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Expression and function of P2X purinoceptors in rat histaminergic neurons

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1 The pharmacology of ATP responses and the expression pattern of seven known subunits of the P2X receptor were investigated in individual histaminergic neurons of the tuberomamillary nucleus (TM).

2 ATP (3–1000 μM) evoked fast non-desensitizing inward currents in TM neurons. 2-methylthioATP (2MeSATP) displayed the same efficacy but a lower potency, EC_{50}s 84 μM versus 48 μM, when compared with ATP. Adenosine-diphosphate (ADP), uridine-triphosphate (UTP) and alpha beta methylene-ATP ($\alpha\beta$-meATP) were inactive.

3 ATP-mediated whole cell currents were potentiated by acidification of the recording solution (pH 7.5 and 6.6 were compared).

4 Single-cell RT–PCR (scRT–PCR) analysis revealed that the $P2X_2$ receptor is expressed in all PCR-positive neurons. Each of the $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_6$ mRNAs were detected in less than 35% of the cells.

5 Suramin antagonized ATP responses with an IC_{50} of 4.2 μM and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 1 μM) reduced ATP responses to 43% of control, when antagonists were pre-applied 90s before the agonist. Cibacron blue (3 μM) given together with ATP potentiated control responses by 67%, but inhibited it to 10% after pre-application.

6 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP–ATP) antagonized ATP responses with an IC_{50} of 7 μM.

7 Pharmacological properties of ATP responses together with scRT–PCR data suggest that $P2X_2$ is the major purinoceptor on the soma of TM neurons, however the presence of heteromeric $P2X_{2/5}$ receptors in some neurons cannot be excluded.

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Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EC_{50}, the ligand concentration producing a half-maximal response; EGTA, *O*,*O*′-bis(2-aminomethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetra-acetic acid; GABA, γ-amino-n-butyric acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; IC_{50}, 50% inhibitory concentration; 2MeSATP, 2-methylthioadenosine 5′-triphosphate; $\alpha\beta$-meATP, α,β-methyleneadenosine 5′-triphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RT–PCR, reverse transcription-polymerase chain reaction; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TM, tuberomamillary; UTP, uridine-5′-triphosphate

Introduction

ATP is found in every cell, where it is a major source of energy. In the nervous system, ATP has additional actions, which include its role in fast synaptic transmission and modulation (North & Surprenant, 2000; Khakh *et al*., 2001). Activity of histaminergic cells in the tuberomamillary nucleus (TM) of the posterior hypothalamus is closely associated with behavioural state (Lin *et al*., 1988): they fire tonically during waking, little during slow wave sleep, and not at all during rapid-eye-movement (REM) sleep. As homeostatic theory of sleep involves ATP depletion and adenosine accumulation in the brain (Huston *et al*., 1996; Benington & Heller, 1995; Arrigoni *et al*., 2001) the role of ATP- and adenosine- receptors in the main sleep-controlling centres needs to be elucidated. The tuberomamillary nucleus receives inputs from the pre-frontal cortex, the pre-optic area, and the septum-diagonal band. Its efferents reach most parts of the brain (Watanabe *et al*., 1984; Schwartz *et al*., 1991; Wada *et al*., 1991), providing, together with other aminergic systems the waking-drive in the brain (Brown *et al*., 2001). High levels of purinoceptor proteins (Loesch & Burnstock, 2001; Collo *et al*., 1996) and mRNA expression (Kanjhan *et al*., 1999) in tuberomamillary neurons are in keeping with ATP-induced rapid inward currents in these neurons (Furukawa *et al*., 1994). ATP is coreleased with different neurotransmitters (Mori *et al*., 2001; Nakatsuka & Gu, 2001; Jo & Role, 2002) and can enhance transmitter release through the presynaptic P2X and P2Y receptors (Jang *et al*., 2001; Norenberg & Illes, 2000). In the lateral hypothalamus ATP is coreleased with GABA (Jo & Role, 2002). We now present a structure-function analysis of P2X-receptors in identified histaminer-

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gic neurons using specific purinoceptor antagonists and single-cell RT–PCR.

Methods

Animals and cell preparation

Housing of rats and all procedures were carried out in accordance with the Animal Protection Law of the Federal Republic of Germany. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used. After decapitation of 3–4-week-old male Wistar rats ($n=20$) the brain was quickly removed and 450 μm thick transverse slices containing the TM region were cut (1 or 2 slices per rat) and incubated for 1 h in a solution containing (mM): NaCl 125, KCl 3.7, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.3, $NaHCO_3$ 23, D-glucose 10, phenol red 0.01%, bubbled with carbogen (pH 7.4). For preparation of isolated cells the lateral part of the TM nucleus was dissected from the slice and incubated with papain in crude form (0.3–0.5 mg ml^{-1}) for 40 min at 37°C. After rinsing the tissue was placed in a small volume of recording solution with the following composition (in mM): NaCl 150, KCl 3.7, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 10, pH adjusted to 7.4 with NaOH. Cells were separated by gentle pipetting. The selected cells were digitally photographed on an inverted microscope and their major axes measured. After whole-cell recording the cytoplasm was sucked into the electrode and cell identification was verified by RT–PCR analysis of histidine decarboxylase (HDC) expression.

Whole-cell current recording

Membrane currents were recorded using the standard whole-cell configuration of the patch-clamp technique. Micropipettes were fabricated from filament-containing thick-wall borosilicate glass tubes using a two-stage puller and had resistances between 2 and 5 MOhm after filling with the following solution adjusted to pH 7.3 (in mM): 140 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES/CsOH. The cells were voltage-clamped by an EPC-9 amplifier. After establishment of the whole cell configuration the series resistance (4–15 MΩ) was compensated (70–90%) and periodically monitored. The holding potential was −55 mV. Data were collected with commercially available software (TIDA for Windows, HEKA, Lambrecht, Germany).

Drug application

A fast perfusion technique was used for application of purinoreceptor agonists (Vorobjev *et al*., 1996). An isolated cell was first patch clamped and then lifted into the application system, where it was continuously perfused with control bath solution. The substances were applied through two glass capillaries, 0.2 mm in diameter. Three solutions flowed continuously, gravity-driven, at the same speed and fast lateral movements of the capillaries exposed a cell either to control- or test-solutions. The system allows brief exposures to agonists alone or in combination with antagonists. Solution exchange at the open tip of a patch electrode developed with a time constant of 0.5 ms or less.

Data analysis

Agonists concentration-response relationships were fitted with the equation (1): $R = R_{max}//(1+(EC_{50}/[ligand])^n)$; where R_{max} is the relative maximal response elicited by the ligand. Agonist-mediated responses were normalized to the maximal agonist-evoked responses. EC_{50} is the ligand concentration producing a half-maximal response, [ligand] is ligand concentration, and n is the Hill slope. The inhibition curves were fitted with the following equation (2): $R = R_{max}/(1+(IC_{50}/[L])n)$, where R_{max} is the maximal degree of block of the ATP-mediated response achieved by the tested blocker, IC_{50} is the concentration of the blocker producing a half-maximal block of control responses, [L] is the blocker concentration and n is the Hill coefficient. Data are given as the mean ± s.e.mean, statistical analysis was done with the non-parametrical Wilcoxon test. Significance level was set at $P<0.05$.

Cellular RNA harvest and reverse transcription

After recording, the cytoplasm of the cell was sucked into the electrode in a stream of sterile control solution. The content of the electrode (8 μl solution with cell) was expelled into an Eppendorf tube, containing 7 μl of a mixture prepared according to the protocol of the 'first strand cDNA synthesis kit' (Pharmacia Biotech, Freiburg, Germany). After incubation for 1 h at 37°C for reverse transcription (RT) this reaction was stopped by freezing at −20°C. PCR analysis was carried out to identify TM neurons and to determine the presence of P2X-receptor types. The primer sequences are listed in Table 1. Primers designed to recognize HDC cDNA were previously published (Sergeeva *et al*., 2001), the expected size of the amplification product was 457 base pairs. Thin-walled PCR tubes contained a mixture of first strand cDNA template (2–5 μl), 10× PCR buffer (5 μl), 10 pM each of sense and antisense primer, 200 μM of each dNTP and 2.5 units Taq polymerase. The final reaction volume was adjusted to 50 μl with nuclease-free water (Promega, Mannheim, Germany). The magnesium concentration was 2.5 mM in all amplifications. The Taq enzyme, PCR buffer, Mg^{2+} solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Amplification was performed on a thermal cycler (Perkin Elmer, GeneAmp 9600, Weiterstadt, Germany). A two round amplification strategy was used in each protocol. In each round 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 s, annealing at 50°C for 48 s, and extension at 72°C for 1 min. For the second amplification round 1 μl of the product of the first PCR was used as a template.

Products were visualized by staining with ethidium bromide and analysed by electrophoresis in 2% agarose gels. Representative products were purified (PCR purification kit from Qiagen, Erkrath, Germany) in water and sequenced in both directions on an automatic sequencing machine (ABI, model 377, Weiterstadt, Germany). They were found to match the published sequences (see Table 1). Care was taken to ensure that the PCR signal arose from the single-cell mRNA. Three negative controls were taken in every experiment: (i) electrode solution was expelled in reaction

Table 1 Individual P2X receptor primers for RT–PCR

Primer	*Sequence*	*Size of product (primer pair)*	*Source**
P2X all			
Dgup:	5′-TGTGA(G/A)(G/A)T(G/C)(T/G/C)(T/C/A/G)(T/A/G)(G/A)(G/C)(T/C/A/G)TGGTG-3′		
Dglo:	5′-GC(A/T/G)(A/T/G)(T/A)(C/T)CTGAA(G/A)TTGTA(GC)CC-3′		
$P2X_1$ lo:	5′-TGCCAGTCCAGATCACACTTC-3′	326 b.p. (+ Dgup)	X80477
$P2X_2$ lo:	5′-TTGGGGTTGCACTCTCTGATTC-3′	341 b.p. (+Dgup)	U14414
$P2X_3$ up:	5′-CCGTGGAGATGCCTATCATGA-3′		
$P2X_3$ lo:	5′-TCTTAATACCCAGAACGCCA-3′	252 b.p.	X90651
$P2X_4$ lo:	5′-CTTGATCTGGATACCCATGATGCCTC-3′	300 b.p. (+Dgup)	X87763
$P2X_5$ up:	5′-GCCGAAAGCTTCACCATTTCCATAA-3′		X92069
$P2X_5$ lo:	5′-CAGAGGAGATGGAGTGTGTGTGT-3′	322 b.p.	
$P2X_6$ lo:	5′-TTTCGTGTCCAGGTTGCAATC-3′	364 b.p. (+Dgup)	X92070
$P2X_7$ lo:	5′-GCCACCTCTGTAAAGTTCTCT-3′	257 b.p.(+Dgup)	X95882

*Sources are described by the accession number in GenBank.

tube as a template for the RT–PCR after submerging of electrode in the recording chamber with isolated neurons but no cell clamped or sucked in; (ii) to ensure that genomic DNA did not contribute to the PCR products, neurons were aspirated and processed in the same manner except that the reverse transcription was omitted; (iii) contamination from extraneous sources was checked by replacing the cellular template with water.

Drugs and chemicals

Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate potassium salt (ADP), 2-methylthioadenosine 5′-triphosphate tetrasodium salt (2MeSATP), α,β-methyleneadenosine 5′-triphosphate lithium salt ($\alpha\beta$-meATP), cibacron blue, suramin sodium salt, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium salt (PPADS), 2′,3′-O-(2,4,6-Trinitrophenyl)Adenosine 5′-Triphosphate (TNP-ATP), uridine-5′-triphosphate (UTP) sodium salt and γ-amino-n-butyric acid (GABA) obtained from Sigma/RBI (Deisenhofen, Germany).

Results

ATP-mediated currents in identified TM neurons

All neurons isolated from the ventral TM ($n=69$) responded to ATP (range from 3 μM to 1 mM) with fast non-desensitizing inward currents, indicating the presence of P2X purinoceptors. Maximal current amplitudes evoked by ATP at a saturating concentration (1 mM) were 0.5 ± 0.06 nA ($n=69$, range 0.05 to 2.3 nA), much smaller than the amplitudes of GABA (500 μM) responses (1 to 26% of the GABA response, 9.0 ± 1.15% ($n=31$) on average).

Single cell RT–PCR and whole-cell recordings

We combined whole-cell recordings with the single-cell RT–PCR (scRT–PCR) analysis of P2X receptor expression. Total mRNA, isolated from the TM nucleus was used as a positive control in RT–PCR analysis. We detected all seven known P2X receptors in this brain region (Figure 1A). Dose-response relations for ATP and the expression pattern of P2X receptors (scRT–PCR) were investigated in 34 neurons. Antagonism by suramin, TNP–ATP and PPADS were tested in some of these cells. A total of 33 were HDC (histidine decarboxylase) -positive, indicating that the majority of isolated neurons were histaminergic. They varied in soma size (16–30 μm) and shape (polygonal or round). One or more types of P2X receptors were detected in 17 neurons

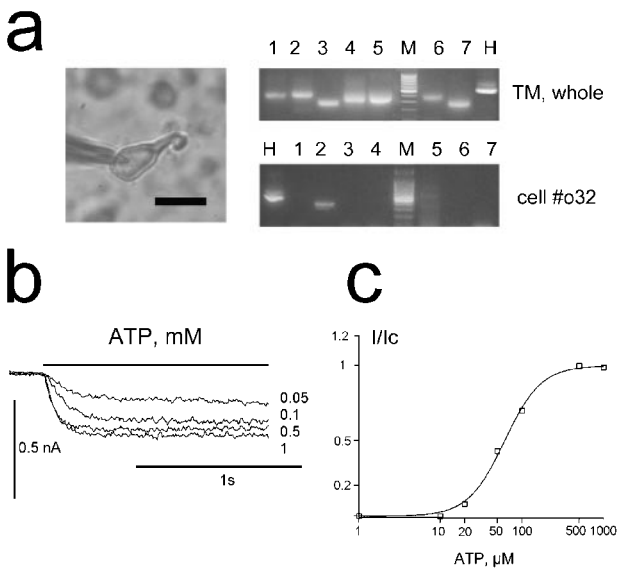

Figure 1 Expression pattern of seven subunits of the P2X receptor in tuberomamillary nucleus (TM) and a single neurone. Example of whole-cell patch-clamp recordings performed in a histaminergic TM neuron (#o32) identified by the expression of histidine decarboxylase (H). (a) Photographs of TM neuron (left, bar scale 20 μm) and ethidium bromide stained agarose gels demonstrating P2X expression in positive control (TM, whole) and in the representative neuron (M: DNA size marker, 100 b.p. ladder, 500 b.p. is the most intense line). (b) ATP-mediated responses in this neuron (0.05–1 mM). (c) Dose-response relation constructed from experimental data obtained in neuron #o32: peak current amplitudes normalized on maximal response amplitude are plotted versus incremental concentrations of ATP and fitted with equation (1). Best fit was obtained with an EC_{50} of 61 μM and Hill coefficient of 1.96.

(Figure 1, Table 2). All cells expressed the $P2X_2$ receptor type, $P2X_7$ was never seen in single neurons. Other P2X types were detected in less than 35% of the cells.

When several concentrations of ATP were applied, the averaged dose-response curve yielded an $EC_{50}=54.0\pm1.6$ μM, and a Hill coefficient, $n=1.6\pm0.07$, $n=15$, for the individual EC_{50} values see Table 2. EC_{50}s were not different between TM cells expressing (48.5 ± 8.5 μM, $n=4$) or lacking (57 ± 3.5 μM, $n=11$) $P2X_1$ and $P2X_3$ receptors (Wilcoxon-test, $P=0.27$). All cells displayed suramin-sensitive ATP-responses. Suramin (5 μM) inhibited ATP (100 μM) responses to $42.5\pm4\%$ ($n=11$, Table 2). There was no difference (Wilcoxon-test $P=0.13$) in the antagonism by suramin of ATP responses in cells expressing or lacking $P2X_4$ and/ or $P2X_6$ receptors. The ATP responses were reduced to $40\pm5.8\%$ ($n=5$) and $44\pm6.1\%$ ($n=6$) of control response respectively.

Pharmacology of ATP-mediated responses

Recombinant homomeric P2X (1–7) receptors have different affinity to agonists (Khakh *et al*., 2001). The potent agonist at homomeric recombinant $P2X_1$, $P2X_3$ and heteromeric $P2X_{2/3}$ purinoceptors alpha beta-methylene-ATP ($\alpha\beta$-meATP) at 300 μM did not evoke any current in TM neurons ($n=3$). The P2 receptor agonist 2-methylthioATP (2MeSATP) demonstrated the same efficacy but a slightly lower potency (EC_{50}) when compared with ATP in the same cells (Figure 2). When dose-response relations for the ATP and 2MeSATP obtained in seven neurons were fitted with the equation (1), the following EC_{50} and Hill coefficient values were obtained: 47.6 ± 1.7 μM, 1.6 ± 0.07 and 83.85 ± 6.96 μM, 1.45 ± 0.15, respectively. Adenosine-diphosphate (ADP, 1 mM, $n=3$) and uridine-triphosphate (UTP, 2 mM, $n=3$) did not evoke a current in TM neurons.

Can protons modulate ATP-responses? Acidification of the recording solution (pH 6.6 instead 7.5 in control conditions) potentiated ATP (30 μM) responses by $179\pm29\%$ ($n=5$, Figure 2B).

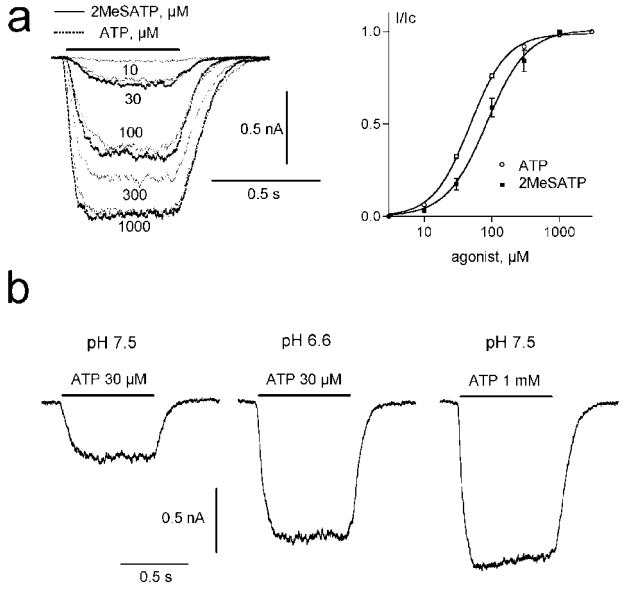

Figure 2 Properties of P2X receptor agonist-evoked responses in TM neurons. (a) Comparison of ATP and 2-methylthioATP (2MeSATP) potencies and efficacies. Representative responses to the same concentrations of both agonists are superimposed (left, ATP responses fat broken line). Dose-response plots for both agonists on the right. (b) Example of pH-dependence of ATP-responses.

Our scRT–PCR analysis revealed that the majority of the TM neurons expressed more than one P2X receptor-type. Are these different P2X receptor sub-populations ($P2X_2$, $P2X_{2/3}$, $P2X_4$, $P2X_{1/5}$, $P2X_6$, $P2X_{2/5}$) functionally present at the somata of TM neurons? In order to answer this question we tested several known P2X receptor-type-specific antagonists. Suramin inhibited ATP (100 μM) responses in a dose-dependent manner. When suramin was given during an ATP application, it inhibited agonist-responses with an $IC_{50}=27.7\pm2.1$ μM (nHill $=1.19\pm0.1$, $n=3$, Figure 3). When suramin in several different concentrations was pre-applied

Table 2 Expression of P2X receptors and pharmacological properties of ATP responses in TM neurons

*cell#**	*P2X$_1$*	*P2X$_2$*	*P2X$_3$*	*P2X$_4$*	*P2X$_5$*	*P2X$_6$*	*P2X$_7$*	*EC$_{50}$, μM*	*SUR 5 μM%*
oa17	–	+	+	–	–	+	–	39	n.m.
oa19	–	+	–	–	–	–	–	70	n.m.
oa9	–	+	–	–	+	–	–	46	50
oc12	–	+	+	–	–	–	–	31	n.m.
oa20	–	+	–	+	+	–	–	57	n.m.
od11	–	+	–	–	–	–	–	62	47
od18	–	+	+	–	–	–	–	70	41
ol2	–	+	+	–	+	–	–	n.m.	35
ot30	+	+	–	–	–	–	–	n.m.	n.m.
ot40	+	+	+	+	–	–	–	54	n.m.
v16	–	+	–	+	+	–	–	36	25
px1	–	+	–	+	+	–	–	52	30
px2	–	+	–	–	+	–	–	47	70
px3	–	+	–	–	–	+	–	64	54
px6	–	+	–	–	–	+	–	77	52
o30	–	+	–	–	–	–	–	61	35
o32	–	+	–	–	–	–	–	56	28

*Cell number has letter code. EC_{50} was obtained by fitting individual ATP dose-response curves with equation (1); Sur 5 μM%: Amplitude of ATP (100 μM, % of control response) in the presence of suramin (5 μM). Antagonist was pre-applied for 90 s. n.m.–not measured.

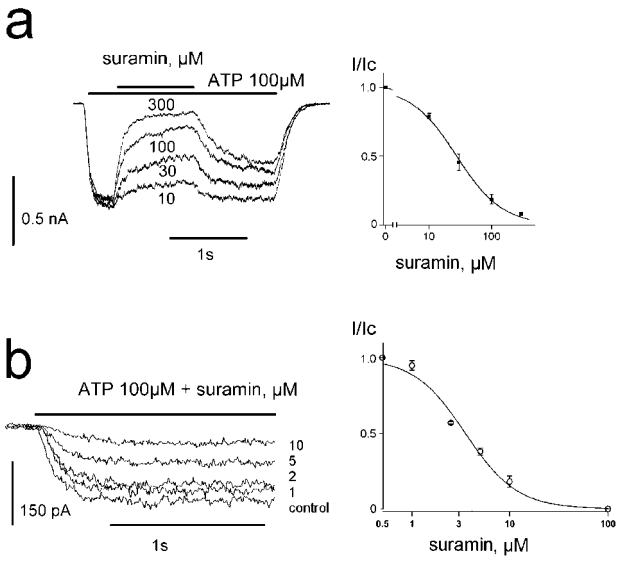

Figure 3 Suramin potency depends on application paradigm. (a) Suramin, 10–300 μM, applied during ATP response reduced control responses with an $IC_{50}=27.7$ μM. The dose-response curve ($n=3$ experiments) on the right was fitted with equation (2). (b) Suramin was preapplied for 90 s at different concentrations before ATP (left traces). The dose-response curve at the right, obtained from eight cells, yielded an $IC_{50}=4.2$ μM.

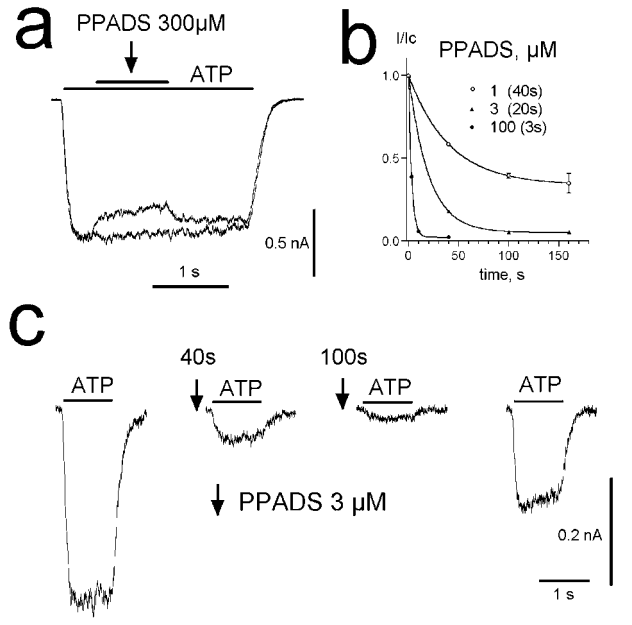

Figure 4 PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) inhibition depends on preapplication time. (a) Example of ATP response reduction by 300 μM PPADS during coapplication. (b) Amplitudes of ATP (100 μM) responses in control and in the continuous presence of PPADS are plotted versus time of PPADS application, demonstrating the time-dependence of the PPADS block. Data points were fitted with an exponential function, time constants (τ) are indicated after the concentrations of PPADS. (c) Example of ATP (100 μM) responses (from left to right: control, after 40 s, after 100 s of PPADS preapplication and after 7 min washout).

for 90 s before ATP (100 μM), the dose-response relation yielded an $IC_{50}=4.2\pm0.3$ μM and a Hill coefficient $=1.6\pm0.15$ ($n=8$).

Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), given during an ATP (100 μM) application slightly reduced the response (to $85\pm5\%$, $n=3$) at a concentration of 300 μM (Figure 4A). PPADS inhibited ATP-evoked currents with much higher potency when it was applied for 1–160 s before the agonist. The longer the pre-application time, the more pronounced was the block. When the resulting ATP response amplitudes were plotted versus time of PPADS pre-application, data points could be fitted with an exponential function, yielding the following time constants and relative amplitudes of responses at maximal inhibition: $\tau=40\pm7$ s, 0.34 ± 0.03 ($n=3$), $\tau=20\pm1$ s, 0.06 ± 0.01 ($n=4$) and $\tau=3\pm0.1$ s, 0.02 ± 0.06 ($n=3$) for the 1, 3 and 100 μM of PPADS respectively (Figure 4B, C). In order to test cell-to-cell variability in their sensitivity to PPADS we perfused 1 μM of this blocker for 90 s prior to the common ATP/PPADS application. All tested neurons were PPADS-sensitive, the average inhibition was to $43.4\pm3.3\%$ of control ($n=9$). Complete recovery of control responses was not observed after prolonged PPADS administration. After a 5 min washout period the ATP responses reached $60\pm3.2\%$ of the control response ($n=9$).

Cibacron blue when given during ATP (30 μM) application at 3 μM potentiated the response by $67\pm0.12\%$ ($n=4$), but inhibited it at 100 and 300 μM to $9\pm4\%$ and $3.5\pm3\%$, respectively ($n=4$, Figure 5A). When cibacron blue was pre-applied for 40 s it inhibited ATP (30 μM) responses to $10\pm2\%$ ($n=3$) and to $3\pm1.5\%$ ($n=2$) at 3 and 30 μM, respectively.

In order to test the functional presence of $P2X_{2/3}$ receptors we used TNP–ATP (2′,3′-O-(2,4,6-Trinitrophenyl)Adenosine 5′-Triphosphate (Virginio *et al*., 1998; Burgard *et al*., 2000) to block ATP (100 μM) responses. As a 90 s pre-application did not change the extent of block, co-application was used in this experiment. When the dose-response curve was fitted with equation (2) we obtained an $IC_{50}=7.1\pm0.5$ μM and a Hill coefficient $n=1.77\pm0.22$ ($n=5$, Figure 6).

Discussion

We used a combination of single cell RT–PCR and patch-clamp electrophysiology to identify the P2X receptors in rat histaminergic neurons. TM neurons responded to ATP and 2MeSATP with a rapid non-desensitizing inward current and

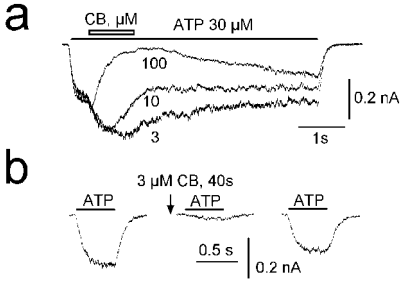

Figure 5 Cibacron blue (CB) modulates ATP responses in a dual way. (a) Cibacron blue (open bar) applied during ATP perfusion either potentiated (at 3 and 10 μM) or inhibited (at 100 μM) the ATP responses. (b) Pre-application of cibacron blue (3 μM) for 40 s (indicated by arrow) causes a strong block of ATP (30 μM) responses (from left to the right: control, block by antagonist and recovery).

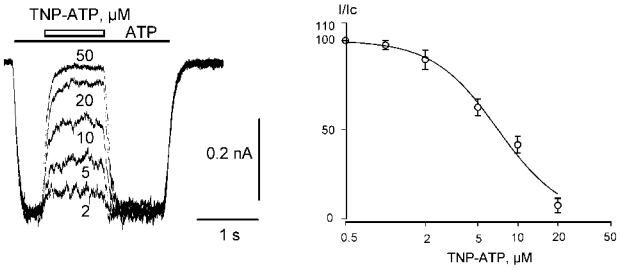

Figure 6 TNP-ATP (2′,3′-O-(2,4,6-Trinitrophenyl)Adenosine 5′-Triphosphate) antagonized ATP (100 μM) responses with an $IC_{50} = 7$ μM. Left: superimposed ATP responses in the presence of different TNP–ATP concentrations (open bar indicates time course of TNP–ATP application). Right: Averaged dose-response curve demonstrating concentration-dependence of TNP–ATP block ($n = 5$).

did not respond to UTP consistent with the activation of P2X channels. UTP is a weak or inactive agonist at P2X receptors, but an agonist at several P2Y receptors (Ralevic & Burnstock, 1998). ADP and alpha beta methylene-ATP ($\alpha\beta$-meATP) did not induce any current in TM neurons. When compared with maximal GABA-currents, the maximal ATP responses represented only a small fraction (9%). We have shown previously (Sergeeva *et al*., 2002) that gabazine (10 μM) completely blocks spontaneous IPSCs in TM neurons, which is different from lateral hypothalamic neurons, where residual spontaneous currents were found to be ATP-mediated (Jo & Role, 2002). Failure to detect such currents in TM neurons may be related to a smaller ATP/GABA amplitude ratio. Physiological activation of P2X receptors in TM neurons needs to be elucidated in the future.

TM neurons responded to exogenously applied ATP with an $EC_{50} = 50$ μM, in keeping with previously reported values for non-identified acutely isolated neurons from the TM region ($EC_{50} = 44$ μM) (Furukawa *et al*., 1994) and for the PC12 cells ($EC_{50} = 45$ μM) (Michel *et al*., 1996). Recombinant homomeric $P2X_1$ and $P2X_3$ and heteromeric $P2X_{2/3}$, $P2X_{1/5}$ and $P2X_{4/6}$ receptors display much higher ATP sensitivity ($EC_{50} = 1-6$ μM), while heteromeric $P2X_{2/6}$ receptors give EC_{50} values (30 μM) (Khakh *et al*., 2001) similar to our study. We did not find a correlation between ATP EC_{50}s and the expression pattern of different P2X receptors in TM neurons. The reason for this finding may be the absence of functional $P2X_1$ and $P2X_3$ receptors in the somata of TM neurons, which is also evident from the lack of sensitivity to $\alpha\beta$-meATP and the low sensitivity of ATP-responses to TNP–ATP (7 μM in our study). TNP–ATP is much more potent at $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors (EC_{50}s are 6, 1 and 7 nM, respectively), than at other subtypes with $P2X_4$ being most insensitive ($EC_{50} = 15$ μM) (North & Surprenant, 2000).

Several groups have reported that 2-methylthioATP (2MeSATP) is a partial agonist and much less potent than ATP in activating $P2X_4$ receptors (Soto *et al*., 1996; Wang *et al*., 1996; Jones *et al*., 2000). In TM neurons 2MeSATP demonstrated the same efficacy and a slightly lower potency than ATP (corresponding EC_{50}s were 84 μM versus 48 μM). In all neurons tested ATP responses were completely blocked by suramin (20 μM) and PPADS (100 μM), while heterologously expressed $P2X_4$ (Buell *et al*., 1996) and $P2X_6$ receptors (Collo *et al*., 1996) were insensitive or only slightly affected by blockers taken in concentration of 100 μM (reviewed in Khakh *et al*. (2001) and Norenberg & Illes, (2000)). Therefore, we can exclude the formation of functional homomeric $P2X_4$ or $P2X_6$ receptors on the somata of TM neurons. The presence of $P2X_{4/6}$ heteromers is also unlikely, these two receptors were never co-expressed in single TM neurons, see Table 2. Recombinant $P2X_2$, $P2X_1$ and $P2X_3$ receptors are more sensitive to pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS, $IC_{50} = 1$ μM), than other P2X subtypes, with $P2X_4$ being the most insensitive (> 300 μM) (North & Surprenant, 2000). In TM neurons 1 μM of PPADS caused half inhibition of ATP responses, when pre-application was used. Heterologously expressed $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ receptors are sensitive to suramin, with half-maximal inhibition at concentrations of 1 to 5 μM (Khakh *et al*., 2001)). Suramin, when applied before ATP, caused half inhibition of control responses in our study (4 μM), which indicates, together with the other pharmacological properties of ATP responses found in TM neurons, the functional presence of $P2X_2$ receptors.

$P2X_2$ receptors show potentiation of ATP responses under acidification of the extracellular solution, as a result of the increased affinity of the channel for ATP in the presence of protons (Ding & Sachs, 1999; King *et al*., 1997). In contrast, $P2X_1$, $P2X_3$, $P2X_4$ receptors are inhibited by lowering pH (Stoop *et al*., 1997). In TM neurons ATP responses were dramatically potentiated by an increase in proton concentration (pH 6.6 versus pH 7.5 in control solution).

The inhibition profile by cibacron blue (an isomer of reactive blue 2) of ATP responses in TM neurons is hard to interpret, as no detailed studies are available on this subject. The effects of cibacron blue on $P2X_4$ receptors appear complex. A recent study has shown that cibacron blue, when co-applied with ATP, is a weak antagonist at the rat $P2X_4$ receptor ($IC_{50} > 300$ μM) while $P2X_4$ currents are enhanced by pre-incubation with low concentrations of cibacron blue (3–30 μM) (Miller *et al*., 1998). The rat $P2X_4$ receptor has been shown in another study to be half inhibited at approximately 120 μM of cibacron blue in rats (Soto *et al*., 1996). We cannot exclude the functional presence of $P2X_{2/5}$ receptors on the somata of some TM neurons as a formation of this heteromer is possible (Torres *et al*., 1999) and its pharmacological features are not known.

Our molecular and pharmacological data show that $P2X_2$ is the predominant receptor type in rat histaminergic neurons and provide a useful basis for elucidating its physiological relevance.

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