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ATP Receptor-Mediated Enhancement of Fast Excitatory Neurotransmitter Release in the Brain

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ABSTRACT

ATP-gated cation channels (P2X receptors) exist on the soma of proprioceptive neurons in the trigeminal mesencephalic nucleus (MNV) in the brain stem. However, these pseudomonopolar neurons seem to receive no synaptic input to their soma; we therefore hypothesized that in MNV neurons, the P2X receptors of importance may be those located on their central terminal projections. Here, we show in trigeminal mesencephalic motor nucleus neurons, which receive their major input from the MNV, that both exogenous ATP (1 mM) and high frequency focal stimulation to evoke endogenous ATP release enhanced the frequency of spontaneous fast excitatory postsynaptic currents (EPSCs) with no change in their amplitude. The enhancement was reduced by the antagonists suramin (300 μM) and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (30 μM) and persisted when action potential conduction was blocked with tetrodotoxin (1 μM). Thus, functional P2X receptors are expressed on nerve terminals in the brain stem, where they increase the spontaneous release of glutamate onto trigeminal mesencephalic motor nucleus neurons.

ATP functions as an extracellular signaling molecule by acting at specific cell surface metabotropic and ionotropic P2 receptors (North and Barnard, 1997). The ionotropic receptors belong to the superfamily of ligand-gated ion channel proteins and are ATP-gated nonselective cation channels that have been termed P2X receptors [see Burnstock (1996) and North (1996) for reviews]. Molecular gene cloning over the last 4 years has been used to identify seven distinct P2X receptor subunits that can form functional channels in heterologous expression systems (North and Barnard, 1997, and references therein). Although our understanding of the molecular identity and structure/function relationships of this family of ion channels has increased rapidly over the last few years, our understanding of the physiological roles of P2X receptors in synaptic transmission is limited. Furthermore, we need to relate the properties of native P2X receptors to those studied in heterologous expression systems.

Evidence that extracellular ATP can function as a neurotransmitter dates back to pioneering work 25 years ago (Burnstock, 1972). More recent studies confirm these early observations and indicate that ATP acts as a fast synaptic transmitter in many peripheral and central neurons (Edwards *et al.*, 1992; Evans *et al.*, 1992; Galligan and Bertrand, 1994; Bardoni *et al.*, 1997; Nieber *et al.*, 1997). In addition, recent evidence shows that P2X receptors also exist on nerve terminals at neuro-neuronal synapses of cultured dorsal root ganglion and spinal dorsal horn neurons (Gu and MacDermott, 1997), which raises the possibility that nerve terminal P2X receptors may also exist in the brain, where they may be a target for endogenously released ATP.

MNV neurons in the brain stem constitute a pure population of proprioceptors; recently, P2X receptors were identified in MNV neurons (Cook *et al.*, 1997; Khakh *et al.*, 1997). We have since attempted to identify P2X receptor-mediated synaptic transmission in the MNV nucleus but have found no evidence to support such a role. A number of studies aimed at mapping the distribution of P2X receptors in the central nervous system indicate that P2X receptors are widespread (Kidd *et al.*, 1995; Collo *et al.*, 1996; Vulchanova *et al.*, 1996) and can be localized to nerve terminals (Vulchanova *et al.*, 1996, 1997; Le *et al.*, 1998). In light of these observations, we hypothesized that in MNV neurons, the P2X receptors of possible physiological importance may be those located on their central nerve terminals in the MoV (Dessem *et al.*, 1997).

In the present study, we have examined the effects of ATP

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ABBREVIATIONS: MNV, trigeminal mesencephalic nucleus; MoV, trigeminal mesencephalic motor nucleus; sEPSC, spontaneous excitatory postsynaptic current; mEPSC, mini excitatory postsynaptic current; TTX, tetrodotoxin; ACSF, artificial cerebrospinal fluid; ATPγS, adenosine-5'-O-[-3-thiotriphosphate]; $\alpha\beta$meATP, $\alpha\beta$-methylene adenosine-5'-triphosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPADS, pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonic acid.

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on spontaneous glutamate release from MNV nerve terminals in the MoV and have sought to determine whether the effects of exogenous ATP can be mimicked by endogenous ATP released upon nerve stimulation.

Materials and Methods

Experiments were performed using 180–200 μm slices of brain stem containing the MoV nucleus (Min and Appenteng, 1996) from Wistar rats of either sex (6–7 days old; killed by cervical dislocation and decapitation), cut in an orientation to ensure that cell bodies of MNV neurons were absent from the slice. Slices were cut at 4° in gassed (95% O_2/5% CO_2) ACSF solution composed of 126 mM NaCl, 2.5 mM KCl, 1.24 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM D-glucose using a Vibratome (Ted Pella, Inc., Redding, CA). Slices were transferred to a recording chamber mounted on a Zeiss Axioskop microscope (0.5-ml chamber volume) and continually superfused (2.5 ml/min) with gassed ACSF at 30°. Only one slice that contained the MoV nucleus could be obtained from each animal. Cells were viewed using Nomarski optics and MoV neurons were identified by the location of the nucleus and by their electrophysiological properties (Min and Appenteng, 1996). Whole cell recordings were made using fire-polished borosilicate glass electrodes of 2.5 MΩ resistance. The pipette filling solution had the following composition: 130 mM K^+ or CsCl, 5 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM Na^+-GTP and 5 mM Mg^{2+}-ATP. Whole-cell recordings were made using the blow-and-seal method on visually identified neurons. Series resistance values were 6–12 MΩ; cells were accepted for analysis only if this did not change by more than 15% during the recording (generally about 20 min). Recordings were filtered at 2–5 kHz using an Axopatch 200B amplifier, saved to digital audio tape and computer for later analysis (digitized at 5–15 kHz). Spontaneous synaptic events were detected semiautomatically using software provided by Dr. Sergei Kasporov (Department of Physiology, University of Bristol) but all were subjected to visual inspection before acceptance. Electrical focal stimu-

lation was applied using a patch clamp electrode filled with ACSF and positioned onto the slice 100 to 200 μm away from the neuron under investigation. We initially varied the number of pulses during focal stimulation and subsequently used 10 pulses (pulse width 0.05 ms, amplitude 100 V using a Grass S88 stimulator) at 200 Hz as standard; these parameters produced a robust increase in the frequency of sEPSCs. These parameters evoked clear excitatory synaptic currents in this nucleus but did not trigger action currents in the postsynaptic cell. We analyzed sEPSCs that occurred during a 6-sec period before and after focal stimulation. The focal stimulation-evoked increase in sEPSCs frequency decayed within 1–10 sec.

All drugs were kept frozen ($-20°$) as stock solutions until required and then were diluted to the appropriate final concentrations and applied in the superfusing fluid. Drug would reach the recording chamber after a time lapse of 30 sec to 1 min after switching and complete exchange took less than 1 min. ATP, ATPγS, $\alpha\beta$meATP, and DPCPX were obtained from Sigma (Poole, Dorset, UK); suramin was a generous gift from Bayer (Newbury, UK) and PPADS was from Tocris (Bristol, UK). Statistical significance was determined using Student's t test and the effect of ATP on frequency and amplitude of mini-EPSCs was tested using cumulative probability plots and Kolmogorov-Smirnoff analysis (Lena and Changeux, 1997).

Results

Whole-cell recordings were made from rat MoV neurons in thin brain stem slices cut in an orientation to ensure that MNV neurons were absent from the brain slice. All studies were performed in the presence of bicuculline (10 μM), strychnine (10 μM), and DPCPX (10 μM) to ensure that γ-aminobutyric acid$_A$, glycine, and adenosine receptors were blocked. Under these conditions, sEPSCs were observed in all cells. These were completely blocked by the ionotropic glutamate receptor antagonists 6-cyano-2,3-dihydroxy-7-nitroquinoxaline and D-2-amino-5-phosphonovaleric acid (10 μM; four of
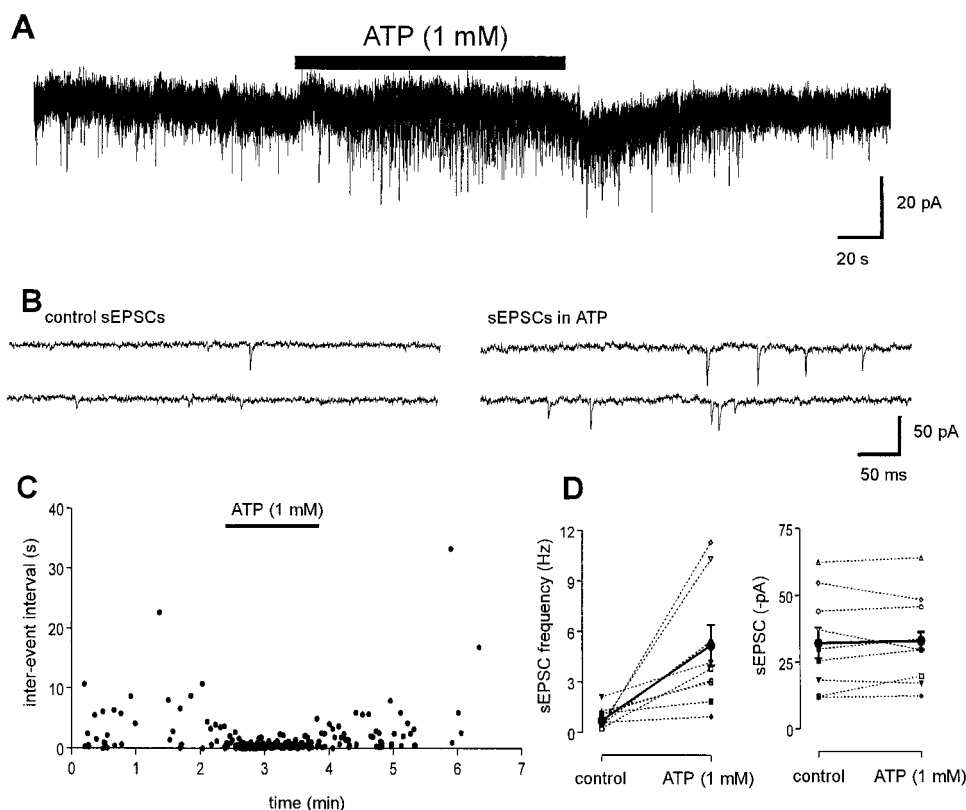

Fig. 1. ATP (1 mM) increased the frequency of glutamatergic EPSCs in MoV neurons. A, A slow time scale recording of the ATP-evoked increase in sEPSCs from a MoV neuron with more frequent downward deflections (EPSCs) occurring during the period of ATP application. B, sEPSCs recorded on a faster time scale before (*left traces*) and during (*right traces*) the application of ATP. C, Graph of interevent interval against time for the cell shown in A. *Points*, sEPSCs. During the application of ATP, the interevent interval was reduced and there was no apparent diminution in the response throughout the exposure to agonist. D, Data from a population of 10 cells showing the increase in sEPSC frequency (*left graph*) with no change in the amplitude (*right graph*). *Solid lines*, mean data ± standard error for the 10 neurons illustrated. For these and subsequent graphs, data were obtained from a 1-min period before and during the peak response to ATP.

four neurons tested; data not shown). The frequency and amplitude of sEPSCs varied from cell to cell with a mean of 0.71 ± 0.20 Hz (range, 0.15–2.12 Hz; $n = 10$ neurons) and -32.1 ± 5.8 pA (-12 to -62 pA; $n = 10$ neurons), respectively.

In 69% of neurons (20 of 29; no effect in nine of 29 neurons), bath application of ATP (1 mM) caused a significant increase in the frequency of sEPSCs (Fig. 1A). This was reflected as a decrease in the inter-event interval (Fig. 1C) and amounted to a 11 ± 4 fold increase in mean frequency (Fig. 1C; $n = 10$; $p < 0.05$). ATP did not change the amplitude of sEPSCs (Fig. 1D). The increase in sEPSC frequency occurred rapidly (within 0.5 to 1 min of exposing the slice to ATP), persisted throughout a 2–3 min application and reversed rapidly on washout of ATP (Fig. 1C). ATP (1 mM) could be applied repeatedly for 3 min every 10 min without any decrease in the evoked increase in sEPSC frequency. The increase in frequency produced by ATP was mimicked by ATPγS (100 μM; 2.6 ± 0.5 fold increase; $p < 0.05$; $n = 4$) and by the P2X receptor-selective agonist αβmeATP (300 μM; 1.5 ± 0.1 fold increase $p < 0.05$; $n = 4$); both these agonists were also effective at evoking inward currents in the cell bodies of MNV neurons (17, 18), the terminals of which innervate the MoV. For both ATPγS and αβmeATP, there was no significant change in the amplitude of the sEPSCs (change = 0.1 ± 0.01-fold; $n = 8$; pooled data for ATPγS and αβmeATP).

We next determined whether antagonists at P2X receptors could block the ability of ATP to increase the frequency of sEPSCs. Suramin (300 μM) itself decreased the frequency of sEPSCs in 50% of neurons (before suramin 0.7 ± 0.3 Hz, in the presence of suramin 0.1 ± 0.03 Hz; $p < 0.05$; $n = 4$; see Nakazawa et al., 1995 for evidence that suramin can directly block glutamate receptor channels). However, in the presence of suramin, ATP (1 mM) did not increase the frequency of sEPSCs (Fig. 2a). PPADS (30 μM) produced no effect on the frequency of sEPSCs (from 0.5 ± 0.3 Hz before PPADS to 0.5 ± 0.5 Hz in the presence of PPADS; $n = 4$) but significantly attenuated the response to ATP (Fig. 2A). Suramin and PPADS did not markedly affect the amplitude of sEPSCs (Fig. 2B).

To test whether ATP could increase the frequency of mEPSCs when action potential conduction was blocked experiments were performed in the presence of TTX (0.5–1 μM). This concentration of TTX can completely block all evoked EPSCs in MoV neurons (data not shown). Applying ATP (1 mM) in the presence of TTX (0.5–1 μM) evoked a 2.6 ± 0.5 fold increase ($p < 0.05$) in the frequency of mEPSCs with no change in their amplitude in seven of seven neurons (Fig. 3, A-D). The ability of ATP to enhance neurotransmitter release was completely blocked by cadmium, a broad-spectrum calcium channel blocker (Fig. 3, E-H; $n = 6$), which, at the low concentration used (100 μM), does not affect calcium influx through P2X receptors (Inoue et al., 1995). Cadmium alone did not alter the frequency or amplitude of mEPSCs (frequency was 1.3 ± 0.6 Hz in control (TTX alone) and 1.2 ± 0.6 Hz in cadmium, the amplitude was -16.5 ± 2 pA in control (TTX alone) and -15.5 ± 1.7 pA in cadmium).

To determine whether it is possible to evoke endogenous ATP release from within the brain slice, to activate receptors on nerve terminals, we applied trains of electrical focal stimulation. In 69% of neurons (11/16) focal stimulation at 200 Hz for 50 msec caused an enhancement of the frequency of

sEPSCs at the end of the stimulation period compared with before stimulation; there was no change in the amplitude of sEPSCs (Fig. 4, A and B). ATP has been shown to be released from neurons in brain slices by focal stimulation (Edwards et al., 1992; Hamann and Attwell, 1996) and we tested whether P2 receptor antagonists could reduce the focal stimulation-evoked increase in sEPSC frequency. Suramin (300 μM; three of five neurons; Fig. 4B) and PPADS (30 μM; six of six neurons) blocked the focal stimulation evoked increase in sEPSC frequency. For these experiments, we concentrated on using PPADS, because suramin itself can affect sEPSC frequency (see above). In control conditions, the sEPSC frequency changed from 2.8 ± 0.4 Hz before focal stimulation to 8.9 ± 1.6 Hz after focal stimulation ($p < 0.05$; $n = 6$). In the same
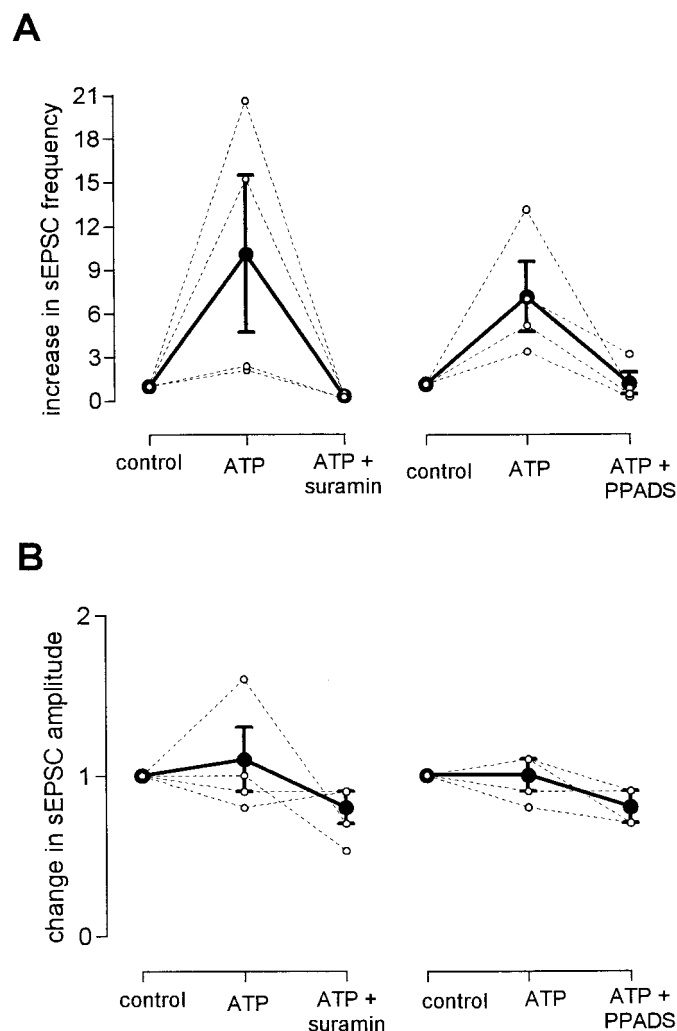

Fig. 2. P2X receptor antagonists block the ATP-evoked increase in sEPSC frequency. A, *Left graph*, increase in frequency produced by ATP (1 mM) and ATP plus suramin (300 μM). The data for these graphs have been normalized to the frequency of control sEPSCs in each cell before the application of ATP. *Right graph*, similar data for PPADS (30 μM). B, Both suramin and PPADS had no effect on the amplitude of sEPSCs in MoV neurons. *Solid lines*, represent the mean data ± standard error. Suramin was applied for 10 min and PPADS for 15–20 min before ATP application. The action of suramin and PPADS was not reversible on washout (up to 20–40 min) in a limited number of cells in which this could be investigated (see Materials and Methods). The data shown are from cells in different brain slices. In parallel studies applying ATP (1 mM) twice to the same neuron produced a consistent increase in sEPSC frequency, with the second application of ATP evoking an increase that was smaller by less than 10%.

neurons, exposing the brain slice to PPADS (30 μM) did not change the basal sEPSC frequency; however, the focal stimulation-evoked increase in sEPSC frequency was abolished

Fig. 3. The ATP-evoked increase in EPSC frequency did not require action potential conduction but required calcium entry. A, Representative recording of mEPSCs recorded in the presence of TTX (0.5–1 μM, *upper trace*) and then in the presence of TTX plus ATP (1 mM, *lower trace*). B, Cumulative probability plot for the cell in A, showing interevent intervals for mEPSCs in control (*solid line*) and then in the presence of ATP (1 mM, *broken line*). C, Data from seven neurons showing that ATP could elevate the frequency of mEPSCs; the data from the same cells are also shown in D, where ATP caused no change in the amplitude of mEPSCs. E, Representative recordings of mEPSCs in the presence of TTX and cadmium (100 μM, *upper trace*) and then in the presence of TTX, cadmium and ATP (1 mM, lower trace). F, Cumulative probability plot for the cell in E showing interevent intervals for mEPSCs in control (*solid line*) and then in the presence of ATP (1 mM, *broken line*); note that the two distributions overlie each other. G, ATP did not elevate the frequency of mEPSCs in the presence of cadmium ($n = 6$). Data from the same cells are also shown in H, where ATP caused no change in the amplitude of mEPSCs in the presence of cadmium. In these experiments, TTX and cadmium were allowed to superfuse the slice for 10–20 min before application of ATP.

(Fig. 4C), which indicates that release of endogenous ATP underlies the focal stimulation-evoked increase in sEPSC frequency.

Discussion

Brainstem MNV neurons are sensory proprioceptive neurons that express P2X receptors (Cook *et al.*, 1997; Khakh *et al.*, 1997). These neurons receive few synaptic inputs (Liem *et al.*, 1991); accordingly, we have been unable to show P2X receptor mediated synaptic currents in MNV neurons (Khakh BS, Henderson G, unpublished observations). In the present study, we have shown that P2 receptors are present in MNV neurons and are located on their nerve terminals in the MoV (Dessem *et al.*, 1997).

The ATP-evoked increase in sEPSC frequency with no change in amplitude provides strong evidence that ATP affects the presynaptic processes of transmitter release rather than altering the postsynaptic sensitivity to glutamate (Lena and Changeux, 1997). The persistence of the facilitatory effect of ATP in the presence of TTX demonstrates that the effect is on presynaptic nerve terminals close to the sites of neurotransmitter release to increase the probability of quantal release. The larger frequency increase observed in the absence of TTX suggests that part of the response to ATP may be mediated by receptors located on preterminal regions (Fig. 5; see Inoue *et al.*, 1992, for a report showing that P2X receptors increase the release of glutamate in the hippocampus in a TTX-sensitive manner; for a review, see Wonnacott, 1997). The inability of ATP to cause an increase in mEPSCs in the presence of cadmium indicates that in the majority of neurons, the receptors located on nerve terminals can cause sufficient depolarization to activate voltage-dependent calcium channels. It is the calcium influx through these calcium channels that then causes an increase in the probability of neurotransmitter release (Lena and Changeux, 1997).

To study responses mediated by P2X receptors on central nerve terminals of MNV neurons, we have used the purine nucleotides ATP, ATPγS, and $\alpha\beta$meATP. All three of these agonists are effective at evoking inward currents in the soma of MNV neurons that cross-desensitize and are equally blocked by suramin, which indicates an action at a common population of P2X receptors (Khakh *et al.*, 1997). We predict that because of enzymatic breakdown, the actual concentration of ATP within the slice is probably around 100-fold less than that of the bathing solution [see Kennedy and Leff, 1995; compare data from Khakh *et al.*, 1997 (using brain slices) with data from Cook *et al.*, 1997 (using cultured neurons)]. An additional consideration may be how readily different agonists penetrate brain slices. Metabotropic ATP receptors are also found in the central nervous system (see North and Barnard, 1997); however, it is unlikely that they contribute substantially to the responses described in the present study for three reasons. First, agonists that are effective at P2Y receptors, but are weak or ineffective agonists at P2X receptors (such as adenosine-5'-*O*-(2-thiodiphosphate) and UTP) do not cause any change in the membrane conductance in the soma of MNV neurons, which indicates that P2Y receptors do not activate or modulate any ion channels in MNV neurons (Khakh and Henderson, 1997). Third, the enhancement of spontaneous transmitter release was blocked by cadmium, which would not block P2Y receptor-
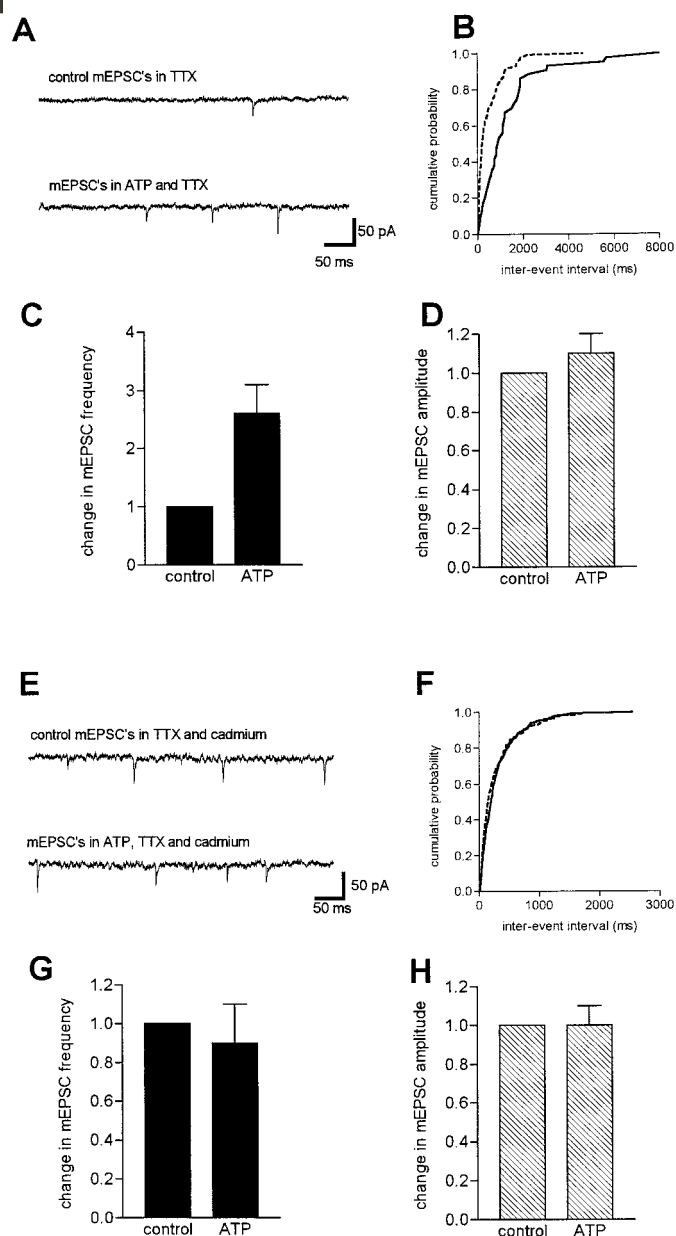
mediated release of calcium from intracellular stores. Third, the ability of αβmeATP to enhance sEPSC frequency is evidence of a P2X receptor-mediated effect (Burnstock, 1996). In future work, it will be important to use potent and subunit-selective antagonists to negate unequivocally the involvement of metabotropic ATP receptors, and to determine which P2X receptor subunits are involved. Suramin and PPADS (see Humphrey *et al.*, 1995) were effective at antagonizing the ATP-evoked inward currents in the soma of MNV neurons and the ATP-evoked enhancement of sEPSC frequency observed in the present study. Importantly, in the present study, the kinetics of action for PPADS (30 μM) are in good agreement with previous studies in which PPADS has been shown to be selective for P2X receptors over other ligand-gated ion channels in sensory neurons (Khakh *et al.*, 1995).

The P2X receptor mediated inward currents in the soma of MNV neurons desensitized over a time course of 2–3 min (Khakh *et al.*, 1997), whereas the increase in sEPSC frequency reported in this study seemed to persist throughout agonist application periods of up to 3 min. Furthermore, although in the present study we have not studied agonist concentration-dependency, it seems that αβmeATP produces

a smaller effect at nerve terminals than it does in the soma of MNV neurons. These differences with regard to desensitization and αβmeATP-sensitivity may indicate that P2X receptors differ in their subunit composition depending on their location along neurons (Vulchanova *et al.*, 1997). The properties of the P2X receptors mediating the increase in the frequency of sEPSCs are similar to those described on synaptosomes prepared from ciliary ganglia (Sun and Stanley, 1996). Early evidence suggested that ATP receptors exist on nerve terminals (Moody and Burnstock, 1982; Fu and Poo, 1991; Kennedy and Humphrey, 1994; Todorov *et al.*, 1994; Allagier *et al.*, 1995) but it was only recently that nerve terminal P2X receptors were unequivocally shown to exist at neuro-neuronal synapses (Gu and MacDermott, 1997). The present paper extends these findings to show that nerve terminal P2X receptors also exist in the brain stem.

In the present study, we have shown that focal stimulation within the brain slice can enhance the frequency of sEPSCs, and that this effect can be blocked by PPADS (30 μM) at concentrations sufficient to block ATP-evoked inward currents in the soma of MNV neurons and the ATP-evoked increase in sEPSC frequency reported in the present study.
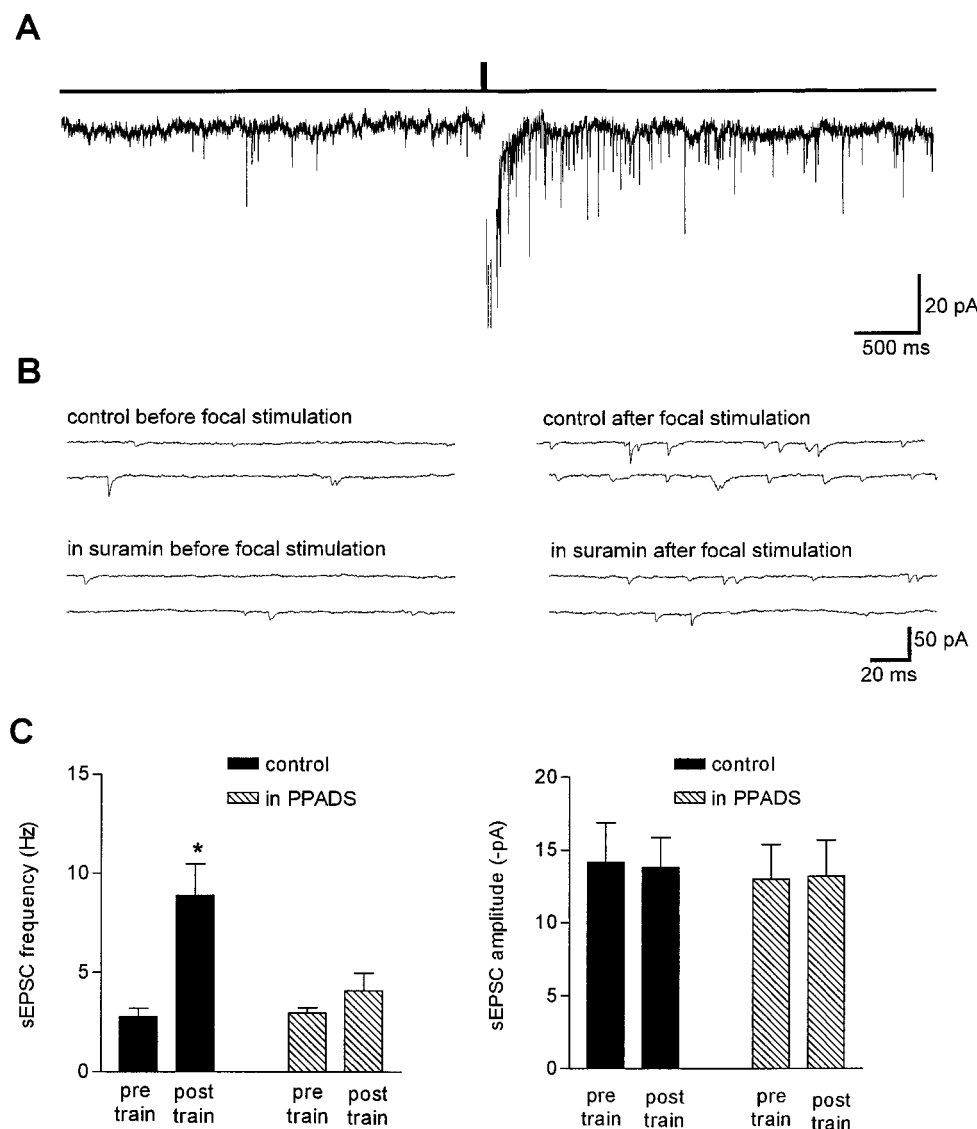

Fig. 4. Focal electrical stimulation enhanced sEPSC frequency. A, *Upper panel*, time of stimulation; ■, period of focal stimulation (10 pulses at 200 Hz). *Lower panel,* recording of membrane current. During the focal stimulation, there was a large inward current that arose from superimposed evoked glutamatergic EPSCs; when this decayed to baseline, there was a clear increase in sEPSC frequency. B, Representative current recordings showing control sEPSCs before focal stimulation, after focal stimulation, and in the presence of suramin (300 μM) both before and after focal stimulation. C, *Bar graph*, results from six cells in which focal stimulation evoked a significant increase in the sEPSC frequency. In the same cells, after the slice was superfused with PPADS (30 μM) for 15 min, there was no evoked increase in sEPSC frequency. D, Data from the same cells as in C showing that focal stimulation or PPADS did not affect sEPSC amplitude. *Bar graphs,* mean ± standard error data for six neurons.
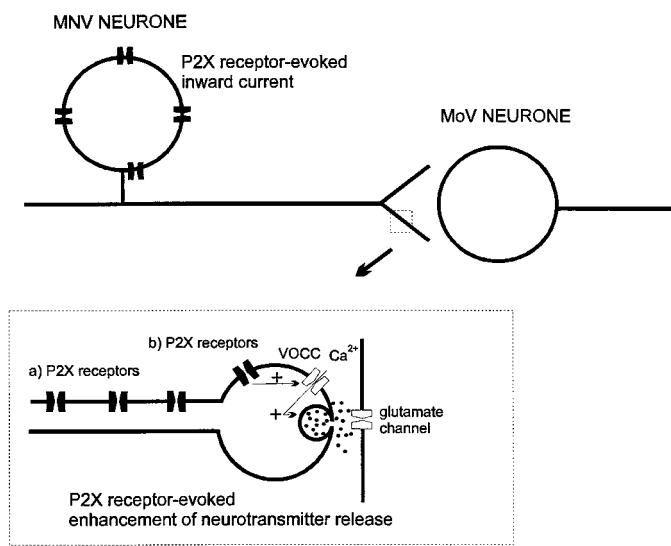
Fig. 5. Schematic illustration of P2X receptors in the soma and central terminals of MNV neurons. P2X receptors in the soma of MNV neurons mediate inward currents (Cook *et al.*, 1997; Khakh *et al.*, 1997), whereas in the nerve terminals, they mediate nerve terminal depolarization, which activates voltage-dependent calcium channels (*VOCC*), which thus enhances the release of glutamate. *Boxed inset*, MoV brain slice does not contain the cell bodies of MNV neurons. *a*), preterminal P2X receptors mediate the TTX sensitive enhancement of sEPSC frequency; *b*), presynaptic P2X receptors mediate the TTX insensitive enhancement of sEPSC frequency.

During electrical stimulation there is sufficient ATP release from within the brain slice to activate presynaptic P2X receptors, which enhance neurotransmitter release. The source of the ATP is unknown at present; the release may be from pre- or postsynaptic neuronal elements or from glial cells [Burnstock, 1972; Edwards *et al.*, 1992; Evans *et al.*, 1992; Galligan and Bertrand, 1994; Bardoni *et al.*, 1997; Nieber *et al.*, 1997; see Hamann and Attwell (1996) for evidence that ATP is also released by electroporation].

In summary, we have provided evidence for presynaptic P2X receptors in the brain stem functioning to increase the release of the fast excitatory neurotransmitter glutamate. It will be important to investigate the effect of P2X receptor activation on evoked transmitter release. Other presynaptic ligand-gated cation channels have been shown to both increase and decrease evoked synaptic transmission (Fu and Poo, 1991; Chittajallu *et al.*, 1996; Clarke *et al.*, 1997; Lena and Changeux, 1997; Wonnacott, 1997).

P2X receptors in the central nervous system mediate fast synaptic transmission (Edwards *et al.*, 1992; Bardoni *et al.*, 1997; Nieber *et al.*, 1997) and act as presynaptic modulators of fast synaptic transmission (this study and Gu and Mac-Dermott, 1997). P2X receptors thus seem well suited to regulate both synaptic strength and efficacy.

Acknowledgments

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