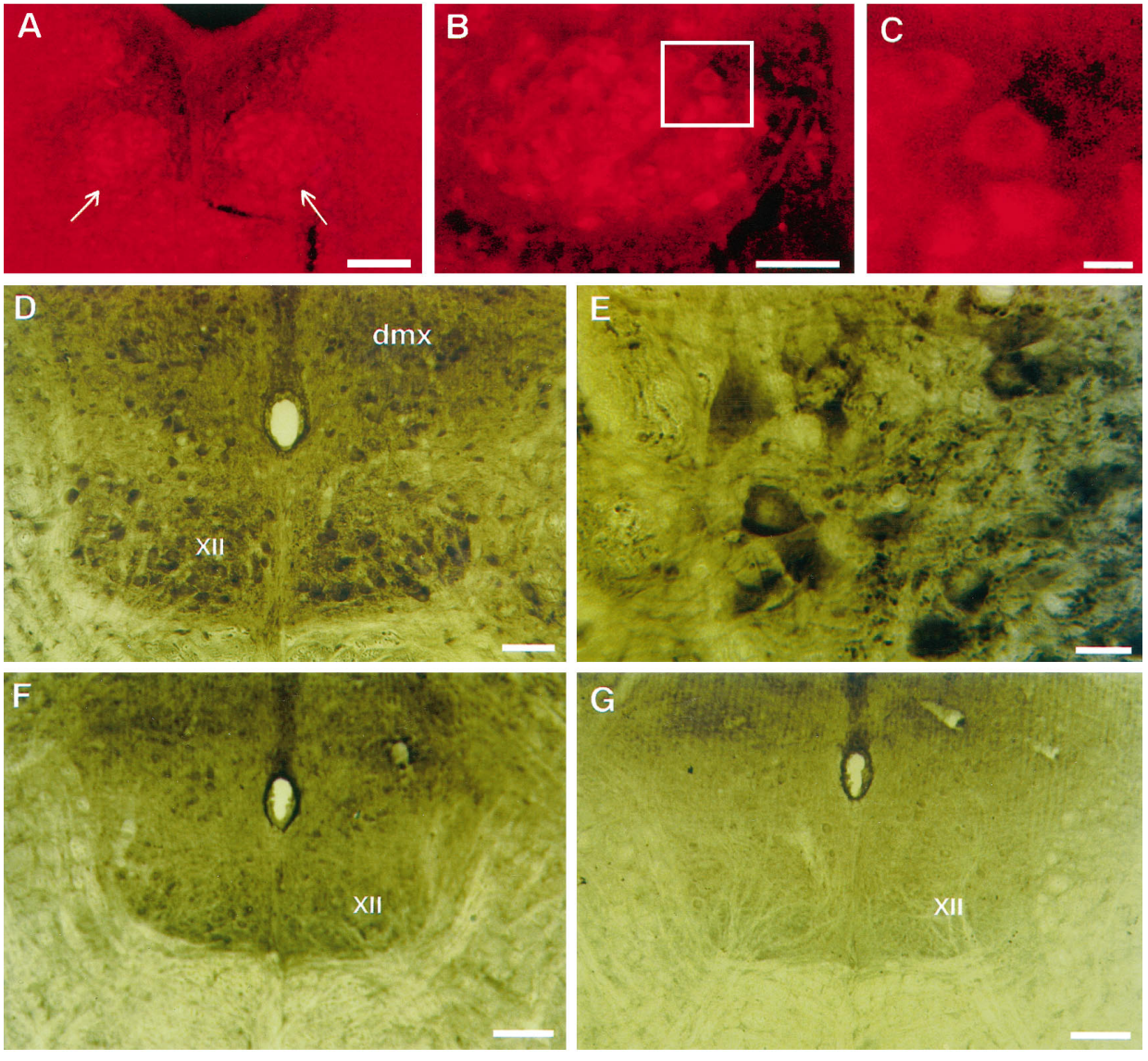

Figure 7. $P2X_2R$ immunostaining in the neonatal mouse and adult rat XII nucleus. *A–C*, Transverse sections through postnatal day 2 neonatal mouse medulla ~100 μm rostral to obex, showing $P2X_2R$ TRITC immunofluorescence (1:100 $P2X_2$96ab) at increasing magnification. The *white box outline* in *B* is the region shown in *C* to illustrate greater cytoplasmic versus nuclear staining. *D*, *E*, Transverse sections through adult rat medulla ~100 μm caudal to obex, showing $P2X_2$ receptor immunoperoxidase staining (1:2000 $P2X_2$96ab) of XII MNs. *F*, *G*, Transverse sections through adult rat medulla immediately caudal to obex, showing $P2X_2$ receptor immunoperoxidase staining of XII MNs under test conditions (1:4000 $P2X_2$R96ab) (*F*) and block of the immunolabeling by antiserum preadsorbed with its target peptide (*G*). Scale bars in *A–G* represent 100, 50, 10, 100, 20, 150, and 150 μm, respectively. *dmx*, Dorsal motor nucleus of vagus; *XII*, hypoglossal motor nucleus (*arrows* in *A*).

nosa (Li and Perl, 1995) are also consistent with postsynaptic P2 receptor potentiation of glutamate responses.

A presynaptic contribution to potentiation of glutamatergic transmission, however, cannot be ruled out (Li and Perl, 1995). Blocking of evoked glutamate EPSCs in CA3 pyramidal neurons, but not glutamate-induced currents, by a P2X antagonist suggests endogenous presynaptic potentiation of glutamate release by P2 receptors (Motin and Bennett, 1995).

Receptors mediating effects of ATP

Inhibition of the excitatory effects of ATP on hypoglossal activity by suramin and PPADS is most consistent with ATP acting on P2 receptors. Suramin inhibition of ectonucleotidase activity (Kennedy et al., 1996) would reduce adenosine production; however, adenosine inhibits glutamatergic inputs to XII MNs (Fig. 5; Bellingham and Berger, 1994).

The kinetics of the ATP-mediated excitation are consistent with activation of an ionotropic mechanism. ATP-mediated excitatory responses in XII nerve were substantially faster in onset and shorter in duration than second-messenger-mediated XII nerve responses elicited by norepinephrine and TRH in identical slice preparations (Funk et al., 1994). *In vivo* responses to ATP were even faster (Fig. 6). The time course data, however, are not conclusive evidence for involvement of P2X receptors because of the limited temporal resolution associated with applying drugs over thick tissue. A P2Y contribution remains possible and may be reflected in the slower time course excitatory response observed *in vivo* (Fig. 6*Ai,B*) and occasionally *in vitro*.

The sensitivity of ATP-mediated responses to PPADS in the low micromolar range provides additional support for $P2X_2$ receptor involvement in XII MN excitation by ATP. PPADS has

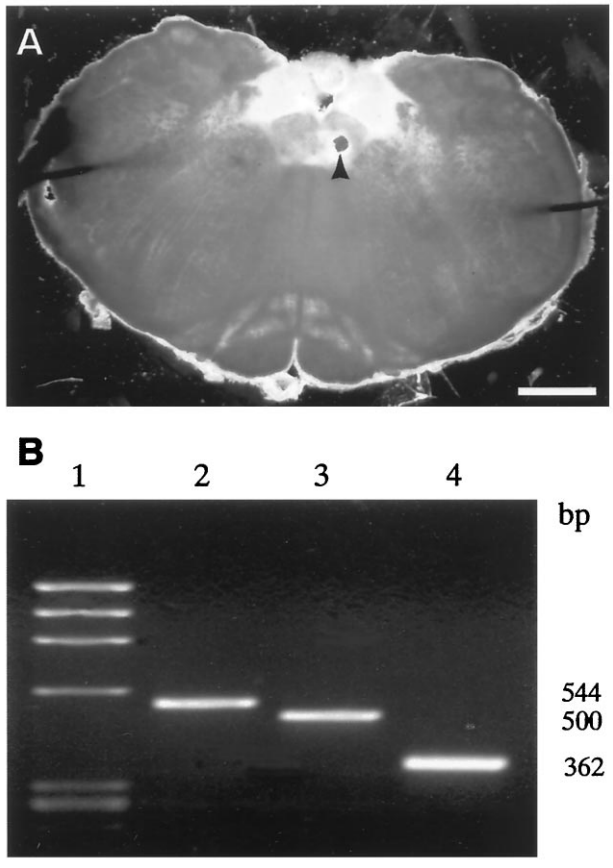

Figure 8. Identification of mRNA for $P2X_2$ receptor subunit isoforms in micropunches from the hypoglossal nucleus. *A*, Dark-field photomicrograph of 240 μm transverse medullary section showing location of micropunch (*arrowhead*). *B*, Ethidium bromide-stained gel showing the PCR products for three $P2X_2$ receptor subunit isoforms isolated from the micropunch. *Lane 1*, Molecular weight marker (ϕX174/*Hae*III; BRL, Bethesda, MD). *Lane 2*, The 85 bp insert isoform (Housley et al., 1995) obtained by using primers 1558as/S4. *Lane 3*, Isoform (Brake et al., 1994) obtained by using primers 1558as/S3. *Lane 4*, Isoform (Brändle et al., 1997) with 207 bp exon deletion obtained by using primers 1558as/S1. Lengths of fragments are indicated on the *right*. Calibration bar in *A*, 1 mm.

been used as a specific P2X receptor antagonist in a number of systems (Lambrecht et al., 1992; Trezise et al., 1994; Ziganshin et al., 1994; Connolly, 1995). However, the recent description of partial P2Y receptor antagonism by PPADS in aortic endothelial cells (Brown et al., 1995) and mesenteric arteries (Windscheif et al., 1994; Ralevic and Burnstock, 1996) questions PPADS selectivity for P2X receptors.

Although pharmacological data remain equivocal, specific involvement of $P2X_2$ receptors in XII MN excitation is supported by (1) $P2X_2$ mRNA within the XII nucleus (Fig. 8; Collo et al., 1996) and (2) $P2X_2$ receptors on ATP-sensitive inspiratory XII MNs (Fig. 4). P2X receptor expression within the XII nucleus is not, however, limited to the $P2X_2$ subunit. $P2X_2$ receptor currents show substantial inward rectification (Brake et al., 1994). Because ATP-gated ion channels are hypothesized to form from pentameric assembly of subunits, minimal rectification of XII MN responses to ATP supports heteromeric assembly of $P2X_R$ subunits, possibly involving $P2X_4$ (Séguéla et al., 1996) and $P2X_6$ (Chang et al., 1995) subunits. Heteromeric assembly is consistent with *in situ* hybridization data (Collo et al., 1996) indicating expression of $P2X_2$, $P2X_4$, and $P2X_6$ receptor subunits on XII MNs.

Mechanisms underlying secondary inhibition

Increases in synaptic adenosine resulting from hydrolysis of ATP (Gibb and Halliday, 1996) primarily inhibit synaptic transmission via activation of presynaptic A_1 receptors (Meriney and Grinnell, 1991; Lupica et al., 1992; Prince and Stevens, 1992; Burnstock, 1995; Dong and Feldman, 1995). The excitatory effects of ATP on XII nerve output *in vitro* are associated with a slowly developing inhibition of inspiratory activity. The secondary depression may result from diffusion of ATP and activation of nearby inhibitory interneurons (Umemiya and Berger, 1995). However, it is more consistent with hydrolysis of ATP to adenosine and $P1(A_1)$ receptor inhibition of glutamatergic activity, as seen in the substantia gelatinosa (Li and Perl, 1995) and striatum (James and Richardson, 1993).

First, excitation of hypoglossal activity was exclusively ipsilateral, indicating minimal diffusion of ATP. Second, suramin blocked the excitatory response but had minimal effects on inhibitory response, consistent with separate receptor mechanisms. Third, the inhibitory component was blocked by preapplication of the specific A_1 adenosine receptor antagonist theophylline (Fig. 5*A–C*). Fourth, adenosine inhibited XII nerve inspiratory output (Fig. 5*D*) and glutamatergic inputs to XII MNs (Bellingham and Berger, 1994).

In vitro versus *in vivo* experiments

ATP potently excited XII nerve discharge *in vitro* and *in vivo*. The amplitude potentiation present *in vitro*, however, was observed only in 2 of 13 trials *in vivo*. Similarly, the post-ATP inhibition of inspiratory burst amplitude was not observed *in vivo*. Several factors may contribute to these differences.

The minimal effects, positive or negative, of ATP on burst amplitude *in vivo* do not seem to result solely from disproportionate activation of noninspiratory MNs *in vivo* (see Results). Attempts to maximize the activation of inspiratory MNs did not enhance amplitude potentiation. It remains possible that longer *in vitro* applications of ATP facilitated drug diffusion, activating a greater portion of the XII MN pool. The higher concentration of ATP used *in vivo* also may have obscured amplitude potentiation through depolarization block.

Alternatively, developmental variation in purinoceptor expression may contribute to differences between neonatal and adult animals (see Evans and Surprenant, 1996). Reduction in the potentiating effect of ATP in the adult may reflect a developmental reduction in a receptor subunit that specifically mediates the inspiratory-potentiating component of the response. Similarly, the reduction in the post-ATP inhibitory response may result from developmental variation in degradation and reuptake systems for ATP and adenosine (Geiger and Nagy, 1990). Postnatal decreases in adenosine receptor expression are unlikely to contribute because A_1 receptor expression within most brain regions, including the XII motor nucleus, does not decrease postnatally (Daval et al., 1991; Rivkees, 1995; Weaver, 1996).

Despite the differences between the *in vivo* and *in vitro* responses to exogenous ATP application, it is clear that in all systems tested ATP potently excites XII nerve activity.

Functional significance of ATP as a transmitter/modulator within the respiratory motor system

Localization of mRNA for P2X receptor subunits within most cranial and spinal motor nuclei (Collo et al., 1996) implicates a general function of purinoceptors in controlling motor outflow

from the CNS. In fact, recent evidence indicates purinergic regulation of motor pattern generation in *Xenopus* embryos (Dale and Gilday, 1996).

A potentially important role for P2 receptor synaptic signaling in respiratory motor control is suggested by the multiple physiological effects of ATP on hypoglossal activity reported here; the presence of $P2X_2$, $P2X_4$, and $P2X_6$ receptor mRNA in nucleus ambiguus and the hypoglossal nucleus (Collo et al., 1996), regions containing upper airway MNs; RT-PCR identification of ATP receptor isoforms in punches of XII nucleus (Fig. 8); and immunohistochemical detection of $P2X_2$ receptor protein on identified inspiratory XII MNs.

The precise function of purinoceptor synaptic signaling in upper airway control, however, remains unclear. ATP may act as a modulator of glutamatergic inspiratory drive to XII MNs (Li and Perl, 1995). Colocalization of ATP and NE in locus coeruleus neurons (Nieber and Illes, 1996) is consistent with ATP modulation of inspiratory XII MN function because the XII nucleus receives NE inputs (Levitt and Moore, 1979; Aldes et al., 1992) and NE excites XII MNs (Parkis et al., 1995) and potentiates inspiratory activity (Funk et al., 1994). However, given that XII MNs also have nonrespiratory functions and that ATP acts as the principal fast excitatory transmitter at other central synapses (Edwards et al., 1992; Evans et al., 1992), ATP also may act as a synaptic mediator for nonrespiratory inputs to XII MNs.

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