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Purinergic P2X Receptors Presynaptically Increase Glutamatergic Synaptic Transmission in Dorsolateral Periaqueductal Gray

Jihong Xing, Jian Lu, and Jianhua Li*

Heart & Vascular Institute and Department of Medicine, The Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, Hershey, PA 17033

Abstract

Purinergic P2X receptors have been reported to present in regions of the midbrain periaqueductal gray (PAG). The purpose of this study was to determine the role of presynaptic P2X receptors in modulating excitatory and inhibitory synaptic inputs to the dorsolateral PAG (dl-PAG), which has abundant neuronal connections. First, whole cell voltage-clamp recording was performed to obtain excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) of the dl-PAG neurons. Our data show that α , β -methylene ATP (a P2X receptors agonist), in the concentration of 50 μ M, significantly increased the frequency of miniature EPSCs without altering the amplitude of miniature EPSCs in eight tested neurons. The effects were attenuated by PPADS, an antagonist to P2X receptors. Furthermore, α , β -methylene ATP increased the amplitude of evoked EPSCs, and decreased the paired-pulse ratio of eEPSCs in ten neurons. In contrast, activation of P2X had no distinct effect on IPSCs. In addition, immunofluorescent methods demonstrate that P2X labeling was co-localized with a presynaptic marker, synaptophysin, in the dl-PAG. The results of the current study provide the first evidence indicating that P2X receptors facilitate glutamatergic synaptic transmission in the dl-PAG via presynaptic mechanisms.

Section

Neurophysiology, Neuropharmacology and other forms of Intercellular Communication

Key Words

P2X; ATP; synaptic transmission; glutamate; midbrain PAG

1. Introduction

Adenosine triphosphate (ATP) is released from nerve terminals in peripheral nervous system and in numerous regions of the CNS (Cunha et al., 1996; Salgado et al., 1996; Sawynok et al., 1993). ATP has been reported to be a mediator involved in synaptic transmission and integration within glial systems in the CNS (Cotrina et al., 2000; Edwards et al., 1992; Khakh, 2001; Stout et al., 2002). A family of ionotropic P2X purinoreceptors including seven P2X

***Corresponding Author:** Dr. Jianhua Li, Heart and Vascular Institute and Division of Cardiology H047, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, 500 University Drive, Hershey, PA 17033, Telephone: (717) 531-5051, FAX: (717) 531-1792, E-mail: jzl10@psu.edu.

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subtypes appears in the brain (Norenberg and Illes, 2000), and mediates the action of ATP as a fast neurotransmitter (Burnstock, 2000; Dunn et al., 2001; North, 2002).

Previous studies suggest that P2X receptors modulate glutamatergic and GABAergic synaptic transmission in the CNS, namely spinal cord, the nucleus tractus solitarius, medial habenula, locus coeruleus, hippocampus, and somatosensory cortex (Bardoni et al., 1997; Evans et al., 1992; Gu and MacDermott, 1997; Hugel and Schlichter, 2000; Jin et al., 2004; Mori et al., 2001; Nakatsuka and Gu, 2001; Nieber et al., 1997; Pankratov et al., 1998). For example, activation of P2X receptors has been shown to presynaptically alter the release of glutamate, GABA, glycine and vasopressin (Jin et al., 2004; Khakh and Henderson, 2000; Nakatsuka and Gu, 2001; Rhee et al., 2000; Troadec et al., 1998). Presynaptic modulation of transmitter release has been thought an important component of P2X receptor function in the brain.

The midbrain periaqueductal gray (PAG) receives abundant neuronal inputs from the dorsal horn of the spinal cord, hypothalamus as well as forebrain (Craig, 1995; Keay et al., 1997; Wiberg and Blomqvist, 1984). Also, the PAG sends descending neuronal projections to the medulla (Hudson and Lumb, 1996; Odeh and Antal, 2001) in regulating pain and autonomic activity (McGaraughty et al., 2003; Tjen-A-Looi et al., 2006; Verberne and Guyenet, 1992). Among regions of the PAG, the dorsolateral region (dl-PAG) contributes to an increase in arterial blood pressure and antinociception (Bandler et al., 1991; Behbehani, 1995).

Purinergic P2X receptors have been reported to appear in the PAG (Worthington et al., 1999). Activation of P2X receptors within the PAG alters parasympathetic activity in the innervation of the internal organs and modifies sympathetic outflow to the cardiovascular system (Rocha et al., 2001). The data suggest that P2X receptors in the PAG play a role in autonomic functions. However, the precise neuronal mechanism of P2X effects is unknown.

Glutamate, the major excitatory neurotransmitter, appears in the dl-PAG region (Beitz and Williams, 1991). The dl-PAG also has the high density of excitatory amino acid binding sites (glutamate receptor subtypes) (Albin et al., 1990; Cotman et al., 1987). Therefore, in this study, we used an in vitro whole cell recording technique in the midbrain slice to determine the role of P2X receptor in modulating glutamatergic synaptic inputs to the dl-PAG neurons.

In addition, GABA-mediated neuronal elements constituting ~50% of the total population of neurons play a crucial role in the intrinsic neuronal circuitry of the PAG (Mugnaini and Oertel, 1985; Reichling, 1991). The GABA synaptic inputs make up ~50% of the synaptic innervation of the PAG neurons and the majority of GABAergic neurons are tonic active interneurons (Barbaresi, 2005). The release of GABA from those neurons may play a role in modulation of the synaptic inputs to the PAG neurons. Studies have further shown that GABA_A receptors are dense within the PAG (Bowery et al., 1987; Chu et al., 1990). Thus the effect of P2X receptor on the inhibitory GABA release to the dl-PAG was also examined in this study.

Finally, immunofluorescence double labeling of P2X₃ receptors and synaptophysin, a specific marker for presynaptic terminals (Hiscock et al., 2000), within the dl-PAG, was examined. We hypothesized that P2X₃ receptors would be located at the presynaptic sites and activation of P2X would increase the release of glutamate within the dl-PAG.

2. Results

2.1. Location of the recording pipette

At the end of each experiment, the location of the recording pipette in the PAG slice was visualized and identified under a microscope using differential interference contrast (DIC, x40 magnification). We have confirmed that all the cells included for data analysis in this report

located in the dl-PAG (Lu et al., 2007; Xing and Li, 2007). Whole cell patch-clamp experiments were performed and experimental data were collected from 68 dl-PAG neurons.

2.2. Role of P2X in modulating glutamatergic excitatory postsynaptic currents (EPSCs)

2.2.1. Effect of α , β -methylene ATP (α , β -me ATP) on miniature EPSCs (mEPSCs)

—The spontaneous mEPSCs were recorded in the dl-PAG in order to determine the effects of P2X activation on synaptic glutamate release onto the neurons (Fig. 1). α , β -me ATP, in the concentration of 50 μ M, was perfused into the recording chamber to stimulate P2X receptors. This significantly increased the frequency of mEPSCs from 2.65 ± 0.31 to 4.66 ± 0.57 Hz ($P < 0.05$, $n=8$), but did not alter the amplitude (17.74 ± 1.46 pA in control vs. 18.12 ± 1.72 pA after α , β -me ATP, $P > 0.05$) and the decay time constant of mEPSCs (7.08 ± 0.59 ms in control vs. 7.03 ± 0.19 ms after α , β -me ATP, $P > 0.05$) in all neurons tested. The mEPSCs recovered during washout of the perfusion solution and were completely abolished with 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, Fig. 1A). The cumulative probability analysis of mEPSCs shows that the distribution pattern of the inter-event interval of mEPSCs shifted toward the left but the distribution pattern of the amplitude was not altered as α , β -me ATP was applied (Fig. 1B&C). Average data of α , β -me ATP effects on the frequency and amplitude of mEPSC of the dl-PAG neurons are also shown (Fig. 1D&E).

2.2.2. Effect of PPADS on mEPSCs—To determine tonic effect of endogenous P2X receptors activation on glutamatergic inputs to the dl-PAG neurons, 20 μ M of PPADS was applied into the recording chamber, and mEPSCs were then examined ($n=8$). PPADS alone decreased the frequency of mEPSCs from 2.80 ± 0.27 to 1.42 ± 0.19 Hz ($P < 0.05$) without affecting the amplitude and decay time constant (Fig. 1A–E).

Furthermore, in order to confirm that the effect of α , β -me ATP on mEPSCs was mediated via P2X receptor, PPADS was perfused prior to α , β -me ATP application ($n=8$). Our results show that α , β -me ATP failed to increase the frequency of mEPSCs of the dl-PAG neurons in the presence of 20 μ M of PPADS (Fig. 1A–E).

2.2.3. Effect of α , β -me ATP and PPADS on evoked EPSCs (eEPSCs)—First, the effects of PPADS on eEPSCs were examined in the dl-PAG neurons (Fig. 2). PPADS alone significantly inhibited the peak amplitude of eEPSCs ($n=11$, $P < 0.05$, Fig. 2A&C). In order to determine whether the effect of PPADS was via presynaptic sites, we further examined the paired-pulse ratio (PPR) of eEPSCs when PPADS was perfused into the recording chamber (Fig. 2B&D). The PPR was increased by 102% in control vs. by 172 % after PPADS ($n=8$, $P < 0.05$).

In the next group of experiments, the effects of α , β -me ATP on eEPSCs were further examined in the dl-PAG neurons (Fig. 3). α , β -me ATP significantly increased the peak amplitude of eEPSCs from 126 ± 25 to 246 ± 47 pA ($n=10$, $P < 0.05$, Fig. 3A&B), and decreased the PPR (113% in control vs. 80 % after α , β -me ATP, $n=10$, $P < 0.05$ shown in Fig. 3C&D). In addition, α , β -me ATP failed to increase the amplitude of eEPSCs of the dl-PAG neurons in the presence of 20 μ M of PPADS in six tested neurons (Fig. 3E&F). This further suggests that the effects of α , β -me ATP on eEPSCs were mediated through P2X receptor.

2.3. Role of P2X in modulating GABAergic inhibitory postsynaptic currents (IPSCs)

2.3.1. Effect of α , β -me ATP on miniature IPSCs (mIPSCs)—The spontaneous mIPSCs were also examined in the dl-PAG neurons in order to determine the effects of P2X activation on synaptic GABA release onto neurons (Fig. 4). α , β -me ATP, in the concentration of 50 μ M, did not produce a significant effect on the frequency and amplitude of mIPSCs in seven dl-PAG neurons. The mIPSCs were completely eliminated in the presence of 20 μ M of

bicuculline (Fig. 4A). The cumulative probability analysis of mIPSCs shows that the distribution pattern of the inter-event interval and amplitude of mIPSCs was not altered as α , β -me ATP was applied (Fig. 4B&C). Average data further show α , β -me ATP had no effect on the frequency and amplitude of mIPSCs of the dl-PAG neurons (Fig. 4D&E).

2.3.2. Effect of α , β -me ATP on evoked IPSCs (eIPSCs)—In another group of experiments, the effects of α , β -me ATP on eIPSCs were examined in eight dl-PAG neurons (Fig. 4F&G). α , β -me ATP had no distinct effect on the peak amplitude and PPR of eIPSCs of the dl-PAG neurons.

2.4. Presynaptic location of P2X₃ receptors in the dl-PAG

To determine the presynaptic location of P2X₃ receptors in the dl-PAG, double immunofluorescence labeling was performed in brain slices of three rats using specific antibodies against synaptophysin and P2X₃ receptors. The confocal images (Fig. 5) show that immunoreactivities for synaptophysin (in red, Fig. 5A) and P2X₃ receptor (in green, Fig. 5B) were present extensively within the dl-PAG. The co-localization of synaptophysin and P2X₃ receptor immunoreactivities was indicated by the color change (in yellow, Fig. 5C) when the synaptophysin and P2X₃ receptor-stained optical sections were digitally merged.

3. Discussion

In the present study, regulatory effects of P2X on excitatory glutamatergic and inhibitory GABAergic synaptic activity in the dl-PAG were determined using in vitro PAG slice preparation. Our results have demonstrated that P2X stimulation with α , β -me ATP significantly increased the frequency of mEPSCs of the dl-PAG neurons, but had no distinct effect on the amplitude of mEPSCs. Furthermore, the effects of α , β -me ATP were eliminated by the P2X receptor antagonist, PPADS. The mEPSCs represent the synaptic quanta release of glutamate that plays a role in modulating the activity of the postsynaptic neuron (Sulzer and Pothos, 2000). Therefore, the data of this experiment indicate that P2X has an excitatory effect on the glutamate release in the dl-PAG neurons. PPADS alone decreased the frequency of mEPSCs of the dl-PAG neurons, suggesting that endogenous P2X receptors tonically influenced glutamatergic inputs to the dl-PAG neurons.

In addition, α , β -me ATP significantly increased the peak amplitude of eEPSCs with decreasing the PPR. Collectively, these data suggest that P2X activation excited the synaptic glutamate release in the PAG and the site of the action was likely at the presynaptic glutamatergic terminals (Sulzer and Pothos, 2000).

In contrast, α , β -me ATP had no distinct effects on the frequency and amplitude of GABAergic mIPSCs, and amplitude of eIPSC recorded from the dl-PAG neurons. This suggests the lack of P2X effects on the synaptic GABAergic terminals in the dl-PAG.

Previous studies have shown that P2X receptors on presynaptic nerve terminals regulate the release of glutamate and GABA in the CNS (Bardoni et al., 1997; Deuchars et al., 2001; Edwards et al., 1992; Evans et al., 1992; Gu and MacDermott, 1997; Hugel and Schlichter, 2000; Khakh and Henderson, 1998). For example, P2X activation can facilitate glutamate release from central terminals of primary afferent in the spinal cord (Nakatsuka and Gu, 2001) and in the nucleus tractus solitarius (Jin et al., 2004). Whether P2X receptors are present on glutamatergic terminals of presynaptic sites of the dl-PAG has not, to our knowledge, been reported although P2X immunoreactivity has been identified in the dl-PAG (Worthington et al., 1999). Our data of the current experiment demonstrated that P2X₃ receptors are likely at presynaptic sites within the dl-PAG using double immunofluorescence labeling. The data also show that activation of P2X receptors increased glutamate release from presynaptic sites. This

provides additional evidence that P2X receptor is likely to appear on presynaptic nerve terminals in the dl-PAG using electrophysiological methods.

There are seven P2X receptor subtypes (P2X₁₋₇) with different tissue distributions (Norenberg and Illes, 2000). Among these subunits, P2X₃ receptors are selectively expressed in small- and medium-diameter of sensory afferent neurons in rats (Novakovic et al., 1999; Vulchanova et al., 1997). Also, P2X₃ receptors have been localized at the central terminals of these sensory neurons (Llewellyn-Smith and Burnstock, 1998). Previous studies have shown that purinergic P2X receptors appear in the PAG (Worthington et al., 1999), and activation of P2X receptors in the PAG plays a role in autonomic functions (Rocha et al., 2001).

In this study, we examined the P2X₃ receptors in the dl-PAG. Our results of immunocytochemical studies have revealed the presence of P2X₃ receptors in the PAG. In addition, α , β -me ATP stimulates mainly P2X₃, P2X_{2/3} and P2X₁ receptor subtypes (Inoue et al., 1996; Ralevic and Burnstock, 1998), and P2X₃ receptors dominate in the PAG. Thus P2X₃ purinoceptors were most likely activated in the present study. However, additional investigation is still necessary to determine the possibility that the effects seen with α , β -me ATP could be due to activation of other subtypes of P2X receptors when specific P2X subtype agonists/blockers become available.

In summary, P2X activation significantly increases the frequency of glutamatergic mEPSCs as well as the amplitude of eEPSCs but not GABAergic IPSCs of the dl-PAG neurons. The effect is likely mediated via activation of P2X receptors on the presynaptic terminals. Our data suggest a mechanism by which P2X modulates glutamatergic synaptic transmission in the dl-PAG. This provides new information that ATP sensitive P2X may play an important role in modulation of physiological functions in the dl-PAG.

4. Experimental procedure

4.1. Brain slice preparations

All procedures outlined in this study were approved by the Animal Care Committee of this institution. Sprague Dawley rats of either gender (4–6 weeks old) were anesthetized by inhalation of isoflurane oxygen mixture (5% isoflurane in 100% oxygen), and then were decapitated. Briefly, the brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid (aCSF). A tissue block containing the midbrain PAG was cut from the brain and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO). Coronal slices (300 μ m) containing the midbrain PAG were dissected from the tissue block in ice-cold aCSF solution. An equilibrium period of 60 min was required to incubate the slices in the aCSF at 34°C before they were transferred to the recording chamber. During the procedures described above, aCSF was saturated with 95% O₂ - 5% CO₂. The aCSF perfusion solution contained (in mM) 124.0 NaCl, 3.0 KCl, 1.3 MgSO₄, 2.4 CaCl₂, 1.4 NaH₂ PO₄, 10.0 glucose, and 26.0 NaHCO₃ (Li et al., 2004).

4.2. Electrophysiological recordings

4.2.1. Postsynaptic currents of dl-PAG neurons—A whole cell voltage-clamp technique was used to record postsynaptic currents in the dl-PAG neurons. Borosilicate glass capillaries (1.2 mm OD, 0.69 mm ID; Harvard Apparatus) were pulled to make the recording pipettes using a puller (Sutter Instrument, Novato, CA). The resistance of the pipette was 4–6 M Ω when it was filled with the internal solution (contained in mM: 130.0 potassium gluconate, 1.0 MgCl₂, 10.0 HEPES, 10.0 EGTA, 1.0 CaCl₂, and 4.0 ATP-Mg) (Li et al., 2004). The solution was adjusted to pH 7.25 with 1 M of KOH and osmolarity of 280–300 mOsm. The slice was placed in a recording chamber (Warner Instruments, Hamden, CT) and fixed with a

grid of parallel nylon threads supported by a U-shaped stainless steel weight. The aCSF saturated with 95% O₂ - 5% CO₂ was perfused into the chamber at 3.0 ml/min. The temperature of the perfusion solution was maintained at 34°C by an in-line solution heater with a temperature controller (Model TC-324; Warner Instruments). Whole cell recordings from the dl-PAG neurons were performed visually using DIC optics on an upright microscope (BX50WI, Olympus, Tokyo, Japan). The tissue image was captured and enhanced through a camera and displayed on a video monitor. A tight giga-ohm seal was subsequently obtained in the dl-PAG neuron viewed using DIC optics. An equilibration period of 5–10 min was allowed after whole cell access was established and the recording reached a steady state. The recording was abandoned if the monitored input resistance changed >15%.

The mEPSCs were recorded in the presence of 1 μM of TTX and 20 μM of bicuculline at a holding potential of −70 mV. The mIPSCs were recorded in the presence of 1 μM of tetrodotoxin (TTX) and 20 μM of CNQX at a holding potential of 0 mV. QX-314 (10 mM) and GDP-β-s (1 mM) were contained in the pipette solution to block sodium current and possible postsynaptic effects mediated through G proteins in this experiment.

In order to examine the eEPSCs and eIPSCs in the dl-PAG neurons, electrical stimulation (0.1 ms, 0.4–0.8 mA, and 0.2 Hz) was induced using a bipolar tungsten electrode connected to a stimulator (Grass Instruments, Quincy, MA). The tip of the stimulating electrode was placed 200–500 μm away from the recorded neuron. The eEPSCs was determined at a holding potential of −70 mV in the presence of bicuculline (20 μM), and eIPSCs at 0 mV in the presence of CNQX (20 μM), respectively. QX-314 and GDP-β-s were also contained in the pipette solution in this experiment.

Single stimuli and paired stimuli at short intervals (40 ms for eEPSCs and 50 ms for eIPSCs) were applied. PPR of eEPSCs and eIPSCs was expressed as percentage (%) of the amplitude of the second synaptic response/the first synaptic response. Ten consecutive responses were averaged for subsequent analysis.

4.2.2. Drugs and their application—TTX, bicuculline, CNQX, GDP-β-s, α, β-me ATP and PPADS were obtained from Sigma Co. QX-314 was obtained from Alomone Labs (Jerusalem, Israel). All drugs were dissolved in the aCSF solution immediately before they were used. According to experimental protocol, the drugs were delivered into the recording chamber at final concentrations using syringe pumps during the experiment (Lu et al., 2007; Xing and Li, 2007). The responses of EPSCs and IPSCs of the dl-PAG to application of drugs were recorded after control data were collected.

4.3. Immunofluorescence double labeling

In order to determine whether P2X₃ receptors presented presynaptically in the dl-PAG, sections from the midbrain were immunolabeled for co-localization of the P2X₃ receptor and synaptophysin, a specific marker for presynaptic terminals (Hiscock et al., 2000) in three rats. Under deep anesthesia with inhalation of isoflurane oxygen mixture (5% isoflurane in 100% oxygen), rats were intracardially perfused with 200 ml of ice-cold normal saline containing 1,000 units of heparin, followed by 500 ml of 4% paraformaldehyde and 250 ml of 10% sucrose in 0.1 M of PBS, pH 7.4. The brain was removed quickly and postfixed for 2 hr in the same fixative solution and cryoprotected in 30% sucrose in PBS for 48 hr at 4°C. Then, sections were cut in 35 μm in thickness and collected free floating in 0.1 M of PBS. The sections were rinsed in 0.1 M of PBS, and incubated in 1% H₂O₂. Next, sections were incubated with the first primary antibody diluted in PBS containing 2% normal goat serum, 0.3% Triton X-100, and 0.05% Tween 20 for 2 hr at room temperature and 48 hr at 4°C. The primary antibodies used in this processing were rabbit anti-P2X₃ polyclonal IgG antibody (dilution, 1:100; Neuromics, Minneapolis, MN), and the labeling was enhanced with tyramide signal

amplification (TSA). Subsequently, sections were rinsed and incubated with biotin-SP-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (dilution, 1:200; Jackson ImmunoResearch Labs, West Grove, PA) for 2 hr at room temperature. The sections were rinsed and incubated with streptavidin-horseradish peroxidase at 1:100 of dilution for 30 min. Finally, the sections were incubated with the FITC conjugated to tyramide (Perkin Elmer, Boston, MA), as well as the second primary antibody (mouse anti-synaptophysin monoclonal IgM antibody, dilution, 1:100; Chemicon, Temecula, CA) for 2 hr at room temperature and 48 hr at 4°C. Then sections were rinsed and incubated with the secondary antibodies (Alexa Fluor-594 conjugated goat anti-mouse IgM, dilution 1:400, Molecular Probes) for 2 hr at room temperature. Finally, the sections were rinsed in PBS for 40 min, mounted on slides, dried, and coverslipped. The sections were then viewed for accurate co-localization of fluorescent markers using a confocal laser scanning microscopy (Leica), and the areas of interest were photographed.

4.4. Data acquisition and analysis

Signals were recorded with a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), digitized at 10 kHz with a DigiData 1440A, and filtered at 1–2 kHz and saved in a PC-based computer using pClamp 10.1 software (Axon Instruments). A liquid junction potential of –15.0 mV (for the potassium gluconate pipette solution) was corrected during off-line analysis (Li et al., 2002; Li et al., 2004). The mEPSCs and mIPSCs of the PAG neurons were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). Detection of events was accomplished by setting a threshold above the noise level. The distribution of cumulative probability of the inter-event interval and amplitude of mEPSCs and mIPSCs was estimated using the Komogorov–Smirnov test (Li et al., 2002; Li et al., 2004). The amplitude of eEPSCs and eIPSCs, and PPR were analyzed using Clampfit 10.1 (Axon Instruments). Experimental data (frequency, amplitude and decay time of mEPSCs and mIPSCs of dl-PAG neurons, and the PPR of evoked currents) were analyzed with one-way ANOVA. Tukey's post hoc analyses were utilized to determine differences between groups, as appropriate. Paired t test was used to analyze amplitude of the eEPSCs and eIPSCs. All values were expressed mean $\pm$ SE. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for windows version 15.0.

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Figure 1.

Effect of P2X receptors on the frequency of glutamatergic mEPSCs of the dl-PAG neurons. This experiment was examined in eight neurons tested. A: Representative tracings from a dl-PAG neuron show that 50μM of α, β-me ATP elevated, but 20μM of PPADS decreased the frequency of mEPSCs. The effects of α, β-me ATP were eliminated by PPADS. The mEPSCs recovered during washout and were completely abolished in the presence of 20 μM of CNQX. B&C: The cumulative probability analysis shows that α, β-me ATP decreased, but PPADS increased the inter-event interval of mEPSCs but did not alter the distribution pattern of the amplitude of the mEPSCs. D&E: Average data show the effects of P2X on the frequency and amplitude of mEPSCs in eight dl-PAG neurons tested. * $P < 0.05$, α, β-me ATP vs. control and

washout. $\dagger P < 0.05$, PPADS vs. control and washout. There was no difference in the frequency of mEPSCs between PPADS and PPADS plus α , β -me ATP.

Figure 2. PPADS attenuated the peak amplitude of eEPSCs of the dl-PAG neurons and increased the PPR of eEPSCs. Typical traces from a dl-PAG neuron (A&B), and summarized data (C&D) show the peak amplitude of eEPSCs during control, PPADS and washout ($n=11$); and the PPR of eEPSCs ($n=8$). * $P<0.05$, vs. control and washout. The traces are average of 10 consecutive responses. Stimulation artifacts are removed and indicated by arrows.

Figure 3.

α , β -me ATP increased the peak amplitude of eEPSCs of the dl-PAG neurons and decreased the PPR of eEPSCs. Typical traces from a dl-PAG neuron (A&C), and summarized data (B&D) show the peak amplitude of eEPSCs during control, α , β -me ATP and washout ($n=10$); and the PPR of eEPSCs ($n=10$). Typical traces from a dl-PAG neuron (E), and summarized data (F) showing that α , β -me ATP failed to increase the amplitude of eEPSCs of the dl-PAG neurons in the presence of PPADS in six tested neurons. * $P<0.05$, α , β -me ATP vs. control and washout in B&D; control vs. PPADS in F. There was no significant difference between PPADS and PPADS plus α , β -me ATP. The traces are average of 10 consecutive responses. Stimulation artifacts are removed and indicated by arrows.

Figure 4.

P2X had no distinct effects on GABAergic IPSCs of the dl-PAG neurons. Representative tracings from a dl-PAG neuron (A), cumulative probability analysis (B&C) and average data (D&E) show that the frequency and amplitude of spontaneous mIPSCs were not altered by bath application of 50 μ M of α , β -me ATP. The results were seen in seven neurons tested. Effects of P2X on eIPSCs were further examined in the dl-PAG neurons. Averaged traces of 10 consecutive responses from a dl-PAG neuron (F) and average data of eight neurons (G) show that 50 μ M of α , β -me ATP did not significantly alter the peak amplitude of eIPSCs. The eIPSCs were completely abolished with bicuculline (F).

Figure 5.

Representative confocal images show presynaptic location of P2X₃ receptors in the dl-PAG. Immunoreactivities for synaptophysin (in red, A) and P2X₃ receptor (in green, B) were present within the dl-PAG. The co-localization of synaptophysin and P2X₃ receptor immunoreactivities was indicated by the color change (in yellow, C) when the synaptophysin and P2X₃ receptor-stained optical sections were digitally merged. Scale bar = 75μm.