Direct Excitation of Hypocretin/Orexin Cells by Extracellular ATP at P2X Receptors

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Wollmann, Guido, Claudio Acuna-Goycolea, and Anthony N. van den Pol. Direct excitation of hypocretin/orexin cells by extracellular ATP at P2X receptors. J Neurophysiol 94: 2195–2206, 2005. First published June 22, 2005; 10.1152/jn.00035.2005. Hypocretin/orexin (hcrt) neurons play an important role in hypothalamic arousal and energy homeostasis. ATP may be released by neurons or glia or by pathological conditions. Here we studied the effect of extracellular ATP on hypocretin cells using whole cell patch-clamp recording in hypothalamic slices of transgenic mice expressing green fluorescent protein (GFP) exclusively in hcrt-producing cells. Local application of ATP induced a dose-dependent increase in spike frequency. In the presence of TTX, ATP (100 μM) depolarized the cells by 7.8 ± 1.2 mV. In voltage clamp under blockade of synaptic activity with the GABA<sub>A</sub> receptor antagonist bicuculline, and ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), ATP (100 μM) evoked an 18 pA inward current. The inward current was blocked by ATP (100 μM) depolarized the cells by 7.8 ± 1.2 mV. In voltage clamp under blockade of synaptic activity with the GABA<sub>A</sub> receptor antagonist bicuculline, and ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), ATP (100 μM) evoked an 18 pA inward current. The inward current was blocked by 

The lateral hypothalamus harbors a variety of functionally distinct neuron populations. One of these cell groups, the hypocretin/orexin neurons (de Lecea et al. 1998; Sakurai et al. 1998), has gained great attention because their loss seems to be the causative factor for narcolepsy, a condition characterized by excessive daytime sleepiness and cataplexy (Peyron et al. 2000; Thannickal et al. 2000; van den Pol 2000). Dogs with mutations in their hypocretin receptor-2 gene (Lin et al. 1999) and hypocretin-knockout mice (Chemelli et al. 1999) present a narcoleptic phenotype. Hypocretin has also been postulated to play a crucial role in energy homeostasis and feeding behavior (Hara et al. 2001; Sakurai et al. 1998; Yamanaka et al. 2003). Hypocretin cells project widely throughout the brain and the spinal cord (Chen et al. 1999; Peyron et al. 1998; van den Pol 1999).

EXTRACELLULAR ATP can modulate synaptic transmission and neuronal excitability in the CNS (Cunha and Ribeiro 2000; Robertson et al. 2001). The effects of ATP can be mediated either through fast-responding ionotropic P2X receptors or through slower metabotropic G protein–coupled P2Y receptors (Edwards 1994; Edwards and Gibb 1993). ATP can also directly inhibit N-methyl-d-aspartate (NMDA) receptor–mediated excitatory currents (Ortinau et al. 2003) or glutamate release (Inoue 1998) in cultured hippocampal neurons. In contrast, the ATP actions in locus coeruleus, cerebellar Purkinje cells, and somatosensory cortex appear excitatory (Brockhaus et al. 2004; Nieber et al. 1997; Pankratov et al. 2003). Additionally, ATP can act intracellularly to modulate or activate a number of ion channels (e.g., the ATP-activated K<sup>+</sup>-channel) (Ballanyi 2004; Liss and Roeppe 2001).

Purinergic receptors have been reported in a number of loci in the mammalian brain (Kanjhan et al. 1999; Norenberg and Illes 2000) including hippocampus, hypothalamus, cerebral cortex, medial habenula, locus coeruleus, and the dorsal horn of spinal cord (Bardoni et al. 1997; Edwards et al. 1997; Jo and Role 2002; Jo and Schlichter 1999; Pankratov et al. 1998; Sorimachi et al. 2001), suggesting a role for external ATP in regulating synaptic transmission in these brain regions. ATP can be released from axon terminals, and there is evidence for synaptic colocalization and corelease of ATP in cholinergic, glutamatergic, and GABAergic neurons (Jo and Role 2002; Mori et al. 2001; Silinsky and Redman 1996). In addition, ATP is involved in intercellular signaling from glia to other glia and to neurons (Bowser and Khakh 2004; Zhang et al. 2003). ATP is also released in a number of pathological conditions, including stroke, trauma, and epilepsy (Ciccarelli et al. 2001; Hansson and Ronnback 2003; James and Butt 2002).

P2X receptors are expressed in the hypothalamus (Xiang et al. 1998), where they may play a role in ATP modulation of electrical activity (Jo and Role 2002; Sorimachi et al. 2001). We studied the effect of extracellular ATP on a single neuronal phenotype, the hypocretin-producing cells, using transgenic mice expressing green fluorescent protein (GFP) exclusively in hypocretin cells. We found substantial direct excitation of hypocretin cells through activation of ionotropic P2X receptors.

METHODS

Animals

Transgenic mice (kindly provided by Dr. T. Sakurai, University of Tsukuba) that express enhanced GFP under control of the hypocretin gene were used. ATP-induced excitation of hypocretin cells was monitored using whole cell patch-clamp recording in hypothalamic slices from mice heterozygous for the transgene. Animals used for electrophysiological recordings were of both sexes and were 4–8 weeks old. The slices were prepared as described below. ATP was applied using a pressure pipette at 0.3 mm away from the recording site, at pressures 2–3 times greater than resting slice perfusion of 0.5–1.0 psi.

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grades of significance (P < 0.01; P < 0.001) are indicated where applicable.

**Preparation and application of reagents**

Most drugs were prepared as a 1,000 times stock solution. In most cases, drugs were locally applied by using a flow pipette with a 250-μm tip diameter aimed at the recorded cell. During control recordings, normal ACSF was applied through the flow pipette. 

- 2-aminooxyacetic acid (AOA), 7-cyano-7-nitrotaurocholic acid (CNTX), 4-aminopyridine (4-AP), CNQX, D-glucose, TTX, and PPADS were purchased from Sigma (St. Louis, MO). TTX was obtained from Alomone Labs (Jerusalem, Israel). Stock solutions for ATP, ATP-γ-S, suramin, and PPADS were prepared before each experiment.

**RESULTS**

**ATP excites hypocretin cells**

To address the effect of extracellular ATP on hypocretin cells, we used acute hypothalamic slices of transgenic mice expressing GFP exclusively in hypocretin-producing cells. Local application of ATP (100 μM) consistently induced a rapid increase in spike frequency by 50.2 ± 8.2% of control (Fig. 1A; range, 8.1–13.5%; P < 0.05; n = 14; ANOVA). This activation significantly decreased within 30 s. after onset of ATP application, even in the continued presence of ATP, as shown in the mean time course of 14 experiments (Fig. 1B). Dose–response analysis revealed significant excitatory effects for 3.0, 100, and 100 μM, respectively (P < 0.05; n ≥ 4; ANOVA). Doses of 0.3 (P = 0.42) and 1.0 μM (P = 0.12) did not evoke a statistically significant increase in spike frequency (Fig. 1C). To determine whether this excitatory action was caused by direct postsynaptic mechanisms, we bath-applied TTX (0.5 μM) to block spike-mediated transmitter release. Under these conditions, current-clamp recordings revealed a rapid ATP-induced depolarization of the cell membrane (Fig. 1D), indicating that ATP exerts direct postsynaptic effects on hypocretin neurons, with most cells presenting signs of a decay of the ATP effect after 30 s. of ATP application. The average peak value for this depolarization in TTX was 7.77 ± 1.23 mV (range, 2.8–13.1 mV; P < 0.001; n = 7; ANOVA). In addition, we evaluated the ATP actions on hypocretin cells in voltage-clamp (~60 mV holding potential). In normal ACSF bath solution, ATP (100 μM) induced an inward current of 15.2 ± 1.7 pA (range, 10.8–27.8 pA; P < 0.01; n = 10; ANOVA). To determine whether this ATP-induced inward current results from direct postsynaptic mechanisms, TTX (0.5 μM), AP5 (50 μM), CNQX (10 μM), and BIC (30 μM) were bath-applied to prevent glutamate- or GABA-mediated synaptic transmission. These experiments revealed an inward current of 17.9 ± 4.1 pA (range, 3.6–34.0 pA; P < 0.01; n = 7; ANOVA) elicited by ATP (100 μM; Fig. 1E), further substantiating the direct nature of the ATP action on hypocretin cells.

The effect of ATP on the whole cell input resistance in hypocretin neurons was examined. In these experiments, a series of short (300 ms) negative current steps from 0 to −140 pA were injected to the cells through the recording pipette in 20-pA increments. To compare the variability of the basic

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**Analysis and statistics**

PulseFit (HEKA Electronik), Axograph (Axon Instruments, Foster City, CA), Igor Pro (WaveMetrics, Lake Oswego, OR), and Kaleidagraph (Synergy, Reading, PA) software were used for analysis. Adobe Photoshop software was used for figure layout. For studying miniature and spontaneous synaptic currents, an event-defining algorithm was used in Axograph (Axon Instruments), and the amplitude threshold was set to >5 pA (Bekkers and Stevens 1995; Gao and van den Pol 2001). Data are expressed as mean ± SE. For statistical evaluation, ANOVA was used either in one-way mode or in conjunction with a Bonferroni post hoc test for multiple comparisons, when indicated. The cumulative distributions of mPSCs under different conditions were compared using the nonparametric Kolmogorov-Smirnov test. For all tests, P < 0.05 was considered statistically significant.
input resistance between cells, resulting voltage amplitudes were normalized to the maximum value at the biggest current step for each cell. The slope for this normalized I/V curve was calculated with a linear curve-fitting function and changed step for each cell. The slope for this normalized were normalized to the maximum value at the biggest current input resistance between cells, resulting voltage amplitudes recovered to 0.72 ± 0.03 after ATP washout, indicating a significant ATP-induced decrease of the normalized input resistance by 10.6 ± 3.3% (P < 0.05; n = 8; ANOVA; Fig. 1F), suggesting ATP opened ion channels. The slope correlates to absolute values for input resistance of 629, 598, and 618 MΩ for control, ATP (100 μM), and recovery, respectively. All effects of ATP were reversible, with values returning to control levels 0.5–3 min after ATP washout.

P2X receptors mediate ATP actions in hypocretin cells

Many brain regions express a variety of ATP receptors that underlie purinergic signaling. Recent reports have highlighted the hypothalamus as a locus for both strong P2X receptor expression as well as functional purinergic transmission (Jo and Role 2002; Xiang et al. 1998). Here, we tested whether the direct excitatory effects of ATP on hypocretin cells were mediated by activation of P2X receptors. In the presence of the P2X receptor antagonist suramin (100 μM), the ATP-induced increase in spike frequency was substantially reduced (Fig. 2A). In these experiments ATP responses were measured in the same cell before and during suramin application. On four cells tested, ATP (100 μM) induced an increase in spike frequency by 45.0 ± 7.9% (P < 0.001) in control conditions and by 15.9 ± 5.2% in the presence of suramin (100 μM). This marks a net suppressive effect of suramin of 59.9 ± 13.9%, a statistically significant inhibition (Fig. 2B; P < 0.05; ANOVA followed by Bonferroni post hoc test for multiple comparisons). After suramin washout, ATP induced excitatory responses similar to those before suramin application (n = 3; data not shown). The application of suramin (100 μM) alone did not significantly alter the spike frequency (increase by 4.7 ± 5.4%; P = 0.48; n = 4; ANOVA).

Although suramin is widely used to study purinergic transmission, some reports have suggested that suramin might also exert an inhibitory effect on glutamatergic transmission (Gu et al. 1998). We therefore tested the P2X receptor antagonist PPADS in additional experiments. In four cells tested, ATP increased spike frequency by 40.2 ± 9.1% (P < 0.01) under control conditions, and this was reduced to 9.2 ± 3.6% in the presence of PPADS (50 μM; Fig. 2, C and D), a significant inhibition of 78.5 ± 9.0% (P < 0.05; ANOVA with Bonferroni post hoc test). Application of PPADS alone had no detectable effect on spike frequency (increase by 4.6 ± 7.6%; P = 0.56; ANOVA). In control experiments (n = 5), repeated application of ATP resulted in consistent peak excitatory responses without signs of a significant peak run-down (data not shown), confirming that the inhibition of the ATP-induced spike frec-
frequency increment by suramin and PPADS was in fact caused by the block of purinergic P2X receptors. The presence of PPADS also reduced the ATP-induced inward current of hypocretin cells held at −60 mV in voltage clamp (Fig. 2E). In 6 cells tested, ATP (100 µM) induced an inward current of 14.6 ± 2.5 pA, which was significantly reduced to 6.8 ± 1.4 pA in the presence of 50 µM PPADS (P < 0.05, ANOVA; Fig. 2F). A recovery of the ATP-induced inward
current was seen in four cells tested after PPADS wash-out (>5 min).

Although suramin and PPADS block P2X receptors, they may also inhibit metabotropic P2Y receptors. To rule out that the ATP-induced activation of hypocretin cells is mediated through P2Y receptors and to examine the subtype composition of the P2X receptors involved, we studied the effect of α,β-MeATP, a selective P2X1 and P2X3 agonist (Lambrecht 2000) and the effect of bath acidification, which potentiates currents through P2X2 receptors (North 2002; Stoop et al. 1997). The effect of α,β-MeATP was assessed in both voltage and current clamp. In six cells tested, α,β-MeATP (100 μM) failed to induce a sizable inward current (1.6 ± 0.6 pA, n = 6; Fig. 3, A1 and A2). The same cells responded to ATP (100 μM) with significant inward currents of 9.9 ± 2.1 pA when applied 2–3 min before α,β-MeATP and 13.2 ± 3.9 pA when applied 2 min after α,β-MeATP wash-out (Fig. 3A2, column ATP 1 and ATP 2, respectively). The effects of ATP and α,β-MeATP application on inward currents differed statistically (P < 0.01; ANOVA with Bonferroni post hoc test). In current clamp and holding the cells at resting membrane potential, application of α,β-MeATP (100 μM) did not significantly change the spike frequency (4.2 ± 3.5%; n = 7) compared with a significant increase by 28.7% ± 5.2 after ATP (100 μM) application (P < 0.01; ANOVA with Bonferroni post hoc test; Fig. 3A3).

We further tested whether the ATP-induced activation of hypocretin cells was pH-dependent. In five cells examined, the mean inward current amplitude on ATP application increased from 12.6 ± 2.5 pA under normal pH (7.4) conditions to 19.2 ± 4.0 pA in acidified ACSF medium (pH 6.8; Fig. 3B1), a significant mean increase by 57.3 ± 14.0% (P < 0.01; n = 5; ANOVA; Fig. 3B2; inset). Together these data substantiate the view that external ATP excites hypocretin cells through purinergic P2X receptors and suggest the involvement of P2X2 subunits.

### Effect of the nonhydrolysable ATP agonist ATP-γ-S on hypocretin cells

Extracellular ATP is subject to rapid hydrolysis by surface-located ectonucleotidases (reviewed in Zimmermann 1996); in addition, ATP can have significant effects intracellularly. We examined the effect of ATP-γ-S, a nonhydrolysable ATP analog, on the activity of hypocretin neurons. Experiments were carried out with local application of ATP-γ-S (100 μM) followed by ATP (100 μM) after a 2- to 3-min wash-out interval. In all cells recorded, ATP-γ-S induced a robust excitatory response comparable to ATP application. Under current clamp, spike frequency increased by 23.8 ± 7.3% after 100 μM ATP-γ-S application. In the same group of cells, the frequency of action potentials was increased by 25.8 ± 9.2% by ATP (100 μM), a nonsignificant difference between ATP and ATP-γ-S (P = 0.87; n = 5; Fig. 4, A1 and A3). Although the peak value for this spike frequency increment was slightly higher after ATP application, the mean increase over the whole 1-min application period was slightly higher after ATP-γ-S application (12.1 ± 2.6 vs. 9.7 ± 5.9% for ATP; Fig. 4A3). A comparison of a typical time-course is shown in Fig. 4A2. The action of ATP-γ-S was also subject to a considerable rundown, suggesting that ATP hydrolysis does not account for the full attenuation. We further compared the inward current induced by ATP-γ-S and ATP in voltage clamp (Fig. 4B1) in normal ACSF solution. Application of ATP-γ-S generated an inward current of 15.1 ± 1.8 pA and ATP generated an 11.3 ± 2.1-pA current (P = 0.2; n = 6; ANOVA; Fig. 4B2).

![FIG. 3. P2X subunit identification. A: selective P2X1 and P2X3 agonist α,β-MeATP did not mimic ATP actions on hypocretin cells. A1: representative trace of inward current induction by ATP, but not by α,β-MeATP. A2: bar graph summarizing the inward current induced by ATP (100 μM) before (ATP 1) and after (ATP 2) α,β-MeATP (100 μM) application, which did not induce a sizable response. **P < 0.01. A3: increase in spike frequency induced by ATP (100 μM) but not by α,β-MeATP (100 μM). ***P < 0.01. B: decrease in bath pH potentiates ATP current, a characteristic feature of P2X2 subunits. B1: typical trace of a cell held at −60 mV in voltage clamp with 2 consecutive ATP applications (100 μM) in normal (pH 7.4) and acidified (pH 6.8) artificial cerebrospinal fluid (ACSF). B2: bar graph shows increase of the ATP-induced inward current under low pH conditions compared with normalized current under control conditions. **P < 0.01. Inset: individual current increase of each of 5 cells tested in absolute values.](http://jn.physiology.org/10.1152/jn.00333.2005)
In addition, we tested whether ectonucleotidase activity may inhibit any intrinsic ATP action on hypocretin cells. In seven cells examined, local application of the ectonucleotidase inhibitor 6-N,N-diethyl-β-γ-dibromomethylene-δ-adenosine-S-triphosphatase 67156 (100 μM) did not change the spontaneous spike frequency (103.9 ± 4.5%; n = 7; not significant; ANOVA; data not shown). The same cells responded to subsequent ATP application (100 μM) by a mean increase in spike frequency of 28.5 ± 5.9%. This may suggest that under these experimental conditions, the intrinsic tone of ATP-mediated purinergic transmission on hypocretin cells is low.

Synaptic input and membrane activity in hypocretin cells

GABA appears to be the primary inhibitory transmitter of the hypothalamus (Decavel and van den Pol 1990). To study the effect of ATP application on GABA-mediated synaptic transmission, we used a KCl pipette solution and bath-applied AP5 (50 μM) and CNQX (10 μM) to block the excitatory ionotropic glutamate receptor–dependent synaptic activity. Under these conditions, voltage-clamp experiments at –60-mV holding potential revealed well-defined brief inward currents characteristic of IPSCs. Application of BIC (30 μM) completely abolished these IPSCs, confirming that they were attributable to GABAA receptor subtype activation (n = 5; data not shown). To determine whether ATP modulates GABAergic transmission in hypocretin cells, we analyzed the frequency of IPSCs before, during, and after ATP application. Representative traces of a typical experiment are shown in Fig. 5A1. ATP (100 μM) did not change the frequency of IPSCs in hypocretin cells (97.8 ± 9.6% of control; 107.1 ± 13.5% recovery; P = 0.78; n = 9; ANOVA with Bonferroni post hoc test; Fig. 5A3). Figure 5A2 shows the mean time-course of this IPSC frequency analysis. We further studied mIPSCs in the presence of TTX (0.5 μM) in the external solution. Representative traces are shown in Fig. 5B1. ATP did not significantly reduce the mIPSC frequency (88.1 ± 8.6% of control; 98.4 ± 21.2% recovery; P = 0.79; n = 6; ANOVA; Fig. 5B2). In addition, the mIPSC amplitude pattern before, during, and after ATP application was compared. The mean amplitude of all events was 37.3 ± 6.4, 40.3 ± 6.1, and 44.5 ± 8.2 pA for control, ATP, and washout, respectively (P = 0.77; not significant; n = 5; ANOVA). The cumulative distribution of the mIPSC amplitude revealed also no significant effect of ATP application (P > 0.05; n = 5; Kolmorogov-Smirnov). Figure 5B3 shows a typical result of such a comparison.

The decay kinetics of mIPSCs were compared before, during, and after ATP application. Detected mIPSCs were averaged and subject to an exponential curve fitting, which revealed the time constant for the event decay. Figure 5C1 shows a typical example of such a comparison with the averaged trace in black, superimposed on the detected raw events in gray. In five cells tested, we found no change of the decay kinetics of mIPSCs with ATP (100 μM) application (decay time constant τ = 15.1 ± 1.2, 14.9 ± 1.0, and 14.5 ± 1.1 ms for control, ATP, and wash-out, respectively; not significant, P = 0.923; n = 5; ANOVA; Fig. 5C2). In addition to the analysis of spontaneous inhibitory synaptic inputs, we tested whether electrically evoked potentials have a BIC-insensitive component that may be attributed to a purinergic effect. This has been previously reported in cultured developing hypothalamic neurons from chickens (Jo and Role 2002). However, in our postnatal brain slice preparation model in the presence of 10 μM CNQX and 50 μM AP5 to block glutamatergic excitatory transmission, evoked potentials were completely abolished in the presence of 30 μM BIC, suggesting that they were entirely

FIG. 4. Nonhydrolysable ATP analogue ATP-γ-S excites hypocretin cells. A1: representative traces showing the increase of spike frequency by ATP-γ-S (100 μM) and ATP (100 μM) at the same cell. A2: time-course of the excitatory action of ATP and ATP-γ-S corresponding to A1. Effect of ATP on spike frequency lasted slightly longer than the one evoked by native drug. A3: bar graph comparing peak values with 1-min mean values for the increase in firing rate of 5 cells. B1: in voltage clamp, ATP-γ-S application induced an inward current similar, but longer-lasting, than the one induced by ATP; representative traces of current responses in the same cell. B2: bar graph showing mean inward currents induced by ATP-γ-S and ATP; n = 6; n.s., not significant.
GABA-mediated \((n = 5\); data not shown), confirming observations from previous studies on hypocretin cells (Acuna-Goycolea et al. 2004; Fu et al. 2004) that evoked potentials are abolished by antagonists of glutamatergic and GABAergic transmission. Together, these results suggest that ATP does not modulate GABAergic transmission to hypocretin neurons.

When ATP was applied to hypocretin cells, an increase in membrane electrical activity was found. To test whether this was caused by a direct effect on the membrane or by excitatory synaptic input, we used KMeSO4 pipette solution and bath-applied BIC \((30 \mu M)\) to block GABA-mediated inhibitory events. Under these conditions, excitatory postsynaptic currents (EPSCs) were detected as fast inward currents at \(-60\) mV holding potentials (Fig. 6A). ATP \((100 \mu M)\) caused a substantial increase in whole cell channel activity (Fig. 6A), and this remained when TTX was added (Fig. 6B), indicating a spike-independent nature of this ATP effect. To further determine whether this ATP effect of increased whole cell channel activity involved modulation of synaptic currents, we first blocked both GABA and glutamate mediated synaptic activity with BIC \((30 \mu M)\), AP-5 \((50 \mu M)\), and CNQX \((10 \mu M)\). In the presence of these antagonists and TTX \((0.5 \mu M)\), hypocretin cells did not show any synaptic activity. Application of ATP still resulted in a similar increase in background activity as seen above, indicating that this ATP effect is independent of activation of ionotropic GABA and glutamate receptors (Fig. 6C). Finally, inhibiting synaptic transmitter release by introduction of a nominally Ca\(^{2+}\)-free extracellular solution did also not abolish the ATP-induced increase in background channel activity (Fig. 6D), supporting the view that a direct activation of purinergic P2X receptors causes this increase in membrane activity. As the large increase in membrane electrical activity obscured the response to ATP of EPSCs, these were not further analyzed.

We found no indication that exogenously applied ATP alters the inhibitory synaptic tone at hypocretin cells. These data further substantiate the view that the excitatory actions of ATP on these cells are based on direct activation of P2X receptors at these cells.

**Na\(^+\)-dependence of the ATP-induced current in hypocretin cells**

To study the ionic mechanisms underlying the ATP-mediated depolarization, we first tested the involvement of extracellular Na\(^+\). To this end, the ATP response was recorded in
voltage clamp before and during choline chloride substitution for extracellular NaCl. Sodium replacement became effective 10 min after switching the bath inflow and was detected by the fading and then absence of action potentials. Figure 7 displays a representative trace before and after exchange of the bath solution. Under these conditions, the mean inward current induced by local ATP application dropped significantly from 20.6 ± 4.1 (in Na⁺) to 4.9 ± 1.6 pA (in choline) (P < 0.01; n = 9; ANOVA; Fig. 7B), indicating that Na⁺-ions are involved in this ATP-induced inward current. To determine the reversal potential of this current, we applied slow voltage ramps (5 s) from −60 to 0 mV to identified hypocretin neurons. A Cs-based pipette solution and 0.5 μM TTX in the bath were used in these experiments to prevent the activation of potassium and sodium channels with depolarizing protocols. The reversal potential for the net ATP-mediated current was −27.6 ± 3.5 mV (range: −36.4 to −14.6 mV; n = 6). This value was negative to the expected Na⁺ reversal potential, but positive to the K⁺ reversal potential, and was suggestive of a nonselective cation current. A typical experiment showing the reversal potential for the ATP-induced current is presented in Fig. 7C.

In addition, studies in Ca²⁺-free conditions revealed that the ATP-induced inward current is not altered under nominal 0 mM extracellular Ca²⁺, consistent with the characteristics of a Na⁺-dependent nonselective cation channel (Liu et al. 2002). In Ca²⁺-free extracellular conditions and high extracellular Mg²⁺, ATP (100 μM) induced a mean inward current of 16.1 ± 4.1 pA (n = 7; Fig. 7B), which was not significantly different (P = 0.454; ANOVA) from inward currents under

![FIG. 6. ATP actions on background channel activity in hypocretin cells. A: typical trace showing increase in background channel activity on ATP application, obscuring the analysis of synaptic currents. This experiment was done in the presence of BIC (30 μM) to block inhibitory synaptic input and clamped at −60 mV. B: ATP-induced effect on whole cell channel activity persisted in the presence of TTX (0.5 μM) in the bath, suggesting that it was not attributable to spike-dependent activity. C: when glutamate- and GABA-mediated activity was blocked (using AP5/CNQX and BIC, respectively), ATP still induced an increase in membrane noise, suggesting mechanisms independent from excitatory postsynaptic current (EPSC) modulation. Traces show a representative experiment. D: ATP-mediated increase in background channel activity persisted under nominally Ca²⁺-free/high magnesium ACSF (conditions that attenuate release of neurotransmitter), further substantiating the independent nature of this ATP effect from GABA or glutamate release. Representative trace from a series of 8 cells recorded. A–D: ATP 100 μM if not otherwise noted.](http://jn.physiology.org/)

![FIG. 7. Na⁺-dependence and reversal potential of ATP-induced current. A: ATP-induced inward current in hypocretin neurons (trace 1) was nearly abolished after exchange of the NaCl bath solution for choline chloride, traces of a representative experiment. B: bar graph summarizing effect of choline substitution on ATP-induced inward current of 9 cells. In nominally Ca²⁺-free external solution, amplitude of ATP-mediated inward current was not significantly decreased. C: reversal potential for net ATP-induced current was near −30 mV in this representative experiment. Voltage-ramp protocols (from −60 to 0 mV during 5 s) were used to determine reversal potential of ATP-mediated current in hypocretin cells. Together, these results are consistent with ATP activation of nonselective cation channels in hypocretin cells. *P < 0.001.](http://jn.physiology.org/)
normal ACSF conditions. Together, these results suggest that ATP activates a nonsynaptic cation current. Such a current has been previously reported with ATP receptor activation in other regions of the brain (Edwards 1994).

**Is there a purinergic component in evoked excitatory potentials on hypocretin cells?**

To evaluate the possibility that purinergic transmission might be involved in electrically evoked excitatory synaptic potentials, cells were current-clamped with a slightly negative current corresponding to a resulting membrane voltage of –65 to –75 mV. A bipolar stimulation electrode placed in the lateral hypothalamus was used to generate controlled electrical stimuli (10–150 μA, 0.5 ms, 0.2 Hz) as described previously (Acuña-Goycolea et al. 2004). BIC (30 μM) was bath-applied to abolish inhibitory GABA-mediated evoked potentials. Local application of suramin (100 μM) reduced the time-voltage integral (area) of the evoked potentials of 10 cells to 70.3 ± 6.9% of control (83.9 ± 8.3% recovery), a statistically significant effect (P < 0.01; n = 10; ANOVA; Fig. 8B), suggesting ATP might be release from axonal terminals. As a control, we examined ionotropic glutamate receptor antagonists under the same conditions. Application of NMDA and AMPA receptor antagonists AP-5 (50 μM) and CNQX (10 μM) completely blocked evoked potentials (Fig. 8A), consistent with the view that they are attributable to glutamate release as was shown previously (Acuña-Goycolea et al. 2004). The inhibition of evoked potentials by suramin may therefore be attributable to suramin effects on glutamate transmission, as has been reported in spinal dorsal horn neurons (Gu et al. 1998; Lambrecht 2000). We therefore used a different receptor antagonist, PPADS, to study the effect on evoked excitatory potentials. Application of 50 μM PPADS did not change the time-voltage integral of evoked potentials in hypocretin cells (97.6 ± 8.2% of control; P = 0.83; n = 11; ANOVA; Fig. 8C). Together, these data indicate that the primary transmitter released during an excitatory evoked potential was probably glutamate. The partial blockade of the evoked excitatory postsynaptic potential (EPSP) by suramin but only a modest statistically insignificant reduction by PPADS suggests that, although a small amount of ATP may be released, the block could also be caused by a nonselective effect of suramin on glutamate receptors.

**DISCUSSION**

In this study, we studied the effect of extracellular ATP on the activity of GFPP-expressing hypocretin cells in mouse hypothalamic slices using whole cell patch-clamp recordings. Local application of ATP induced a substantial excitatory action on hypocretin cells with a positive response in 154 of 170 recorded cells (89.6% response rate), showed by membrane depolarization, an increase in spike frequency, a decrease in input resistance, and an induction of a Na⁺-dependent inward current that reversed near –27 mV. This excitatory effect was direct and postsynaptic in nature and sensitive to the ATP receptor antagonists PPADS and suramin. Application of the nonhydrolyzable ATP agonist ATP-γ-S mimicked these excitatory action, further substantiating activation of extracellular receptors. Insensitivity to α,β-MeATP and pH dependence of the inward current suggest involvement of purinergic P2X receptors that include P2X2 subunits.

Direct excitation of hypocretin cells by ATP through activation of P2X receptors

In the CNS, ATP can modulate synaptic transmission through the activation of ionotropic (P2X family) or metabotropic (P2Y family) receptor subtypes (Edwards and Gibb 1993). ATP can also exert an intracellular effect on membrane K⁺ channel activity (Ballanyi 2004; Liss and Roeper 2001). P2X receptors show a prominent expression throughout the hypothalamus (Kanjhan et al. 1999; Xiang et al. 1998). Here we found that local ATP application increased the spike frequency of hypocretin cells in a dose-dependent fashion. This excitatory reaction was independent of synaptic input, because even in the presence of TTX, ATP produced depolarization and an inward current. In addition, these effects correlated with a decrease in input resistance suggestive of an opening of ion channels in the postsynaptic membrane.

When choline was exchanged for sodium in the external solution, the ATP-induced inward current was substantially suppressed. A small inward current remained under nominally Na⁺-free conditions and might reflect a Ca²⁺-component of the ATP-induced current, as previously reported in dorsomedial hypothalamic neurons (Matsumoto et al. 2004). The reversal potential for this inward current was near –27 mV, part...
way between the values suggested by the Nernst equation for reversal of Na\(^+\) or K\(^+\) currents, suggesting a mixed current, probably a nonelective cation current, a type that has been reported elsewhere for ATP actions (Edwards 1994; Sorimachi et al. 2001). In parallel, G protein–coupled nonelective cation currents underlie the response of hypocretin cells to glucagon-like peptide 1 (Acuna-Goycolea and van den Pol 2004). Finally, use of a nominally Ca\(^{2+}\)-free extracellular buffer did not block the current, suggesting that it was not primarily caused by a calcium dependent mechanism, for instance a sodium/calcium exchanger (Liu et al. 2002).

The response of hypocretin neurons to ATP was reduced during the course of continuous application (see Fig. 1B). One mechanism that might account for this effect is the action of ectonucleotidases, which can rapidly degrade extracellular ATP (Zimmermann 1996). The nonhydrolysable ATP analog, ATP-\(\gamma\)-S, induced a depolarization and an inward current that lasted slightly longer than the one evoked by native ATP. However, even in the ongoing presence of ATP-\(\gamma\)-S we found a reduction in the postsynaptic responses, suggesting that receptor desensitization mechanisms also contribute to this effect (Ralevic and Burnstock 1998). Activity of ectonucleotidases has also been shown to diminish the action of endogenously released ATP (Westfall et al. 1996). However, using the ectonucleotidase inhibitor ARL 67156, we did not find any effect on the spontaneous spike frequency, suggesting a low intrinsic tone of extracellular ATP on hypocretin cells.

The question of what receptor class and subunit composition may mediate the ATP actions on hypocretin cells was addressed pharmacologically by using a number of purinergic agonists and antagonists. P2X channels form homomultimers or heteromers and different subtypes can be coexpressed in the same cell. Among the seven P2X subunits, P2X1–7, that have been described, CNS receptors seem to be predominantly comprised of P2X1, P2X2, P2X3, P2X4, and P2X6 subunits (Illes and Ribeiro 2004; Norenberg and Illes 2000; Robertson et al. 2001). On the other hand, 10 members of the G protein–coupled P2Y receptor family have been described, of which a number have been detected in the CNS (Illes and Ribeiro 2004). Our results show that postsynaptic ATP actions could be blocked by two antagonists of purinergic transmission, suramin and PPADS. Although these antagonists appear to be nonselective in terms of P2X and P2Y receptor specificity (Ralevic and Burnstock 1998), a number of P2X and P2Y subunits, i.e., P2X1, P2X4, P2X7, P2X4/6, P2Y3, P2Y4, P2Y5, and P2Y11, can be excluded based on their insensitivity toward these drugs (Lambrecht 2000; North and Suprenant 2000). Molliver et al. (2002) showed a suramin-sensitive P2Y2 receptor mediated depolarization in sensory neurons.

In this regard, additional tests were used to study purinergic receptors on hypocretin cells. The nonhydrolysable ATP agonist ATP-\(\gamma\)-S has been shown to be active at homomeric P2X1–6 and heteromeric P2X2/3 and P2X1/6 subunits as well as on P2Y1, P2Y2, and P2Y11. In contrast, \(\alpha,\beta\)-MeATP is a potent agonist only at P2X1 and P2X2 subunits and heteromeric combinations thereof (Lambrecht 2000). Because \(\alpha,\beta\)-MeATP was ineffective on hypocretin cells, those subunits can be excluded, suggesting an involvement of either P2X2 or P2Y1. Although no specific agonists or antagonists for P2X2 are available, the potentiation of the ATP current in the presence of an acidic environment seems to be a characteristic feature of those subunits (North 2002; Stoop et al. 1997). Our data show clearly an increase in ATP current amplitude when the extracellular medium is acidified to a pH of 6.8, thereby indicating ionotropic P2X2 receptor involvement and excluding mediation through metabotropic P2Y receptors.

Additional support for the nature of P2X2 receptor expression can be found in the desensitization kinetics of the ATP effects. Compared with P2X1 and P2X3, which desensitize rapidly (time constant, \(\tau < 100\) ms), receptors composed of P2X2 subunits are considered slow desensitizing, with \(\tau > 10\) s (North 2002). Also, P2X2 and P2X3 subunits require a considerable recovery time after ATP application for a reproducible response (>15 min), whereas P2X2 subunits can mediate ATP currents on repetitive agonist application in the frequency range of seconds, even responding with an increased current amplitude (Khakh et al. 2001; North 2002). Figures 1, 2, and 4 clearly show the slow but consistent desensitization of the ATP response on hypocretin cells. In addition, repetitive ATP application throughout our studies resulted in reproducible current responses as exemplified in Fig. 3A2. Consistent with the idea of P2X2 receptor involvement, the amplitude of the second ATP application was increased compared with the initial response. Taken together, the data suggest that hypocretin cells express predominantly P2X2 subunits. However, the expression of other subunits as part of heteromeric P2X channels cannot be ruled out and may account for the incomplete inhibition of the ATP action by PPADS and suramin (Jo and Role 2002). Our data are consistent with previous reports that at least three different ionotropic purinoceptors are expressed in the hypothalamus: P2X1, P2X2, and P2X3 (Gurin et al. 2003; Kanjhan et al. 1999; Xiang et al. 1998). We previously showed that hypothalamic melanin-concentrating hormone (MCH) cells are also excited by ATP (van den Pol et al. 2004). However, the subunit composition of ATP receptors in those cells was not addressed.

In other parts of the brain, ATP has both direct and indirect effects on synaptic activity (Bowser and Khakh 2004). In contrast to the strong direct excitatory action of ATP on hypocretin cells, analysis of both spontaneous and miniature synaptic activity did not reveal any detectable effect on the synaptic input to hypocretin cells by ATP. Hypocretin neurons do show an increase in electrical activity on ATP stimulation, but because this activity was blocked by choline substitution for sodium but not by GABA and glutamate receptor antagonists, it was most likely caused by an increase in somatic ion channel activity independent of synaptic activity.

Source of ATP and functional considerations

As described above, hypocretin cells show robust excitatory responses to extracellular ATP. What might be potential sources of ATP that could stimulate hypocretin neurons? An interesting report showed that ATP may be released by developing GABAergic axons of lateral hypothalamic cells during a period when GABA has excitatory effects caused by the elevated Cl- reversal potential (Jo and Role 2002). In the presence of the ATP receptor antagonist suramin, evoked potentials recorded in hypocretin neurons were diminished in amplitude; however, when a different ATP receptor antagonist, PPADS, was used, only a minor depression was noted. Whereas this could suggest a low level of ATP was released...
from stimulated axons, an alternative possibility is that the suramin attenuation of the evoked potential was not caused by blockade of the ATP receptor but could be caused by a reported nonselective attenuation of ionotropic glutamate receptors (Gu et al. 1998; Lambrecht 2000). This would be consistent with our finding both here and previously (Acuna-Goycolea et al. 2004; Li et al. 2002) that selective ionotropic glutamate receptor antagonists eliminate both evoked and spontaneous EPSPs in hypocretin neurons. It is noteworthy that Jo and Role found ATP release from cultured lateral hypothalamus cells, but not slices. The lack of detection in hypothalamic slices (here and in Jo and Role 2002) could be caused by developmental differences, to endogenous extracellular enzymes that break ATP down, or to different parameters regulating ATP release. Along similar lines, in spinal cord slices, <5% of the neurons showed clear ATP synaptic responses (Bardoni et al. 1997). ATP concentration is highest in neuronal vesicles and other cellular organelles, reaching ≤100 μM; free cytosolic ATP levels can reach high millimolar levels (Zimmermann 1996). The latter is true not only in neurons, but also in glia, from which ATP can be released to act as an intercellular signaling molecule (James and Butt 2002). ATP released by neurons into the synaptic cleft may reach local concentrations of ≤100 μM. Local stimulation of retinal astrocytes evoked ATP release with extracellular concentrations reaching 78 μM (Newman 2001). ATP can initiate glial Ca2+ and ATP waves able to travel several hundred microns (Hansson and Ronnback 2003; Wang et al. 2000). This glia–neuron communication, potentially mediated by ATP, can modulate or underlie homo- and heterosynaptic suppression at the astroglia-nerve terminal interface in other brain regions (Zhang et al. 2003), interneuron excitation (Bowser and Khakh 2004), and extrasynaptic neuron-glia signaling (Fields and Stevens 2000). Acute pathological conditions in the brain, such as seizures, hypoxia, ischemia, or trauma, can induce a dramatic increase in extracellular ATP, reaching millimolar levels (Ciccarelli et al. 2001; Hansson and Ronnback 2003; James and Butt 2002). In these conditions, elevated extracellular ATP would lead to P2X receptor activation, which in turn would excite hypocretin cells, resulting in enhanced secretion of hypocretin from axon terminals. This putative link between local cell injury and activation of the hypocretin system gets additional support from the finding that hypocretin cells appear to express mainly P2X2 subunits. Those subunit typically potentiate their response at a lower pH, and could do so in an acidified milieu of local brain ischemia or injury. Thus an increase in ATP within the lateral hypothalamus, either from neurons, glia, or from pathological conditions, may play a role in activation of the hypothalamic arousal system.

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REFERENCES


