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P2X7 Receptors in Neurohypophysial Terminals: Evidence for their Role in Arginine-Vasopressin Secretion

Adolfo E. Cuadra¹, Edward E. Custer¹, Elizabeth L. Bosworth¹, and José R. Lemos^{1,2,*}

¹Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, Massachusetts, 01605-2324

²Program in Neurosciences, University of Massachusetts Medical School, Worcester, Massachusetts, 01605-2324

Abstract

Arginine-vasopressin (AVP) plays a major role in maintaining cardiovascular function and related pathologies. The mechanism involved in its release into the circulation is complex and highly regulated. Recent work has implicated the purinergic receptor, P2X7R, in a role for catecholamine-enhanced AVP release in the rat hypothalamic-neurohypophysial (NH) system. However, the site of P2X7R action in this endocrine system and whether or not it directly mediates release in secretory neurons have not been determined. We hypothesized that the P2X7R is expressed and mediates AVP release in NH terminals. P2X7R function was first examined by patch-clamp recordings in isolated NH terminals. Results revealed that subpopulations of isolated terminals displayed either high ATP-sensitivity or low ATP-sensitivity, the latter of which was characteristic of the rat P2X7R. Additional recordings showed that terminals showing sensitivity to the P2X7R-selective agonist, BzATP, were further inhibited by P2X7R selective antagonists, AZ10606120 and brilliant blue-G. In confocal micrographs from isolated terminals of the NH showed that P2X7R-immunoreactivity was localized in the plasma membranes. Lastly, the role of P2X7R on AVP release was tested. Our results showed that BzATP evoked sustained AVP release in NH terminals, which was inhibited by AZ10606120. Taken together, our data lead us to conclude that the P2X7R is expressed in NH terminals and corroborates its role in AVP secretion.

Keywords

Depolarization-secretion coupling; P2X receptor; Neuropeptide; Exocytosis; posterior pituitary; ligand gated ion channel; Neuronal; Positive feedback

INTRODUCTION

An important role for the hormone arginine-vasopressin (AVP) is in cardiovascular homeostasis by acting as both a pressor and an antidiuretic agent (Brownstein, 1983). AVP

*Correspondence to: Prof. José R. Lemos, Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Albert Sherman Center, 368 Plantation Street AS8-1041, Worcester, MA 01605-2324, Tel.: (508)856-8567, Fax: (508)856-5920, Jose.Lemos@umassmed.edu.

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and the hormone oxytocin (OT) are released by the hypothalamic-neurohypophyseal (NH) system (HNS). This system consists of the magnocellular neurons of the paraventricular nuclei (PVN) and the supraoptic nuclei (SON), in the hypothalamus, which produce, process and transport hormones along their axonal projections that terminate in the NH (Brownstein, 1983; Lemos, 2012). Purinergic ionotropic receptors (P2XR) are involved in the secretion of AVP and OT (Gomes et al., 2009; Knott et al., 2008; Troadec et al., 1998). ATP mediates AVP release by stimulation of the P2X2R in NH terminals (Knott et al., 2008; Troadec et al., 1998). ATP-stimulated neuropeptide release is synergistically enhanced by catecholamine stimulation (Sladek and Song, 2008). Recent work has implicated the P2X7R in this process since the selective antagonist, brilliant blue-G (BBG), inhibited secretion from the HNS, but only upon co-application of ATP with the catecholamine analog, phenylephrine (Gomes et al., 2009). These observations could have important implications regarding AVP and catecholamine's role in cardiovascular pathology.

Based on these findings, we asked two key questions in this study: *i*) what is the site of P2X7R action for AVP release within the HNS; and, *ii*) does the P2X7R act directly in magnocellular neurons to mediate secretion. It has been suggested that the effects of P2X7R may be mediated at the somata, in the hypothalamus (Sladek and Song, 2008; Song and Sladek, 2006). However, investigations of the magnocellular-neurosecretory cell somata in this region have not revealed P2X7R activity (Knott et al., 2012; Shibuya et al., 1999; Vavra et al., 2011). Our laboratory has reported P2X2R- and P2X3R-mediated currents in isolated NH terminals, showing sensitivity to P2X7R inhibitors suramin and Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (Knott et al., 2005). More recently our work has revealed purinergic ionotropic currents with resistance to suramin and further show immunoreactivity to P2X7R in the nerve endings of the NH (Knott et al., 2012; Knott et al., 2005). This finding is consistent with earlier reports showing P2X7R immunoreactivity in presynaptic nerve terminals of the brain and neuromuscular junction (Deuchars et al., 2001; Marcoli et al., 2008; Moores et al., 2005). Based on these and other observations, we hypothesized that the P2X7R is expressed in NH terminals and that it is capable of mediating hormone release. In this study we directly assessed P2X7R function in the NH with electrophysiology experiments and then examined the role of this receptor in AVP secretion in neuropeptide release assays.

MATERIAL AND METHODS

Materials

Monoclonal, ecto-P2X7R antibody (Kim et al., 2001) was generously provided by Prof. R.A. North, of the University of Manchester; rabbit polyclonal anti-P2X7 antibody was obtained from Millipore (Billerica, MA); and guinea pig anti-AVP antibodies was obtained from BACHEM, Torrance, CA. Magnetic Dynal bead-protein-G and alexafluor-conjugated secondary antibodies were obtained from Invitrogen (Grand Island, NY). HRP-conjugated goat anti-rabbit secondary antibody and BS³ cross-linking reagent were obtained from Thermo Fisher Scientific (Rockland, IL). AZ10606120 was purchased from Tocris (Ellisville, MO) and BBG obtained from Biorad (Hercules, CA). Unless otherwise indicated all other materials used in this study were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

All studies were performed using rat brain tissue preparations acquired according to guidelines approved by the Institutional Animal Care and Use Committee IACUC (docket # A-1031) of the University of Massachusetts Medical School.

Isolated NH terminals-Isolated

NH terminals were prepared using previously described methods (Lemos and Nordmann, 1986). Briefly, adult male Sprague-Dawley rats were sacrificed, brains removed and the pituitary was collected and further dissected to isolate the NH in low divalent cation (LDv) buffer (3 μ M free Ca^{2+}) containing, in mM: NaCl, 140; KCl, 5; MgCl_2 , 0.01; HEPES, 10; CaCl_2 , 1.9; EGTA, 2; with osmolarity of 310 mOsm when pH was adjusted to 7.3 with NaOH. The NH was transferred to buffer containing, in mM: sucrose, 270; tris-EGTA, 0.01 and HEPES, 10; with pH adjusted to 7.3 with trizma base, and mechanically dissociated with plastic pestle using gentle pressure. This suspension was then plated for use in electrophysiological and immunocytochemical experiments.

Preparation of NH sections

Tissue sections were prepared by fresh frozen method. Briefly, Adult Male Sprague Dawley rats were sacrificed, the pituitary collected and the NH was further dissected. The NH was embedded in Tissue Tek OCT compound (Sakura, Torrance, CA) and frozen. Tissue sections (10 μ m) were cut, set on slides, and air dried overnight at room temperature. Sections were fixed using Zamboni's fixative (American Mastertech, Lodi, CA) (Stefanini et al., 1967) for 10 minutes and washed in standard PBS. In order to minimize autofluorescence sections were exposed to standard PBS containing 300 mM glycine for 30 min, and then to PBS containing 100 mM NH_4Cl for 10 min. Tissue slices were washed, air dried and then stored at 4°C until ready to use.

Immunocytochemistry

NH sections and isolated terminals, plated onto a dish, were immunostained in two stages to optimize ecto-P2X7R labeling (Kim et al., 2001). In the first stage samples were treated in non-permeabilizing media, initially by blocking with 10 percent normal donkey serum (Abcam, Cambridge, MA) in standard PBS for 30 minutes at room temperature. Then, block solution was aspirated, replaced with PBS containing monoclonal ecto-P2X7R antibody (0.01 μ g/mL) and incubated overnight at 4°C (Kim et al., 2001). Afterwards, tissues were washed with PBS before the second stage of immunostaining, which started with permeabilization in standard PBS containing 1.0 percent Triton X-100 (PBST) for 15 minutes. Tissue were, once again, blocked with PBST containing 10 percent donkey serum for 30 minutes and then incubated overnight at 4°C in PBST containing guinea pig anti-AVP antibodies (1:10,000;). Samples were subsequently washed and exposed to PBST containing a mixture of secondary antibodies including Alexafluor 488 donkey anti-mouse IgG (1:200) and Alexafluor 594 donkey anti-guinea pig (1:200) for 2 hours, at room temperature. Slides were washed and coverslips were mounted using Fluormount-G (Southern Biotech, Birmingham, Alabama) supplemented with 4 percent N-propyl-gallate. Images were acquired with a Leica TCS SP5, laser scanning confocal microscope.

Immunoprecipitation

80 ng of Ecto-P2X7R antibody was bound to 10 μ L of magnetic Dynal bead-protein-G and further cross-linked using BS³ reagent as directed by manufacturer's instructions. The NH of two rats were dissected, transferred to a tube containing 250 μ L of ice cold solubilization buffer (PBS containing 20 mM dodecyl β -D-maltoside, protease inhibitor cocktail and 4 mM phenylmethylsulfonyl-floride) and set on ice for 30 minutes. Tissues were homogenized with a plastic pestle, and then centrifuged at $10,000 \times g$ for 30 minutes at 4°C. 200 μ L of supernatant containing membrane was transferred to a tube containing ecto-P2X7 antibody linked Dynal beads and the remainder was transferred to a fresh tube. Antibody complex was incubated in membrane preparation overnight, with rotation at 4°C. Immunoprecipitated complex was isolated by attaching the tube to a magnet to retain beads, supernatant was aspirated, samples were resuspended, transferred to a new microfuge tube and further washed. Afterwards, samples were resuspended in standard Towbin's sample buffer, heated to 80°C for 10 minutes and then set on ice. Lysate and immunoprecipitated samples were separated on Novex 10 percent tris-glycine PAGE (Novex, Invitrogen) and then electroblotted onto PVDF membrane. Membrane was blocked with 5 percent milk in Tris buffered saline containing 0.5 percent tween-20 for 30 minutes, then exposed to rabbit polyclonal anti-P2X7 antibody (1:1000) overnight. Membrane was washed, exposed to goat anti-rabbit secondary antibody, conjugated with HRP (1:2,000) for 30 minutes, then subjected to a final wash prior to exposure to Supersignal West Dura chemiluminescent reagent (Thermo Pierce Scientific). Immunoreactive bands were digitally acquired using Syngene G:Box Chemi XL system (Syngene, Frederick, MD).

Electrophysiology

All experiments were performed at room temperature (~22°C). Freshly dissociated NH terminals were deposited onto a 35 mm culture dish that was then filled with LDv bath solution, which was followed by perfusion with normal Locke's bath solution containing, in mM: NaCl, 145; KCl, 5; glucose, 10; HEPES, 10; CaCl₂, 2.2; MgCl₂, 1.0; osmolarity was 310 mOsm with pH adjusted to 7.3 using NaOH. NH terminals were chosen that ranged between 7–10 μ m in diameter as determined using an eyepiece micrometer. Recording electrodes were pulled from borosilicate glass (Warner Instruments) and the resistance of pipettes were 3–5 M Ω when filled with pipette solution, which contained, in mM: CsGlutamate, 135; HEPES, 10; tetraethyl-ammonium chloride, 10; MgCl₂, 1.0; osmolarity was 300 mOSM with pH adjusted to 7.2 with trizma base. Recordings from terminals were performed in the whole cell voltage clamp mode. Access to the cytosol was obtained by perforated patch technique using Amphotericin-B. Amphotericin-B was prepared in 3 mM stock concentration, dissolved in dehydrated DMSO, using sonication. Nine volumes of stock amphotericin-B solution were mixed with one volume of DMSO, containing 20 percent pluronic F-127. 10 μ L of the amphotericin-B-pluronic F-127 solution was added to 1 ml of pipette solution, for a final concentration of 27 μ M amphotericin-B. Amphotericin-B solutions were prepared fresh after every hour. Pipettes were front-filled, using negative pressure with plain pipette buffer and back-filled with amphotericin-B containing pipette solution. Access to the cytosol was typically achieved between 10 – 20 minutes after attaining a G Ω seal. All experiments were sampled at 10 kHz using HEKA EPC 9/2 amplifier and data digitally acquired with HEKA Pulse 8 software (HEKA, Germany).

Terminals were stimulated by applying agonists one dose at a time, from low concentration to high concentration, for 2 seconds using SF77B fast step superfusion apparatus (Warner instruments) and then allowed 2–3 minutes for recovery in between exposures. Concentration-response experiments were performed with either normal Locke's bath or LDv Bath solutions. ATP was tested in both buffer conditions, while the agonists 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) was tested in LDv bath solution. The P2X7R selective antagonists, AZ10606120 or BBG, were applied in between batteries of concentration-response experiments in the presence of normal Locke's buffer. Bath solution was switched to LDv media, with antagonist application, two minutes prior and during concentration-response experiments. This promoted stability of pipette seal with terminals during recordings (Surprenant et al., 1996).

AVP release assay

The NH from three rats were used in each experiment. NH were isolated and dissociated according to procedures described above, followed by centrifuged at $2,400 \times g$ for 3 min. The resulting pellets, containing purified neurohypophysial terminals, were resuspended in Normal Locke's buffer and equally loaded onto 6 filters (Acrodisc, LC13, 0.45 μm , Pall Corporation, Ann Arbor, MI). The filters were then perfused for 30 minutes with Normal Locke's buffer at a flow rate of 50 μL per minute. After a 30 minute equilibration period the perfusion buffer was switched to a reduced divalent solution containing, in mM: NaCl, 140; CaCl_2 , 1.2; MgCl_2 0.01; KCl, 5; HEPES-tris, 10; glucose, 10; buffered at pH 7.25; 300–305 mOsm/L. The flow rate of the reduced divalent solution was slowly increased to the final perfusion rate of 250 μL per minute. Eluted samples were collected at 1.5 minute intervals and the first five samples were used to determine basal AVP release. Both ATP and BzATP treatment were each perfused for 12 minutes. P2X7R antagonist AZ10606120 (10 μM) was applied 30 minutes prior to agonist application and maintained for the duration of the experiment. Eluted samples were frozen and stored at -20°C until AVP content could be determined with an AVP ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA), as directed by manufacturer's instructions (Custer et al., 2007).

Data Analysis

For electrophysiological experiments, results from 83 NH terminals were evaluated in this study. Current density, obtained by dividing total current with NH terminal capacitance (equivalent to cell capacitance), was used for statistical analysis. Average NH terminal capacitance was 1.4 ± 0.1 pF and average diameter was 7.5 ± 0.1 μm . Electrophysiology data were analyzed using Igor Pro 6.0 software (Portland, OR), Microsoft Excel (Redmond, WA) and Graphpad Prism 5.4 (San Diego, CA). Current traces, concentration-response plots, non-linear regression and statistical analysis were performed with Graphpad Prism 5.4. Regression analysis to determine pEC_{50} ($-\text{Log of EC}_{50}$ concentration, which is agonist [Ag] concentration that stimulates half maximal current) and E_{Max} (Maximal receptor mediated Effect = Maximal current or I_{Max}) values were obtained from each experiment with "Logarithmic [Agonist] - response analysis" (equation1) from Graphpad Prism.

$$I = \left(\frac{I_{Max} - I_{Min}}{1 + 10^{(LogEC_{50} - [Ag])}} \right)^{nH} \quad (\text{Eq. 1})$$

Where (I) is the current response, I_{Min} and I_{Max} defines current range obtained from the nerve-terminal, and nH is the Hill coefficient. A population of terminals showed a complex response pattern as described in results. As detailed in results, these terminals were grouped as intermediate ATP-sensitivity (IS) population and analyzed with “Biphasic Logarithmic [Agonist] - response analysis” (equation 2) from Graphpad Prism.

$$I = \left[\left(\frac{I_{Max} - I_{Min}}{1 + 10^{(LogEC_{50} - [Ag])}} \right)^{nH} \right]_H + \left[\left(\frac{I_{Max} - I_{Min}}{1 + 10^{(LogEC_{50} - [Ag])}} \right)^{nH} \right]_L \quad (\text{Eq. 2})$$

Analysis were performed assuming the concentration response relationships included a combination of two distinct response, where the sum of high ATP-sensitive response (IS_H) and a low ATP-sensitive responses (IS_L) results in the total current or the total effect.

Statistical analysis comparing the pEC_{50} values between high and low sensitive responses to ATP was performed using unpaired Student's T-test (2-tailed assuming equal variance). The effects of P2X7R selective antagonists against BzATP stimulated currents were compared with one-way analysis of variance (ANOVA) to determine interaction between groups followed by Dunnett's post-hoc analysis to determine which group was significantly different from controls. Confocal micrographs were formatted using NIH Image J (Abramoff et al., 2004) and Adobe Photoshop (San Jose, CA).

RESULTS

Differential sensitivity of ATP-generated currents in isolated NH terminals

Previous reports from our laboratory have reported that isolated NHT exhibit high ATP-sensitive purinergic currents, with an EC_{50} of 10 μM ($pEC_{50} = 5$), but have also noted that a proportion of terminals failed to respond at doses of up to 10 μM (Knott et al., 2012; Knott et al., 2005). Therefore, we re-examined purinergic currents in isolated NH terminals by increasing the concentration range of ATP applications. Voltage-clamp recordings were performed on NH terminals using a normal Locke's bath solution, which contained standard divalent cation concentrations (2.2 mM Ca^{2+} and 1 mM Mg^{2+}). Figure 1A depicts representative current traces obtained from different NH terminals subjected to two doses (10 μM and 300 μM) of ATP. Our results demonstrate different responses between subpopulations of NH terminals. Current traces show that most terminals respond with high sensitivity to ATP ($pEC_{50} = 5$). These terminals exhibited moderate current amplitude when stimulated with 10 μM ATP, compared to 300 μM ATP. A second population of terminals showed low sensitivity to ATP ($pEC_{50} \gg 5$). As shown in figure 1A, these terminals responded with negligible currents in response to 10 μM ATP applications when compared to currents stimulated with 300 μM ATP. Plotted results of concentration-response experiments (Fig. 1B) demonstrate differential ATP-sensitivity between two populations of NH terminals (Table 1), with marked differences in ATP-sensitivity ($P < 0.001$, Student's T-

test, $n = 6 - 15$). The high-sensitivity population was consistent with previous results showing the presence of P2X2R and P2X3R in NH terminals (Knott et al., 2005). The potency of ATP in generating currents in the low sensitivity population was reduced by over one magnitude (Table 1), with values that are characteristic of the rat P2X7R subtype (EC_{50} of approximately 120 μM or pEC_{50} of 3.9) (Surprenant et al., 1996).

The P2X7R is inhibited by divalent cations in an allosteric manner (Michel et al., 1999; Virginio et al., 1997; Yan et al., 2011). Therefore, to determine if purinergic currents in NH terminals showed a similar effect, experiments were performed using a low divalent cation (LDv: 3 μM free Ca^{2+} and 10 μM Mg^{2+}) bath solution. In contrast to results from experiments using normal Locke's bath solution, LDv experiments demonstrated three different sets of responses. Figure 1C shows representative current traces from NH terminals responding to stimulations with 3 μM , 30 μM and 1000 μM ATP. Representative traces exemplify the differential sensitivity to ATP in these three populations. Figure 1D plots concentration-response results for currents generated by ATP in the LDv bath solution. Once again, the high ATP-sensitive and low ATP-sensitive subpopulations showed significantly different pEC_{50} values (Table 1; $P < 0.001$, one-way ANOVA; $n = 8 - 28$). In addition, results exhibited a small leftward shift in ATP potency in both subpopulations, compared with normal Locke's experiments (Fig. 1B,D; Table 1). The shift in potency was greater for the low ATP-sensitive population of terminals, which, again, is characteristic of the P2X7R (Duan et al., 2003). The third population of responses appeared to show intermediate-sensitivity to ATP (Figure 1 C,D). The majority of high ATP-sensitivity terminals demonstrated maximal current amplitude with 30 μM ATP. However a subset of terminals displayed high ATP-sensitivity by responding to ATP applications below 30 μM , but continued to demonstrate increasing current amplitude with increasing concentration of ATP, up to 3 mM (Figure 1C). Similar responses have been reported by others (Casas-Pruneda et al., 2009; Valdez-Morales et al., 2011) and suggested that this subset of terminals contained both low ATP-sensitive and high ATP-sensitive P2XR. Regression analysis, with equation 2, revealed two distinct pEC_{50} values for this group (Table 1). The pEC_{50} values for each components in these terminals differed by 1.5 orders of magnitude. However, the mean pEC_{50} values of the low ATP sensitive-component and the high ATP-sensitive component were within one order of magnitude of the corresponding values for the high ATP-sensitive and low ATP-sensitive terminals, respectively (Table 1; $P < 0.001$, one-way ANOVA; $n = 8 - 28$).

P2X7R Immunoreactivity in the HNS

Our laboratory has previously reported P2X7R immunofluorescence in the NH of rats (Knott et al., 2012; Knott et al., 2005). However, P2X7R-immunoreactivity in brain tissue is controversial due to concerns regarding the specificity of commercially available antibodies (Anderson and Nedergaard, 2006; Sim et al., 2004). To address this issue we employed an ecto-P2X7R antibody, which recognizes the extracellular epitope of the receptor in a conformation-dependent manner (Kim et al., 2001). Antibody-specificity was tested in NH tissue with immunoprecipitation experiments. Lysates and samples immunoprecipitated with ecto-P2X7R antibody were immunoblotted with a carboxyl-terminus targeted antibody (Fig. 2A). Our results showed that both lysates and immunoprecipitated samples displayed

immunoreactive-bands at approximately 75 kDa, which corresponds to the monomeric subunit of the rat P2X7R (Nicke, 2008). The blot also showed a faint band at approximately 140 kDa that is consistent with the dimer conformation of the receptor.

Next, we examined P2X7R localization in the NH using the ecto-P2X7R antibody. This antibody was applied to NH sections in non-permeabilized conditions to promote extracellular labeling, followed by labeling with AVP-antibody in permeabilized conditions (see Material and Methods). Confocal micrographs acquired from the NH displays immunofluorescence for both ecto-P2X7R and AVP (Fig. 2B). While co-localization was extensive in the NH, it was not evenly distributed. This would be in agreement with electrophysiological results that some nerve terminals exhibit P2X7R current properties, while others do not. Magnified sections showed that AVP-immunoreactive structures co-localized with the ecto-P2X7R label (Fig. 2b', b''). As further corroboration, ecto-P2X7R labeling was examined on isolated NH terminals. Figure 2C shows punctate ecto-P2X7R-immunofluorescence in an AVP-immunoreactive terminal at both middle and bottom confocal optical-sections. P2X7R labeling was observed mainly on the membrane region of isolated NH terminals.

Ecto-P2X7R localization was examined on the cell bodies of magnocellular neurons in the SON and PVN. Ecto-P2X7R antibody was used in non-permeabilized conditions on hypothalamic sections. This was done to preferentially label receptors on the plasma membrane since the ecto-epitope of the receptor should only be available either on the surface or inside transport vesicles, the latter of which should be inaccessible. Our results reveal abundant P2X7R immune-labeled puncta in both the PVN and the SON (Fig. 2 D,E). However, receptor-immunoreactivity was absent from AVP-containing magnocellular neurons. Instead, ecto-P2X7R-immunoreactivity was found in elongated shaped structures throughout both nuclei. These data suggest that functional P2X7R are restricted to the neurosecretory terminals of magnocellular neurons.

BzATP-sensitive currents are inhibited by P2X7R antagonists

Immunofluorescence results place the P2X7R in AVP-containing NH terminals, which is in agreement with our electrophysiology results. To further corroborate that NH terminals contain *bona fide* P2X7Rs, additional electrophysiological studies were conducted. The P2X7R shows increased sensitivity and increased efficacy to the selective agonist, BzATP (North and Surprenant, 2000; Surprenant et al., 1996). Therefore, experiments were performed with BzATP. In addition, the effects of the selective P2X7R antagonist AZ101606120 were tested (Michel et al., 2007; Michel et al., 2008; Xia et al., 2012; Yan et al., 2010). These experiments were performed in the LDv bath solution to maximize BzATP responses (Michel et al., 1999; Virginio et al., 1997; Yan et al., 2011). A subgroup of terminals showed high-sensitivity to BzATP (Figure 3A,B, Table 2), which were subsequently treated with 10 nM, then 100 nM AZ10606120. Antagonist pretreatment significantly attenuated BzATP-stimulated currents (Fig. 3A,B; Table 2; $P < 0.01$, 1 way ANOVA, $n = 3-7$) with reductions to 71.3 ± 11.6 and $42.3 \pm 1.3^*$ percent of control (*significant reduction in E_{Max} with 100 nM dose of AZ10606120; percentage values = average of percent reduction $\pm$ SEM within each experiment, to their respective control).

AZ10606120 had no effect on pEC₅₀ values, which is consistent with the non-competitive properties of this antagonist (Michel et al., 2008).

We then compared the effects of another P2X7R inhibitor, BBG, on BzATP-stimulated currents. Terminals that showed high-sensitivity to BzATP were subsequently treated with 10 nM, then 300 nM BBG (Fig. 4A,B; Table 2). Pretreatment with both doses of BBG resulted in markedly inhibited BzATP-stimulated currents compared to controls ($P < 0.001$, 1 way ANOVA, $n = 4-6$), with current reductions to $54.8 \pm 11.3^*$ and $23.8 \pm 7.5^*$ percent of control (* significant reductions in E_{Max} with 10 nM and 300 nM BBG; percentage values = average of percent reduction $\pm$ SEM within each experiment, to their respective control). This reduction of P2X7R currents by BBG at these concentrations is comparable to results obtained in recombinant cell experiments as well as in astroglia (Jiang et al., 2000; Norenberg et al., 2010). BBG pretreatment exerted no effect on pEC₅₀ values, which is consistent with the non-competitive properties of this antagonist (Jiang et al., 2000).

Taken together, the high-sensitivity response to BzATP, coupled with potent, non-competitive, inhibition by AZ10606120 and BBG, strongly suggest that a P2X7R component is present in, at least, a subpopulation of NH terminals.

BzATP-stimulated AVP release from NH terminals is inhibited by AZ10606120

Results from electrophysiological and immunocytochemical experiments place the P2X7R in NH terminals. Therefore, our next objective was to determine if the P2X7R can stimulate AVP release from these terminals. Previous work in HNS explants showed that the stimulation of AVP release with ATP (100 μM) was unaffected by the P2X7R-selective antagonist, BBG, unless it was co-stimulated with the catecholamine analog, phenylephrine (Gomes et al., 2009). Therefore, to see if P2X7R activation can directly mediate AVP release in NH terminals, we compared the effects of the agonists ATP and BzATP in a modified divalent solution (1.2mM Calcium and 10uM Magnesium). Since divalent cations allosterically inhibit the P2X7R (Michel et al., 1999; Virginio et al., 1997; Yan et al., 2011), we compared the effects of both agonists at a 200 μM concentration. In these experimental conditions (see Material and Methods) 200 μM ATP would be greater than the EC₅₀ for the P2X7R (Table 1), and 200 μM BzATP would be at a saturating concentration. Isolated NH terminals perfused with ATP showed a rapid, transient, increase in AVP secretion to 224.61 ± 27.21 percent of basal levels. By 6 minutes AVP release returned to basal levels, to 100.36 ± 8.9 percent, where it was maintained for the remainder of ATP treatment (Fig. 5A). Isolated terminals perfused with BzATP showed an increase in AVP levels, which was slow to peak, by three minutes, to 138.10 ± 6.49 percent of basal levels. The peak levels of AVP with BzATP increased to only 30.6 percent of the maximal levels induced by ATP (Student's T-test; $P < 0.05$; $n = 3$). Following the peak, BzATP-induced AVP release showed some decline, but remained significantly elevated, at an average of 123.64 ± 5.37 percent of basal levels for the duration of BzATP application (Fig. 5A). This sustained phase of AVP release, induced by BzATP, may indicate the participation of the P2X7R.

Previous studies have already reported inhibition of AVP and OT release by the P2X7R inhibitor BBG in the HNS (Gomes et al., 2009). For this study we decided to compare the effect of the highly potent P2X7R antagonist AZ10606120 on ATP- and BzATP-stimulated

AVP release. Terminals were pretreated for 30 minutes with or without 10 μ M AZ10606120 prior to the application of either 200 μ M ATP or 200 μ M BzATP. This concentration of AZ10606120 has previously been used by others to selectively inhibit the P2X7R (Michel et al., 2007; Michel et al., 2008; Xia et al., 2012). Pretreatment with AZ10606120 had no effect (Fig. 5B) on ATP-induced AVP release. In contrast, AZ10606120 pretreatment suppressed BzATP-induced AVP release. In controls, BzATP-stimulated release of AVP to 128.46 ± 2.14 percent of basal. In the AZ10606120-pretreated terminals, the levels of AVP release stimulated by BzATP were lowered to 99.23 ± 2.45 percent of basal, which is a reduction of 30.8 percent, compared to release levels of the BzATP-stimulated controls (Student's T-test; $P < 0.05$; Fig. 5B; $n=3$). Taken together these results suggest that a component of the BzATP-induced AVP release in rat NH terminals was mediated by the P2X7 receptor.

DISCUSSION

In the HNS the P2X7R is suggested to play a role in catecholamine-mediated AVP and OT release (Gomes et al., 2009). In that study, the P2X7R effect on release, defined by inhibition with BBG, was evident only upon exposure to ATP and the catecholamine analog phenylephrine. In the present study P2X7R involvement in NH terminals was examined by three approaches: *i*) directly testing for electrophysiological properties of the receptor; *ii*) immunoreactivity of the receptor; and *iii*) showing that the P2X7R-selective agonist, BzATP, stimulated AVP release in the absence of catecholamine-preconditioning, which was further inhibited by the P2X7R selective antagonist AZ10606120. Our results lead us to conclude that P2X7R is functionally expressed in NH terminals and corroborates earlier findings that it participates in the release of AVP (Gomes et al., 2009).

Concentration-response experiments with ATP in normal Locke's bath solution show that NH terminals respond with either high- or low-sensitivity to ATP (Fig. 1A,B). Because the P2X7R is sensitive to divalent cations, the intermediate ATP-sensitive population (Fig. 1C,D) observed in LDv bath experiments, appears to have been obscured by the presence of Ca^{2+} and Mg^{2+} in the standard divalent bath experiments (Jiang, 2009; Virginio et al., 1997). Furthermore, the altered concentration-response results (Fig 1D) from the intermediate ATP-sensitive population of NH terminals appeared to show a mix of both high- and low-sensitive components. This resembles results from other studies showing mixed sensitivity in either endogenously expressing cells or in recombinant cells (Casas-Pruneda et al., 2009; Valdez-Morales et al., 2011).

Currents in terminals that respond with high sensitivity to the P2X7R-selective agonist, BzATP, were markedly inhibited by nM concentrations of the selective antagonists, AZ10606120 and BBG (Figs. 3–4). In recombinant expression cells, or in cell types with a homogenous P2X7R population, E_{Max} values with BzATP are greater than that for ATP (Norenberg et al., 2010; Surprenant et al., 1996). However, in NH terminal preparations, E_{Max} values for high BzATP-sensitive currents were not significantly different from low ATP-sensitive currents. We believe that this is very likely due to attenuation of the non-P2X7R current component in the mixed-sensitivity population. BzATP is a partial agonist, showing poor efficacy, in high ATP-sensitive P2XRs (Allgaier et al., 2004; North and

Surprenant, 2000), which has also been reported in NH terminals (Knott et al., 2005). Consequently, the non-P2X7R component may be obscured in the concentration-response experiments with BzATP, which would then translate into a diminished P2X7R response when compared with ATP concentration response experiments. Therefore, without an effective method of isolating low ATP-sensitive NH terminals from the high or mixed ATP-sensitive terminals, comparisons of E_{Max} between ATP and BzATP as a test for P2X7R function are not yet feasible.

In this study we took efforts to validate the specificity of the ecto-P2X7R antibody. We have previously described P2X7R immunolabeling of magnocellular neurosecretory cells in the HNS at both the NH terminals and somata of the SON and PVN (Knott et al., 2012; Knott et al., 2005). However, the specificity of commercially available P2X7R antibodies in brain tissue has been disputed (Anderson and Nedergaard, 2006; Sim et al., 2004). We addressed this by employing a previously described P2X7R antibody, which is targeted to the ecto-epitope of the P2X7R in a conformation-specific manner (Kim et al., 2001). Our results are in agreement with previous reports in showing that this antibody immunoprecipitates a protein band, at approximately 75 kDa, which is characteristic of the rat P2X7R subunit (North and Surprenant, 2000). Taken together, these results allow us to be confident of our immunofluorescence results.

Our results show that the P2X7R-selective agonist, BzATP, stimulated AVP release from NH terminals, which was inhibited by the P2X7R-selective inhibitor AZ10606120 (Fig. 5). This is consistent with similar findings in HNS explants co-stimulated with ATP and phenylephrine (Gomes et al., 2009; Sladek and Song, 2008). These results suggest that the P2X7R may play a role in AVP secretion from the NH. However, our electrophysiology results, as well as those from others (Michel et al., 1999; Virginio et al., 1997; Yan et al., 2011), suggest that P2X7R may play a greater role in release after depletion of extracellular Ca^{2+} , which is a phenomenon that occurs in the neurohypophysis during physiological bursts of action potentials (Leng et al., 1988; Shibuki, 1990).

In conclusion, our work shows P2X7R function in a subset of NH terminals. Using selective agonists and antagonists, we also demonstrate that the P2X7R is capable of mediating AVP release from NH terminals (Fig. 5). This is in agreement with previous reports showing that P2X7R stimulation promotes, and triggers, exocytosis of large dense core vesicle in Neuro2a cells (Gutierrez-Martin et al., 2011). However, the large ATP concentration requirement for P2X7R activation would suggest that its participation would be more likely in either stressed or pathological conditions (Sladek and Song, 2008). Noradrenergic input has been implicated in facilitating P2X7R activation in the HNS (Gordon et al., 2005). Conditions that result in increased catecholamine levels coincide, and may even have a causal link, with increased AVP levels (de Wardener, 2001; Stocker et al., 2006). These conditions include exercise, stress and chronic hypertension (Bakris et al., 1997; Benarroch, 2005; Hew-Butler et al., 2008). How catecholamines may promote P2X7R involvement is not entirely clear (Sladek and Song, 2008). This is complicated by reports that noradrenergic receptors show limited expression in hypothalamic neurons (Sawyer and Clifton, 1980). An explanation has been offered from studies showing that catecholamine stimulation of astroglia, in the PVN, mediates release of ATP, which triggers an increase in EPSP activity

in magnocellular neurons (Gordon et al., 2005). This “gliotransmitter” effect is inhibited by BBG, suggesting P2X7R participation. Astrocytes of the pituitary, which are called pituicytes, are abundantly associated with NH terminals (Troade and Thirion, 2002). These cells express catecholamine receptors (Hosli and Hosli, 1982; Hosli et al., 1982). Recently, pituicytes have also been reported to express pannexin channels (Li et al., 2011), which are high-conductance channels reportedly capable of releasing high concentrations of ATP and may further be involved in P2X7R activation (Kim and Kang, 2011; Pelegrin and Surprenant, 2006; Xia et al., 2012). Future studies will determine if catecholamines stimulate gliotransmission of ATP, from pituicytes, to trigger hormone release in the NH.

Finally, association of the P2X7R with enhanced AVP secretion may make it a target for treatment of dysregulated AVP secretion, which would be relevant for essential hypertension (Bakris et al., 1997; Luft, 2007). Pertinent to these findings is that the NH is a circumventricular organ of the brain and, thus, has a “leaky” blood-brain-barrier (Duvernoy and Risold, 2007). While this property facilitates hormone release into the blood, it can also serve to simplify treatment options targeting the NH (Begley, 2004). Therefore, localization of the P2X7R in the NH may prove to be an important therapeutic target to regulate AVP levels in the circulation.

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Figure 1.

Purinergic currents in isolated NH terminals exhibit differential sensitivity to ATP. Isolated terminals were tested in concentration-response experiments with ATP in standard divalent or LDv bath solutions. **A**, Representative traces of currents stimulated with 10 μ M and 300 μ M ATP (bars) while exposed to standard divalent cation bath solution (2.2 mM Ca^{2+} and 1 mM Mg^{2+}). Traces shown are examples from terminals with high ATP-sensitivity (HS: left) and low ATP-sensitivity (LS: right). Traces display currents generated from these two doses of ATP to emphasize difference in sensitivity. **B**, Plot of concentration-response results from experiments with NH terminals stimulated with ATP, in standard divalent bath solution. Results detail the two different populations of responses (see Table 1). **C**, Representative traces of currents stimulated with 3 μ M (grey), 30 μ M (grey-dotted) and 1000 μ M (black) ATP while exposed to LDv bath solution (3 μ M free Ca^{2+} and 10 μ M Mg^{2+}). Current traces show examples from NH terminals responding with either high ATP-sensitivity (HS: left), intermediate ATP-sensitivity (IS: middle) or low ATP-sensitivity (LS: right). Traces display currents generated from these three doses of ATP to emphasize difference in sensitivity. **D**, Plot of concentration-response results from experiments in NH terminals stimulated with ATP in LDv bath solution. As in “C”, results reveal three different levels of responses, by population, in NH terminals (see table 1). (Results presented as mean $\pm$ SEM of currents normalized to cell capacitance; * $P < 0.001$ Student’s T-test comparing pEC_{50} obtained from each experiment with non-linear regression curve fit)

Figure 2.

Ecto-P2X7R-immunoreactivity in the HNS. **A**, Monoclonal ecto-P2X7R antibody was used to immunoprecipitate P2X7R protein from membrane lysate prepared from the NH. Lysate (lane 1; 1/8 volume of lysate) and immunoprecipitated (lane 2) samples were subjected to western blot analysis and immunoblotted with polyclonal anti-P2X7R antibody targeted to carboxyl-terminus (cX7). Figure shows P2X7R immunoreactivity at ~75 kD corresponding to monomeric P2X7R subunit. **B**, Extracellular labeling of P2X7R was evaluated in sections of the NH. NH terminals identified by immunolabeling for AVP (red) show co-localization with P2X7R immunoreactivity (green). (Scale bar = 20 μ m) Insert, selected regions (**b'**, **b''**), displayed according to indicated immunoreactivity and in merged image. Arrowheads indicate co-localized immunofluorescence (in yellow) for ecto-P2X7R and AVP. **C**, ecto-P2X7R labeling (green) is observed in punctate formations (arrows) on isolated NH

terminals showing AVP immunoreactivity (red) in confocal optical-sections of the middle and bottom of the terminal. (Scale bars = 2 μ m) Extracellular P2X7R-immunoreactivity was evaluated within the SON and the PVN using ecto-P2X7R antibody. **D**, Confocal Micrographs showing an AVP-immunoreactive-rich region of the PVN. Images show P2X7R-immunoreactivity (green; indicated by arrowheads) displayed in the PVN, mostly in elongated spindle shaped structures, in punctate formation. However, P2X7R-immunoreactivity is absent from AVP-labeled (red) cell bodies. **E**, Micrographs of magnified region of the SON displays P2X7R-immunostaining (green; indicated by arrowheads) similar to the PVN. P2X7R-label is seen proximally localized with AVP (red), however, a lack of color merge with P2X7R-label (in yellow) indicated that there was no co-localization with AVP labeled cell bodies. (OpT; Optic Tract; Scale bars for all images are 30 μ m).

Figure 3.

BzATP-sensitive currents in NH terminals are inhibited by the P2X7R-selective antagonist, AZ10606120. Terminals were screened for BzATP-sensitive response in LDv bath solution. Terminals that showed high sensitivity to BzATP were then treated with 10 nM or 100 nM AZ10606120 (AZ), for 20 minutes prior to subsequent battery of concentration-response applications. **A**, Representative current traces stimulated (bars) by 100 μM BzATP prior to (black) and after (grey) indicated AZ10606120 pretreatment. **B**, Plot of concentration-response experiments from NH terminals. Figure shows average of non-linear regression fit for each group. Compared to controls, pretreatment with 10 nM and 100 nM AZ10606120 showed reduction in E_{Max} to 71.3 ± 11.6 and 42.3 ± 1.3 percent of control, respectively. (* $P < 0.01$, 1 way ANOVA, $N = 3-7$ each; results presented as mean $\pm$ SEM)

Figure 4. BzATP-sensitive currents in NH terminals are inhibited by the P2X7R-selective antagonist, BBG. Terminals were screened for BzATP-sensitive responses in LDv bath solution. Terminals that showed high sensitivity to BzATP were treated with 10 nM or 300 nM BBG, for a minimum of 20 minutes, prior to subsequent battery of concentration-response applications. **A**, Representative current traces stimulated (bars) with 100 μM BzATP prior to (black) and after (grey) indicated BBG pretreatment. **B**, Plot of concentration-response results from NH terminals. Figure shows average of non-linear regression fits for each group. Compared to controls, pretreatment with 10 nM and 300 nM BBG showed reduction in E_{Max} to 54.8 ± 11.3 and 23.8 ± 7.5 percent of control, respectively. (* $P < 0.001$, 1 way ANOVA, $N = 4-6$; Results presented as mean $\pm$ SEM)

Figure 5.

Differential patterns of AVP secretion from NH terminals in response to P2X7R-selective agonists and antagonist. **A**, Plot of time course for AVP release in response to stimulation with indicated agonist. Perfusion of 200 μM ATP caused a transient increase in AVP release. Afterwards, AVP release returned to basal levels for the duration of ATP application. In contrast, 200 μM BzATP stimulated a blunted transient increase, which was only 30.6 percent of the levels stimulated by ATP. Then, AVP levels declined, but remained significantly elevated compared with ATP, for the remainder of BzATP application. (Data presented as mean percentage of AVP release levels $\pm$ SEM; dotted line and arrow indicates the period of agonist stimulation; * Time-cross comparison of AVP levels were significantly different between BzATP and ATP; $P < 0.05$). **B**, Effect of the P2X7R-selective antagonist, AZ10606120, on ATP- and BzATP-stimulated AVP release. NH terminals were perfused in the presence or absence of 10 μM AZ10606120 for 30 minutes prior to stimulation with either 200 μM ATP or 200 μM BzATP. Pretreatment with 10 μM AZ10606120 had no effect on ATP-stimulated AVP release from NH terminals. AZ10606120 pretreatment suppressed BzATP-stimulated AVP release by 30.8 percent compared to BzATP treatment alone (t-test, $P < 0.05$; $n=3$ each). (ATP- and BzATP-induced release is averaged over the entire time that agonist is present; Data presented as mean percentage of AVP release levels $\pm$ SEM).

Table 1
Concentration-response parameters for ATP-generated currents in NH terminals

Isolated NH terminals were tested for purinergic current responses to ATP in either normal Locke's (NL) or LDv bath solutions.

Bath solution	Sensitivity response	pEC ₅₀	E _{Max} (pA/pF)	N
NL	HS	5.1 ± 0.1	41.5 ± 9.7	15
	LS	3.8 ± 0.1 ^a	34.8 ± 9.5	6
LDv	HS	5.4 ± 0.1	48.7 ± 6.2	28
	LS	4.2 ± 0.1 ^b	54.0 ± 7.2	8
	IS		56.4 ± 11.8	8
	(IS _H)	5.8 ± 0.2	(65.2 ± 6.4 %) ^c	
	(IS _L)	3.3 ± 0.5 ^b	(34.8 ± 6.4 %) ^c	

Results presented as mean value ± SEM for pEC₅₀ and for E_{Max}.

^a In NL bath, pEC₅₀ were significantly different between high ATP-sensitive (HS) and low ATP-sensitive (LS) population of terminals (values obtained from fit with equation 1); P<0.001; Student's T-test.

^b In LDv bath, pEC₅₀ values were significantly different between high ATP-sensitive (HS) and low ATP-sensitive (LS) population of terminals (values obtained from fit with equation 1). In intermediate sensitivity (IS) terminals the high ATP-sensitive component (IS_H) was significantly different from low ATP-sensitive component (IS_L) (values obtained from fit with equation 2); P<0.001; one way ANOVA.

^c Results are presented as the component's percentage, of the maximal current, ± SEM from the IS population. IS population showed a combination of high ATP-sensitive component (IS_H) and low ATP-sensitive (IS_L) component (values obtained from fit with equation 2).

Table 2
Effect of P2X7R-selective inhibitor on BzATP-generated currents in NH Terminals

Concentration-response parameters from isolated NH terminals tested for response to BzATP in LDv bath solution in the absence, or presence of P2X7R-selective inhibitors, AZ10606120 (AZ) or BBG.

Stimulus Condition	pEC ₅₀	E _{Max} (pA/pF)	N
BzATP	5.3 ± 0.0	48.2 ± 7.0	7
+ AZ 10 nM	5.6 ± 0.1	31.0 ± 3.4	5
+ AZ 100 nM	5.5 ± 0.2	19.4 ± 4.6 ^a	3
BzATP	5.6 ± 0.0	54.9 ± 5.0	6
+BBG 10 nM	5.7 ± 0.6	39.0 ± 5.8 ^b	5
+BBG 300 nM	5.4 ± 0.4	12.0 ± 4.0 ^b	4

Results presented as mean ± SEM for pEC₅₀ and E_{Max} (values obtained from fit with equation 1).

^aE_{Max} values are significantly different between control and 100 nM AZ10606120 pretreatment; one way ANOVA (P<0.01).

^bE_{Max} values are significantly different between control, 10 and 300 nM BBG pretreatment; one way ANOVA (P<0.001).