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The phenothiazine-class antipsychotic drugs prochlorperazine and trifluoperazine are potent allosteric modulators of the human P2X7 receptor

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ABSTRACT

P2X7, an ATP-gated cation channel, is involved in immune cell activation, hyperalgesia and neuropathic pain. By regulating cytokine release in the brain, P2X7 has been linked to the pathophysiology of mood disorders and schizophrenia. We here assess the impact of 123 drugs that act in the central nervous system on human P2X7. Most prominently, the tricyclic antipsychotics prochlorperazine (PCP) and trifluoperazine (TFP) potently inhibited P2X7-mediated Ca^{2+} entry, dye permeation and ionic currents. In divalent cation-containing bath solutions or after prolonged incubation, ATP-evoked P2X7 currents were inhibited by 10 μM PCP. This effect was not related to dopamine receptor antagonism. Surprisingly, PCP co-applied with ATP enhanced inward currents in bath solutions with low divalent cation concentrations. Intracellular perfusion with PCP did not substitute for the extracellularly applied drug, indicating that its binding sites are accessible from the extracellular space. Since P2X7 current potentiation by PCP was voltage-dependent, at least one site may be located within the electrical field of the membrane. While the channel opening and closure kinetic was altered by PCP, the apparent affinity of ATP remained unchanged (potentiation) or changed slightly (inhibition). Measurements in human monocyte-derived macrophages confirmed the PCP-induced inhibition of ATP-evoked Ca^{2+} influx, Yo-Pro-1 permeability, and whole cell currents. Interestingly, neither heterologously expressed rat or mouse P2X7 nor native P2X7 in rat astrocyte cultures or in mouse bone marrow-derived macrophages were inhibited by perazines with a similar potency. We conclude that perazine-type neuroleptics are potent, but species-selective allosteric modulators of human but not murine P2X7 receptors.

1. Introduction

Purinergic signalling in the CNS is involved in a plethora of adaptive responses. These responses may range from dynamic and spatially confined effects on synaptic co-transmission to long-lived alterations of brain function e.g. in traumatic, ischaemic or hemorrhagic brain injury or in neuroinflammatory or neurodegenerative diseases (Burnstock and Kennedy, 2011; Khakh and North, 2012). The receptors for ATP and related nucleotides divide into the families of G-protein-coupled P2Y and ion channel-linked P2X receptors. Within the latter family, P2X7 is characterized by a low ATP binding affinity and mediates non-inactivating cationic currents that are poorly selective and voltage-independent. In the continuous or repeated presence of ATP, P2X7 forms or recruits large pores that, in addition to Ca^{2+} and Na^{+} entry or K^{+} efflux, allow organic ions to penetrate into or out of the cell (recently reviewed in Coddou et al., 2011; Khakh and North, 2012). Strong and long-lasting P2X7 stimulation may eventually trigger apoptosis or Ca^{2+} overload-induced cell death (Burnstock and Verkhratsky, 2010). Since ATP concentrations of 0.1–10 mM are required to elicit a robust activation of P2X7 (Jarvis and Khakh, 2009), its stimulation by vesicular release of ATP, leading to appropriately high extracellular ATP

Abbreviations: 5-BDBD, 5-(3-bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; AZ10606120, *N*-[2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-ylacetamide; CaM, calmodulin; BzATP, 2′(3′)-*O*-(4-benzolybenzoyl)adenosine-5′-triphosphate; DIC, divalent cations; HBS, HEPES-buffered solution; hMDM, human monocyte-derived macrophages; PCP, prochlorperazine; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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concentrations (Pankratov et al., 2006) is presumably spatially and temporally restricted. In the vicinity of damaged cells, leakage of ATP acts as a danger signal to trigger immunological responses (Franke et al., 2012). In the continuous presence of high extracellular ATP concentrations, immune cell activation and proliferation is triggered by P2X7-mediated release of proinflammatory cytokines such as IL-1β or TNFα (Ferrari et al., 1997).

In the CNS, P2X7 expression has been reported in microglia (Chessell et al., 1997), astrocytes (Oliveira et al., 2011) and oligodendrocytes (Domercq et al., 2010), but also in specific subsets of neurones (Deuchars et al., 2001; Sperlágh et al., 2002). Recent evidence points to a seminal role of P2X7 activation in mediating microglia activation (He et al., 2012) and modulation of astroglial proliferation after local brain injury (Franke et al., 2012), but also in affecting neuronal functions within the context of chronically diseased states. Indeed, there is a growing body of evidence, that P2X7 receptors are involved in the pathophysiology of Alzheimer's disease, mood disorders and schizophrenia (Basso et al., 2009; Skaper et al., 2009, 2010; Volonté et al., 2012). Variations in the P2X7-encoding gene that are correlated with the occurrence of affective disorders (Roger et al., 2010) or schizophrenia (Hansen et al., 2008) further corroborate the link between P2X7 and psychiatric diseases.

We have recently observed that human P2X7 is modulated by many drug-like or natural compounds. The activity of another member of the P2X subfamily, P2X4, has been shown to be strongly antagonized by the antidepressants paroxetine and fluoxetine (Nagata et al., 2009), presumably by reducing receptor cell surface expression (Toulmé et al., 2010). We here investigated whether psychoactive drugs interfere with P2X7. To this end, we screened a focussed library, comprising a total of 123 centrally acting drugs, including antipsychotics, antidepressants, anticonvulsives, sedatives, psychostimulants and nootropics, for possible modulatory activities on recombinantly expressed human P2X7 channels. We provide evidence for an as yet unknown species-selective inhibition of human P2X7 by prochlorperazine (PCP) and trifluoperazine (TFP). Thus, these CNS-penetrating perazine-type drugs may be instrumental to assign pathophysiological roles of P2X7 and, moreover, to develop experimental therapies.

2. Material and methods

2.1. Cell culture

Human embryonic kidney (HEK) 293 cells stably expressing the human P2X7 (HEK_{hP2X7}) or P2X4 (HEK_{hP2X4}) or the rat P2X7 (HEK_{rP2X7}) were cultured as described previously (Nörenberg et al., 2011, 2012), and used for experiments one (P2X4) or two (P2X7) days after splitting. Similarly, the rat P2X2 was transfected to a HEK293 cell line (HEK_{rP2X2}) and maintained in DMEM, supplemented with 10% foetal calf serum, 2 mM L-glutamine, 15 μg/ml blasticidin, and 100 μg/ml hygromycin in a humidified 5% CO_2 incubator. A cell line, stably expressing the mouse P2X7 was generated by subcloning the mouse (C57BL/6 strain) P2X7 into pcDNA3.1-Zeo (Invitrogen), and transfecting HEK293 cells with lipofectamine. Positive clones were selected, expanded and maintained in a growth medium supplemented with 500 μg/ml zeocin. Preparation and cultivation of human monocyte-derived macrophages was done according to the protocol of Davies and Gordon (2005), with modifications as described by Nörenberg et al. (2011). Mouse monocytic cells were obtained by flushing the bone marrow of the femoral and tibial bones of euthanized C57BL/6 mice. The isolation procedure, differentiation into macrophages, and culture conditions are described by Nörenberg et al. (2011). Astrocyte cultures were prepared as reported by Vermeiren et al. (2005), and Nörenberg et al. (2012). All animal procedures were approved by the governmental animal care committee of Saxony, Germany. Procedures were optimized to reduce the number of sacrificed animals and their suffering, according to the regulations of the German Animal Welfare Act. Human materials were obtained after positive evaluation by the local ethical committee.

2.2. Fluorometric $[Ca^{2+}]_i$ measurement

Changes of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in HEK_{hP2X7} cell suspensions was assessed by loading cells with Fluo-4/AM (4 μM) and monitoring the time-course of fluorescence intensities during ATP injection either with a filter-based plate reader device (POLARstar Omega, BMG Labtech, Offenburg, Germany) or with a custom made fluorescence plate imaging device (Nörenberg et al., 2012). To compensate for uneven loading or fluorescence detection sensitivity, fluorescence intensities were corrected for background signals, and normalized to the basal intensity (F/F_0). To eliminate contaminating signals arising from P2Y receptor-triggered Ca^{2+} mobilization from internal storage organelles, Fluo-4-loaded HEK_{rP2X2} and HEK_{hP2X4} cell suspensions were incubated with 2.5 μM thapsigargin for 5–8 min prior to ATP stimulation. Non-inactivating $[Ca^{2+}]_i$ signals in HEK_{hP2X7} cells were exclusively attributable to P2X7 (without thapsigargin treatment) when assessed > 2 min after agonist application.

2.3. Yo-Pro-1 uptake assay

The permeability of large organic molecules was monitored with a Yo-Pro-1 accumulation assay. $HEK293_{P2X7}$ cells were harvested, washed, resuspended in HBS with low divalent cation concentrations (no $MgCl_2$ and 0.1 mM $CaCl_2$; low-DIC HBS), and dispensed into pigmented clear-bottom 384-well microplates. Yo-Pro-1 (1 μM, Invitrogen) was added. The fluorescence was monitored, and ATP was added with a plate reader device, or in the plate imaging system described above. For single cell imaging of Yo-Pro-1 accumulation in hMDM, cells were grown on 25 mm glass coverslips, mounted in a bath chamber, and viewed under an inverted microscope (Fluar 10×/0.5; Axiovert 100 microscope, Carl Zeiss, Jena, Germany). For excitation a monochromator (Polychrome V, Till-Photonics, Gräfelfing, Germany) was used, creating a wavelength of 470 nm. Emission was recorded with a cooled CCD camera (Sensicam HR, PCO, Kelheim, Germany) through a dichroic beam splitter (DCXR-510, Chroma, Rockingham, VT) and filtered with a 515 nm long-pass filter (OG 515, Schott, Jena, Germany). After imaging 10 cycles (one image every 5 s) Yo-Pro-1, modulators and ATP were subsequently added. To permeabilize the cells and for determination of regions of interest covering single cells as well as subsequent quantitative analysis of fluorescence intensity, Triton X-100 (0.1% w/v) was added at the end of each experiment.

2.4. Electrophysiology

Whole-cell recordings were performed at room temperature and at a holding potential of −60 mV, using an EPC9 amplifier controlled by Pulse software (HEKA, Lambrecht, Germany). The extracellular solution (standard divalent cation concentration = standard DIC) contained 147 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 13 mM D-glucose, and 10 mM HEPES (~305 mOsm l^{-1}; pH 7.3 with NaOH). In a fraction of experiments, a modified extracellular solution (low divalent cation concentration = low DIC; 0 mM Mg^{2+}, 0.1 mM Ca^{2+}) was used instead. In experiments with astroglial cells and human monocyte-derived macrophages, respectively, the low DIC bath solution was augmented with either carbenoxolone (100 μM) in order to block a gap junction-like conductance or 5-BDBD (5 μM) to suppress residual currents mediated by coexisting P2X4 receptors (Nörenberg et al., 2012). Patch pipettes had a resistance of 2–4 MΩ, when filled with intracellular solution of the following composition: 147 mM KCl, 10 mM HEPES, and 10 mM EGTA (~300 mOsm l^{-1}; pH 7.3 with KOH). Drugs were focally delivered in vicinity (~50 μm) of the cell under investigation by means of the application cannula (100 μm inner diameter) of a solenoid valve-driven pressurized superfusion system (DAD-12, Adams and List, New York, USA). Experiments during which series resistance (routinely compensated by 60–80%) changed by more than 20% were not included in the analysis. ATP-induced currents, sampled at 5 kHz and filtered at 1.7 kHz, were measured as peak amplitudes and normalized for differences in cell size (membrane capacitance) to obtain current densities (pA pF^{-1}).

2.5. Drugs

ATP, carbenoxolone, antipsychotic and antidepressive drugs were from Sigma–Aldrich (Taufkirchen, Germany). AZ10606120, 5-BDBD, BzATP, and W-7 were from Tocris (Bristol, UK). Stock solutions of drugs (1–100 mM) were prepared with the appropriate standard or low DIC bath solution (ATP and BzATP), in deionized water (carbenoxolone) or in DMSO (AZ10606120, 5-BDBD, W-7, and all psychoactive drugs). With the exception of carbenoxolone, which was always freshly prepared at the experimental day, aliquots of stock solutions were stored at −20 °C, and freshly diluted with the appropriate extracellular saline. The final DMSO content in the bath never exceeded 0.1%, a concentration that had no effect on ATP-induced currents in HEK_{hP2X7} cells (Nörenberg et al., 2012). ATP stock solutions were routinely readjusted to pH 7.3. Calculations of physicochemical data and van-der-Waals volumes were obtained from www.chemicalize.com.

2.6. Statistical analysis

All data were expressed as mean ± SEM, obtained in *n* cells or experiments. Statistical significance was tested using the Mann–Whitney *U*-Test or the Kolmogorov–Smirnov test to proof for normal distribution followed by a Student's *t*-test. In case of multiple comparisons, the Kruskal–Wallis one-way ANOVA on ranks was